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Transcytosis of Polymeric Immunoglobulin A in Polarized Madin-Darby Canine Kidney Cells

Asli Oztan, **Christine Rondanino**, and **Gerard Apodaca**

Summary

The transcytotic pathway allows for the bidirectional transport of endocytosed solutes, lipids, and proteins between the two membrane domains of polarized epithelial cells while maintaining the functional integrity of the epithelial tissue. A method is described to measure basolateral-to-apical transcytosis of immunoglobulin A (IgA) in polarized Madin-Darby canine kidney (MDCK) cells expressing the polymeric immunoglobulin receptor (pIgR). The cells are grown on porous Transwell filter supports, and radiolabeled ^{125}I-immunoglobulin A (IgA) is internalized from the basolateral pole of MDCK cells. During a subsequent 2-h chase, the amount of ^{125}I-IgA that is recycled, degraded, or transcytosed is quantified. This assay can be adapted to follow the postendocytic fate of other ^{125}I-labeled ligands and proteins.

Keywords

Apical; basolateral; immunoglobulin A (IgA); iodination; polarized Madin-Darby canine kidney (MDCK) cells; polymeric immunoglobulin receptor (pIgR); transcytosis

1 Introduction

Epithelial tissues are comprised of sheets of adherent cells that cover surfaces, line cavities, and form glands. They establish a selective barrier that modulates the exchange of ions, solutes, and macromolecules between a contacting basolateral surface that is bound by cell-cell and cell-matrix interactions and a free apical surface that faces the extracellular milieu. In addition to pathways that allow for direct transport of newly synthesized cargo from the *trans*-Golgi network to either apical or basolateral plasma membrane domain, epithelial cells also have a characteristic pathway called *transcytosis*. This key process is universally found in all epithelial cells examined and allows for the transport of endocytosed proteins, lipids, and solutes from one pole of the epithelial cell to the other while maintaining the epithelial barrier (1). In some epithelial cells, such as hepatocytes, transcytosis is the predominant route for membrane proteins to reach the apical cell surface (2,3).

A well-studied example of transcytosis is the transport of immunoglobulin A (IgA) across various mucosae (4). IgA is the principal class of immunoglobulin found in the mucosal secretions of the gut, respiratory and urogenital tracts, and exocrine gland secretions (e.g., milk, saliva, and tears). Dimers of IgA are produced by submucosal plasma cells. Following secretion, IgA binds to the polymeric immunoglobulin receptor (pIgR), which is located on the basolateral surface of the epithelial cells that form the mucosa. The pIgR-IgA complex is then endocytosed, transported through a series of endosomal compartments (described below), and then on arrival at the apical membrane the extracellular domain of the pIgR is proteolytically cleaved, releasing it and bound ligand into the secretions. The cleaved

extracellular domain of the pIgR is known as the secretory component. Once in secretions, IgA forms the first specific immunologic defense against infection (reviewed in *Ref*. 5).

The intracellular pathway for transcytosis of pIgR-IgA complexes has been extensively studied in polarized Madin-Darby canine kidney (MDCK) cells transfected with rabbit pIgR complementary deoxyribonucleic acid (cDNA). Our current understanding of the transcytosis pathway is shown in Fig. 1. Transcytosing proteins such as IgA and its receptor pIgR are endocytosed and delivered to the basolateral early endosomes (BEEs) (step 1). The pIgR-IgA complexes are subsequently transported to common endosomes (CEs; step 2), then to Rab11-positive apical recycling endosome (ARE; step 3) (6,7), and finally to the apical surface (step 4), where proteolytic processing occurs.

We describe here a method to measure basolateral-to-apical IgA transcytosis in polarized MDCK cells expressing the rabbit pIgR. For this assay, MDCK cells are grown on porous filter supports to form well-polarized monolayers with tight junctions. IgA is radiolabeled with ^{125}I to allow for its sensitive detection and quantification. The ^{125}I-IgA is internalized from the basolateral pole of MDCK cells, and the cells are then washed with ligand-free medium to allow nonadherent ligand to dissociate from the cell surface pIgR. During a subsequent 2-h chase, the amount of ^{125}I-IgA released at the apical (transcytosed) or basolateral (recycled) pole of the cell is quantified with a gamma-counter. The amount of degraded ^{125}I-IgA is measured upon trichloroacetic acid (TCA) precipitation. This assay can be easily adapted to follow the postendocytic fate of other ^{125}I-labeled ligands, proteins, and antibodies.

2 Materials

2.1 Iodination

1. D-Salt excellulose plastic desalting column: column containing 2 mL of preswollen, beaded 12% cellulose; exclusion limit 5000 MW (Pierce, Rockford, IL).
2. Phosphate-buffered saline (PBS, 10X): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 80.6 m*M* Na_2HPO_4, 14.7 m*M* KH_2PO_4. Store at room temperature. Prepare working solution by diluting one part stock with nine parts water. The pH of 1X PBS is 7.4.
3. PBS/bovine serum albumin (BSA): 1% (w/v) BSA in 1X PBS buffer. Prepare fresh.
4. Human polymeric IgA (*see* Note 1). For long-term storage, keep at -80 °C. For short-term storage (<6 mo), keep at 4 °C.
5. 1 *M* Tris-HCl, pH 8.0. Store at room temperature.
6. 2 *M* NaCl. Prepare fresh.
7. Iodine monochloride (ICl) (8,9): The preparation of ICl should be carried out in a fume hood. Because ICl is a corrosive chemical, gloves and a lab coat should be worn during preparation. Dissolve 0.15 g solid NaI in 8 mL 6*N* HCl. Next, forcibly inject 2 mL $NaIO_3$ (49.5 mg/mL in H_2O) into the NaI solution using a 5-mL syringe fitted with a 20-gage needle (*see* Note 2). Bring the solution up to 40 mL with water and place in a clean separatory funnel. Add 10 mL CCl_4 to the mixture and shake

[1]The pIgR does not bind to monomeric IgA, such as that found in serum; instead, it binds to polymeric IgA complexed with J chain. Dimeric, trimeric, and tetrameric IgAs are all transcytosed with comparable efficiencies (11). We used IgA prepared by Dr. J.P. Vaerman, Catholic University of Louvain, Brussels, Belgium; however, he has since retired. An alternative source of polymeric IgA is to purify it from hybridoma supernatants (12).

[2]It is very important that you rapidly and forcibly inject the $NaIO_3$ into the NaI solution. After mixing, there should be no precipitate, and the resulting liquid should be a clear light yellow solution. Discard any preparation that is not a clear yellow solution.

vigorously (*see* Note 3). Carbon tetrachloride is a carcinogen: Avoid breathing vapors or skin contact. Discard the lower pink organic phase and repeat the extraction with CCl_4 until the organic phase is colorless. Transfer the extracted aqueous layer to a clean Erlenmeyer flask and aerate with moist air for 8-10 h to remove any trace of CCl_4 from the aqueous phase (*see* Fig. 2). Prepare 0.5-mL aliquots and store away from light at -20 °C. The solution is stable for several years at -20 °C.

8. $Na^{125}I$ (350 mCi/mL, 17.4 Ci/mg) supplied by PerkinElmer (Boston, MA). $Na^{125}I$ is a volatile radioactive compound that should be handled in a certified fume hood. Store megacurie quantities of ^{125}I in lead pigs either at room temperature or at 4 °C. If necessary, the lead pigs can be further surrounded by 3-mm thick lead sheeting.
9. NaI/PBS/BSA: Dissolve NaI (5 mg/mL) in PBS/BSA. This solution can be aliquoted and stored at -20 °C.

2.2 Cell Culture

1. MDCK type II cells expressing the pIgR (10). An MDCK tet-off cell line that expresses the pIgR is available from Clontech (cat. no. 630913, Clontech, Mountain View, CA).
2. Complete minimum essential medium (MEM): MEM with Earle's salts and L-glutamine (MEM; Cellgro®, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; HyClone; Logan, UT) and 1% antibiotic-antimycotic (100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 250 ng/mL amphotericin B as Fungizone®; Gibco-Invitrogen; Grand Island, NY).
3. Sterile PBS (Gibco-Invitrogen).
4. Trypsin/ethylenediaminetetraacetic acid (EDTA): 0.25% (w/v) trypsin with 1 m*M* EDTA (Gibco-Invitrogen).
5. Freezing medium: 10% (v/v) dimethyl sulfoxide (DMSO) and 90% (v/v) FBS.
6. 70% (v/v) ethanol.
7. Transwells Costar®, 12-mm diameter, 0.4-μm pore size (Corning, Corning, NY).

2.3 Postendocytic Fate of Basolaterally Internalized ^{125}I-IgA

1. MEM/BSA (*see* Note 4): In a 1-L beaker, add 20 mL 1 *M* HEPES-NaOH, pH 7.6, 10 mL penicillin/streptomycin (10,000 U/mL penicillin G, sodium salt, and 10,000 μg/mL streptomycin sulfate; Gibco-Invitrogen) to 900 mL of water. Add 10.7 g MEM with Hanks' salts, L-glutamine, and nonessential amino acids and without sodium bicarbonate (cat. no. M1018-10X1L, Sigma-Aldrich, St. Louis, MO); 1.4 g sodium bicarbonate; and 24 g BSA. Adjust to pH 7.4 (using either HCl or NaOH) and store at -20 °C in clean, autoclaved bottles. Thaw prior to use. It is important to swirl the bottles to disperse the components after thawing the medium. Thawed MEM/BSA is stable at 4 °C for approx 1 wk.

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[3] CCl_4 is added to extract the unreacted I_2. After the initial extraction, a biphasic solution forms; the top layer contains ICl and has a clear yellow color, whereas the lower CCl_4 layer is pink. Depending on the amount of I_2, it will take at least two to four extractions to generate a clear CCl_4 layer.

[4] MEM/BSA contains a tenth the amount of bicarbonate found in standard culture medium. It is buffered with HEPES to maintain a physiological pH; MEM/BSA is not intended for use inside of a cell culture incubator that is gassed with CO_2. MEM/BSA shelf life at 4 °C is largely limited by how rapidly it becomes contaminated with microbes. Contamination is apparent when the solution becomes turbid or changes to a yellow color (a result of acidification). The addition of penicillin/streptomycin is intended to suppress bacterial growth, but the antibiotics can be omitted if needed.

2. Tissue culture plates: 12-well, flat-bottom Falcon® (Becton Dickinson, Franklin Lakes, NJ).
3. ^{125}I-IgA (see Subheading 3.1.): ^{125}I-IgA is stored at 4 °C in a leaded pig. It is stable for approx 1 mo.
4. Collection tubes: 1.2-mL polypropylene microdilution tubes with conical bottom (USA Scientific, Ocala, FL).
5. PBS^{+}: In 100 mL ice-cold 10X PBS (see Subheading 2.1.2.), add 0.1 g anhydrous $CaCl_2$ and 0.1 g $MgCl_2 \cdot 6H_2O$. Bring up to 1 L with ice-cold water and stir to dissolve. Store at 4 °C.

2.4 Trichloroacetic Acid Precipitation

1. TCA: 100% (w/v) in water (Sigma). TCA is very caustic; handle with gloves in a ventilated enclosure. Store at 4 °C.
2. 12 × 75 mm polypropylene test tubes and 12-mm natural hollow top caps (Fisher Scientific, Pittsburgh, PA).
3. Fisherbrand corks (cat. no. 07782B, Fisher Scientific).

3 Methods

In this assay, iodinated polymeric IgA is internalized from the basolateral surface of polarized MDCK cells cultured on Transwell filter supports, and the percentage of internalized ligand that is recycled, degraded, or transcytosed is quantified. Efficient IgA transcytosis requires recently iodinated IgA (< 4 wk old) and freshly thawed MDCK cells that are not more than 2 wk old. The same method can be modified to study the postendocytic traffic of other apically or basolaterally internalized ligands, including those that recycle at the cell surface or those that are targeted to lysosomes and degraded.

3.1 Iodination

1. Remove top and bottom caps of desalting column and then secure column in a vise clamp. Place a waste container under the column to catch the eluant. Wash the column four times by filling the column buffer reservoir with PBS/BSA and allowing the eluant to flow through the column by gravity. At the end of the final wash, allow the buffer reservoir to empty and the eluant to completely enter the column (*see* Note 5).
2. All the reagents required for the iodination reaction are prepared on ice. Three 1.5-mL Eppendorf tubes are labeled as “ligand,” “reaction,” and “waste” and then inserted into a container containing approx 4 inches of ice. One 2-mL screw-cap tube, which is used to hold the final iodinated IgA ligand product, is similarly placed on ice.
3. Place 25-50 μg polymeric IgA in the tube labeled “ligand.” The total volume of ligand should not exceed 10-20 μL for an efficient iodination reaction. Keep the ligand on ice.
4. Add 100 μL Tris-HCl (pH 8.0) to the tube labeled “reaction.” Place the ice container with labeled tubes in a fume hood. The hood is typically certified to perform iodination reactions. The investigator should wear a protective lab coat and lead apron, eyewear (e.g., goggles), and a double pair of gloves in the following steps. A Geiger counter

[5]The column contains a white permeable fret above the cellulose matrix that prevents the column from running dry. It also prevents the matrix from disturbance when loading the column with sample. The void volume of the column is approx 0.7 mL.

with scintillation probe is useful for monitoring the dose of radiation and potential contamination. Lead foil or lead-impregnated Plexiglass can be used to further shield the investigator from the ^{125}I.

5. Prepare a 1:100 dilution of ICl reagent in 2 *M* NaCl and then add 20 μL of the diluted ICl/NaCl reagent to the "reaction" tube containing 100-μL Tris-HCl (pH 8.0). The contents should be mixed by tituration and then placed on ice.

6. Add 4 μL Na^{125}I (~1.4 mCi) to the "reaction" tube, mix by tituration, and incubate the reaction for 1 min (*see* Note 6). At the end of the 1-min incubation, transfer the IgA ligand to the "reaction" tube. Mix gently by titurition and incubate for 10 min on ice.

7. At the end of the 10-min incubation, stop the reaction by adding 100 μL NaI/PBS/BSA to the "reaction" tube.

8. Carefully transfer the iodination mixture from the "reaction" tube and place on the surface of the washed desalting column. Once the iodination mixture has entered the column, wash the column by adding 300 μL PBS/BSA to the column bed. Collect any eluant in the tube labeled "waste." This initial eluant usually contains little iodinated ligand (this can be confirmed with the Geiger counter) and can be discarded.

9. Replace the "waste" tube with the screw-cap tube (with cap removed) and collect the ^{125}I-IgA by eluting the column with an additional 600 μL PBS/BSA.

10. Gently mix the tube containing ^{125}I-IgA to disperse the ligand equally in the eluant and then count a 1-μL aliquot in a γ-counter. A typical reaction contains 500,000 to 1 million counts per minute ^{125}I-IgA/μL reaction (*see* Note 7). Store iodinated ligand at 4 °C for no longer than 4 wk.

3.2 Cell Culture

1. We use MDCK type II cells expressing the wild-type rabbit pIgR (10). Cells are maintained in complete MEM in a 37 °C tissue culture incubator with 5% CO_2/95% air. We routinely culture cells in 10-cm diameter tissue culture-treated dishes, but flasks are an acceptable alternative.

2. Cell passaging: The medium is aspirated from a confluent 10-cm dish of cells, and the cells are washed with 10 mL warm (37 °C) PBS. The PBS is aspirated, 2 mL trypsin are added, and the cells are incubated at 37 °C in the tissue culture incubator until the cells detach from the plate (*see* Note 8). When the cells detach from the plate, 8 mL complete MEM is added, and the cells are resuspended by tituration using a 10-mL pipet until there are no visible cell clumps remaining. For routine passaging, 1 mL of cell suspension is added to a 10-cm dish containing 9 mL complete MEM (1:10 dilution). The cells take approx 4-5 d to reach confluence (*see* Note 9).

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[6] In this step, the Na^{125}I is oxidized to $^{125}I_2$ (molecular iodine) in preparation for the iodination reaction. This step is time sensitive and should be accurately timed. Because reduction occurs prior to the addition of ligand, the ICl method works for a broad range of proteins (e.g., IgA, IgGs, transferrin, and recombinant proteins) and ligands, such as low-density lipoproteins that are sensitive to oxidation.

[7] If the reaction is less than 100,000-200,000 cpm/μL, it is usually considered poor and is discarded. In a typical reaction, more than 99% of the recovered iodinated products are TCA precipitable, indicating that there is little free iodine in the final eluate. This test can be performed by adding 1 μL eluate to 0.5 mL MEM/BSA, adding TCA to 10% (v/v), incubating for 30 min on ice, centrifuging for 15 min at 4 °C, and counting the resulting supernatant and pellet (see Subheading 3.4. for additional details).

[8] Trypsinization usually takes 10-30 min and depends on how long the cells have been cultured on the dish. An easy way to test if the trypsinization reaction is complete is to rock the dish slowly back and forth. When the cells are trypsinized, they readily come off the dish and enter the moving fluid phase. Do not overtrypsinize the cells as this can lead to cell death.

[9] The cell dilution affects the time it takes for the cells to become confluent. At a dilution of 1:2, the cells will become confluent overnight. When diluted 1:5, the cells take approx 3 d to reach confluence. At 1:20, the cells take approx 7-8 d to reach confluence.

3. Freezing and thawing cells: To freeze cells, a confluent dish of MDCK cells is trypsinized, the cells are resuspended in 8 mL complete MEM, and the cells are then recovered by centrifugation at 100 *g* for 5 min at room temperature. Following aspiration of the medium, the cells are carefully resuspended by titurition in 1.5 mL warm (37 °C) freezing medium. Aliquots (0.5 mL) of the cell suspension are placed in freezing vials, capped, and then placed in a room temperature Styrofoam container. The Styrofoam container is placed in a -70 °C freezer. The cells are stable at -70 °C for some time but can be transferred after 2-3 d to liquid nitrogen for extended storage. Cells are thawed by partially immersing the cell vial containing the frozen cells in a 37 °C water bath. The cell vial is removed from the water bath, dried, rinsed with ethanol, and the cap removed in a cell culture hood. The thawed cell suspension is transferred to a 10-cm dish, and 10 mL complete MEM are slowly added to the cells. To minimize osmotic shock, the first 2 mL of MEM should be added at a rate of approx 1 mL/min. The next day, the medium is aspirated and replaced with 10 mL complete MEM. The cells typically achieve confluence by the second day of thawing. To maintain a high level of pIgR expression, new cells are thawed every 2 wk.

4. Plating cells on Transwell filters: A confluent dish of MDCK cells is trypsinized, and the cells are resuspended in 10 mL complete MEM. The cells are then recovered by centrifugation at 100 *g* for 5 min at room temperature. The trypsin-containing medium is aspirated, and the cell pellet is resuspended in 12 mL complete MEM (*see* Note 10). Place 0.5 mL cell suspension (containing ~ 1×10^6 cells) into the apical chamber of each 12-mm Transwell. Add 2 mL complete MEM to the well that faces the basolateral surface of the Transwell unit. The cells typically form a tight, polarized monolayer by 3 d postplating (*see* Note 11). After the third day, the cells are fed daily by aspirating the basolateral, then apical medium and adding 0.5 mL complete MEM to the apical chamber and then 1.5 mL medium to the basolateral chamber. The order of medium aspiration and addition is important to prevent hydrostatic pressure-induced cell damage. Cells are typically used 3-4 d postplating on the Transwell units.

3.3 Postendocytic Fate of Basolaterally Internalized ^{125}I-IgA

1. Prepare a 37 °C circulating water bath (Fig. 3). We use a 30.5 × 50.8 × 21.6 cm (width × length × height) bath with temperature regulator/circulator (VWR, West Chester, PA). A 22.5 × 26 cm aluminum plate is placed on top of test tube racks, and the water in the bath is adjusted so that the aluminum plate is covered by approx 5 mm H_2O.

2. Place MEM/BSA in the water bath and warm to 37 °C. Label a total of eleven 1.2-mL collection tubes for each Transwell filter: tubes for apical medium collected at 7.5, 15, 30, 60, 120 min; tubes for basolateral medium collected at 7.5, 15, 30, 60, 120 min; and a tube for the filter, which is cut out at the end of the experiment.

3. Place 500 μL MEM/BSA into the wells of a 12-well plate. You will need 5 wells of MEM/BSA for each individual Transwell filter (one for each of five timepoints). Perform each experimental treatment group in triplicate or quadruplicate. If performing multiple treatment groups, it is easier to use a separate 12-well plate for each time-point. Place the 12-well plates containing MEM/BSA atop the aluminum plate in the water bath to keep them warm before sample collection starts.

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[10] It is important to make sure the cell pellet is completely resuspended to ensure proper monolayer formation. Improper resuspension or plating too many cells results in cells that adhere to the apical surface of the monolayer or stratification.

[11] The formation of a tight monolayer can be assessed by monitoring the transepithelial resistance across the monolayer using an Epithelial Volt Ohm Meter (EVOM) (Warner Instruments, Hamden, CT). A Transwell lacking cells but otherwise incubated in complete MEM medium is used to correct for series resistance. Although there is some variation between clones, type II MDCK cells typically achieve a transepithelial resistance of 110-150 ohm·cm^2.

4. Prepare a humid labeling chamber by placing a piece of moistened filter paper in a storage container with a removable lid. A sheet of Parafilm is cut and placed on top of the moistened filter paper. For each filter, dilute 3-4 μL ^{125}I-IgA in 27 μL MEM/BSA and place the drop of diluted ^{125}I-IgA on the sheet of Parafilm, avoiding air bubbles. Close the chamber and place it in the water bath to keep it at 37 °C.

5. Wash the Transwell-grown MDCK cells with warm MEM/BSA two times to remove the cell culture medium. Washing is accomplished by transferring Transwells to a 12-well dish, aspirating the medium (basolateral first, then apical), and then adding 1 mL MEM/BSA to the apical and then basolateral chambers of the Transwell. Following washing, the MEM/BSA is aspirated, and 300 μL MEM/BSA are added to the apical chamber of the Transwell. A Kimwipe is used to carefully remove any MEM/BSA adhering to the rim of plastic surrounding the porous filter on the basolateral side of the Transwell. The Transwell filter is placed on the 30-μL drop of ^{125}I-IgA in the humid labeling chamber (*see* Note 12) and pulse labeled for 10 min at 37 °C. Lead foil can be placed over the labeling chamber to shield the investigator during this incubation.

6. Next, remove unbound ligand by washing. Return the Transwell filters to a 12-well plate and add 1 mL MEM/BSA to the basolateral surface of each Transwell filter. Remove the basolateral medium by aspiration. Wash the basolateral surface an additional two times (*see* Note 13). Discard the MEM/BSA wash liquid generated in this and subsequent steps in suitable radioactive waste disposal containers. Aspirate any remaining apical and basolateral media and then add 1 mL MEM/BSA to the apical and then basolateral chamber of each Transwell. Place the 12-well plate containing Transwells in the 37 °C water bath. Incubate the cells for 3 min to allow for nonspecifically bound ligand to dissociate from the filter and for cell surface ligand to be internalized and “chased” into the cells. The total wash and chase should take 5 min to complete. During the chase, discard the sheet of Parafilm containing residual ^{125}I-IgA (in step 4) in a suitable radioactive waste container.

7. At the end of the 3-min chase, aspirate the medium from both sides of the filter and add 500 μL MEM/BSA to the apical chamber. Place the filters in the 12-well plates containing MEM/BSA prepared in step 3. Start a timer as the filters are placed in the wells of MEM/BSA.

8. After 7.5 min, begin collecting samples. Lift an individual Transwell from the 12-well plate, quickly recover the apical medium using a 1-mL pipetor tip (taking care not to puncture the Transwell filter), and transfer the contents to the labeled 1.2-mL collection tubes prepared in step 2 (these are the 7.5-min apical samples). Eject the tip and use a new tip to gently transfer 500 μL MEM/BSA to the apical chamber of the Transwell. Place the same Transwell in a new well containing 500 μL MEM. Repeat the process for all Transwells in the experiment (*see* Note 14). After all of the Transwells are transferred to new wells, collect the MEM/BSA from the basal wells of the previous time-point and place in the 1.2-mL collection tubes (these are the 7.5-min basolateral samples).

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[12]It is important to avoid air bubbles during the pulse-labeling step. This can be accomplished by first touching the edge of the Transwell near the edge of the drop and then slowly releasing the Transwell onto the drop of ligand.

[13]The washing step is crucial to the success of the experiment. The initial three washes should take approx 2 min to complete. If these washes are performed too rapidly or with insufficient MEM/BSA, there will be a large pool of loosely adherent ligand that dissociates from the plastic associated with the Transwell cup and filter.

[14]It is important to be rhythmic when collecting the samples. A useful strategy is to stagger the collection of each sample by approx 10-15 s.

9. Repeat the collection process described in step 8 at 15, 30, 60, and 120 min. At the end of 120-min chase period, you will have five apical samples (7.5, 15, 30, 60, 120 min) and five basolateral samples (7.5, 15, 30, 60, 120 min) for each Transwell filter. Finally, wash the filter and associated cells with ice-cold PBS^+ two times to remove MEM/BSA, cut the filter from its holder using a scalpel, and place the filter into a 1.2-mL counting tube (this is the cell-associated IgA fraction for each sample).

3.4 Trichloroacetic Acid Precipitation

1. Place the collection tubes containing medium from Subheading 3.3., step 9 on ice. Add 50 μL TCA to each tube. Vortex each tube for 15 s to thoroughly mix the sample. The TCA will precipitate intact ^{125}I-IgA ligand but not degraded fragments of ^{125}I-IgA released from the cell into the culture medium (*see* Note 15).
2. Incubate samples on ice for 30-45 min and centrifuge the samples in a microfuge at 14,000 *g* for 10 min at 4 °C (*see* Note 16).
3. The collection tubes are placed in a rack, and the supernatant is carefully removed using a 3-mL syringe fitted with a 22-gage needle. The pellets are retained as these contain the undegraded ^{125}I-IgA. If quantifying the total amount of degraded ^{125}I-IgA, then pool all of the basolateral and apical supernatants for each Transwell in 12 × 75 mm polypropylene test tubes. If the aim is to measure the kinetics of degradation, then place the supernatant of each apical and basolateral time-point into separate collection tubes. Cap all collection tubes (containing pellets, supernatants, or filter) with a cork stopper.
4. Place the samples in a γ-counter and count the ^{125}I associated with the filter (cellassociated IgA), apical TCA pellets (transcytosed IgA), basolateral TCA pellets (recycled IgA), and the TCA supernatants of the apical and basolateral samples (degraded IgA).

3.5 Calculation of Data

1. For each Transwell, sum the total counts per minute associated with the filter, apical and basolateral TCA pellets, and the apical and basolateral TCA supernatants (Table 1). This is the total ligand that was retained by the cell during the pulse-chase labeling protocol.
2. For each Transwell, calculate the percentage of total ligand associated with the filter, the apical and basolateral TCA pellets for each time-point, and the pooled apical and basolateral TCA supernatants (Table 1). If examining the kinetics of degradation, then calculate the individual percentages associated with the TCA supernatant for each time-point.
3. Calculate a running sum of percentages for the apical TCA pellets (transcytosis) and then for the basolateral TCA pellets (recycling) (Table 1). If examining the kinetics of degradation, then also calculate the running sum of the apical and basolateral TCA supernatants.
4. Calculate the averages and standard deviation for each experimental treatment and plot the data using a graphing program such as Excel or Prism (Fig. 4).

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[15]Degraded iodinated peptides are membrane permeable and diffuse from the cell into the MEM/BSA. We have found that the majority of cell-associated ligand remaining in the filter at the end of the experiment is TCA precipitable, so we do not routinely perform TCA precipitation of the filter-associated cells.

[16]We place the counting tubes in 1.5-mL Eppendorf tubes with their caps removed. The Eppendorf tube containing the counting tube is then placed in a microfuge for centrifugation.

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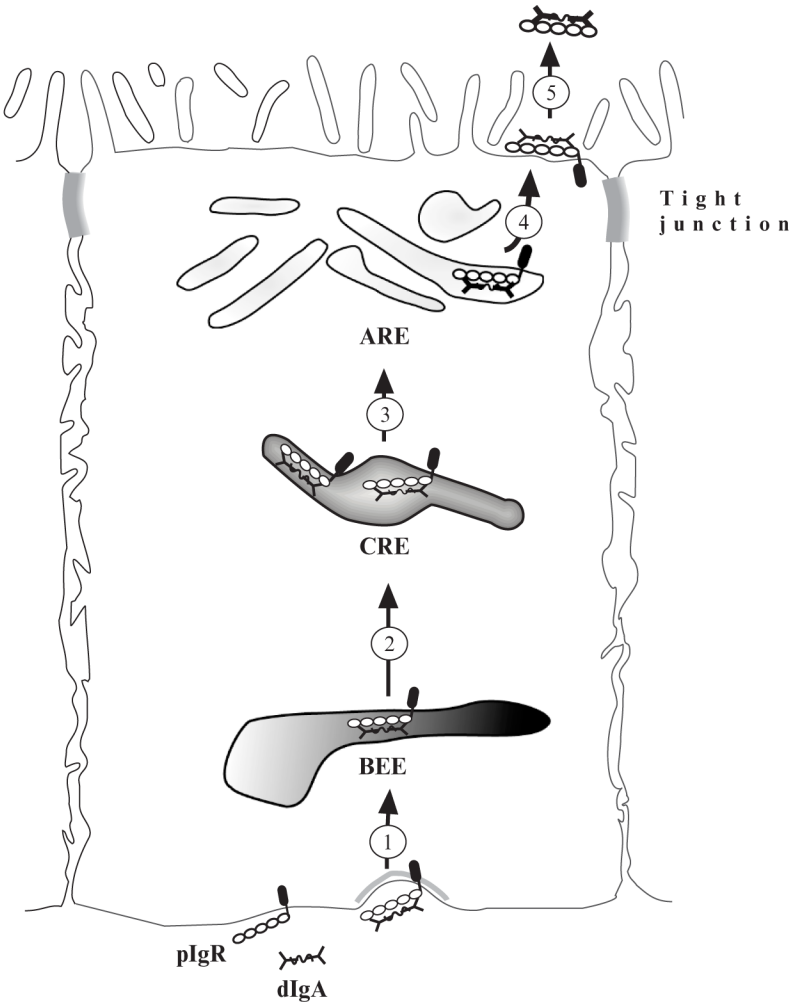

Fig. 1.

Transcytosis of pIgR and its dimeric IgA (dIgA) ligand in polarized MDCK cells. The pIgR binds dIgA at the basolateral surface of the cell and is rapidly endocytosed via clathrin-coated pits (step 1). On entry in basolateral early endosomes (BEEs), pIgR-dIgA is delivered to the common recycling endosomes (CRE; step 2) and then to apical recycling endosomes (AREs; step 3). On exocytosis at the apical pole of the cell (step 4), a proteinase cleaves the extracellular domain of the receptor, releasing it and its ligand into the apical secretions (step 5).

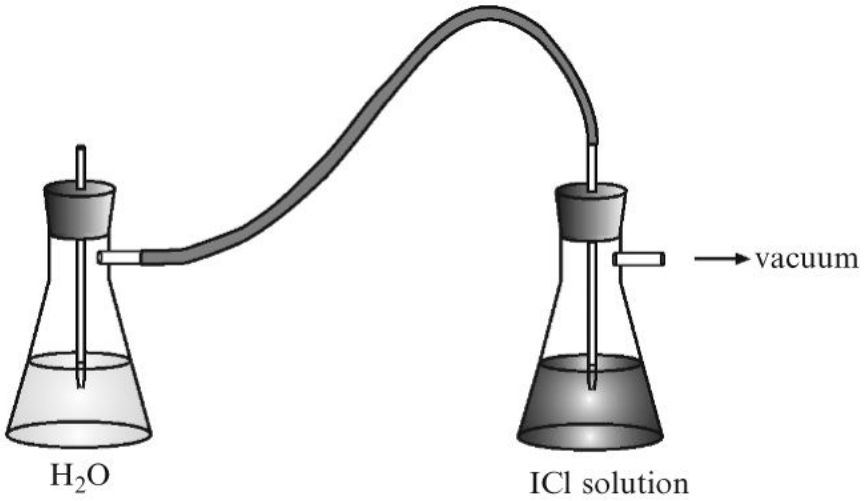

Fig. 2.

Apparatus to aerate the ICl solution with moist air. The first flask contains distilled water, and the vacuum draws moist air into the second flask, which contains the ICl solution.

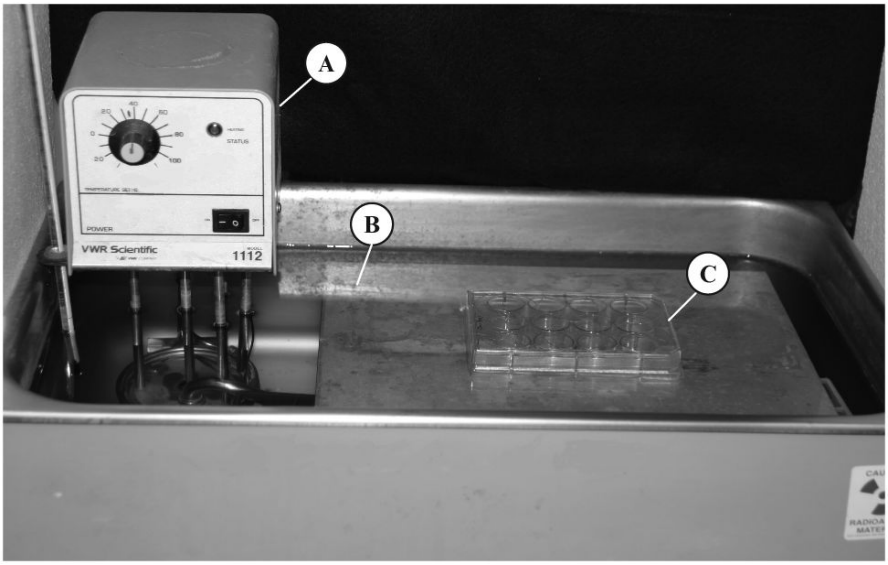

Fig. 3.

Water bath used to perform the postendocytic fate assay. The water bath is equipped with a temperature regulator/circulator **(A)**. A 22.5 × 26 cm aluminum plate **(B)** is placed on top of test tube racks, and the water in the bath is adjusted so that the aluminum plate is covered by approx 5 mm H_2O. A 12-well plate containing Transwell filters **(C)** is placed on top of the aluminum plate.

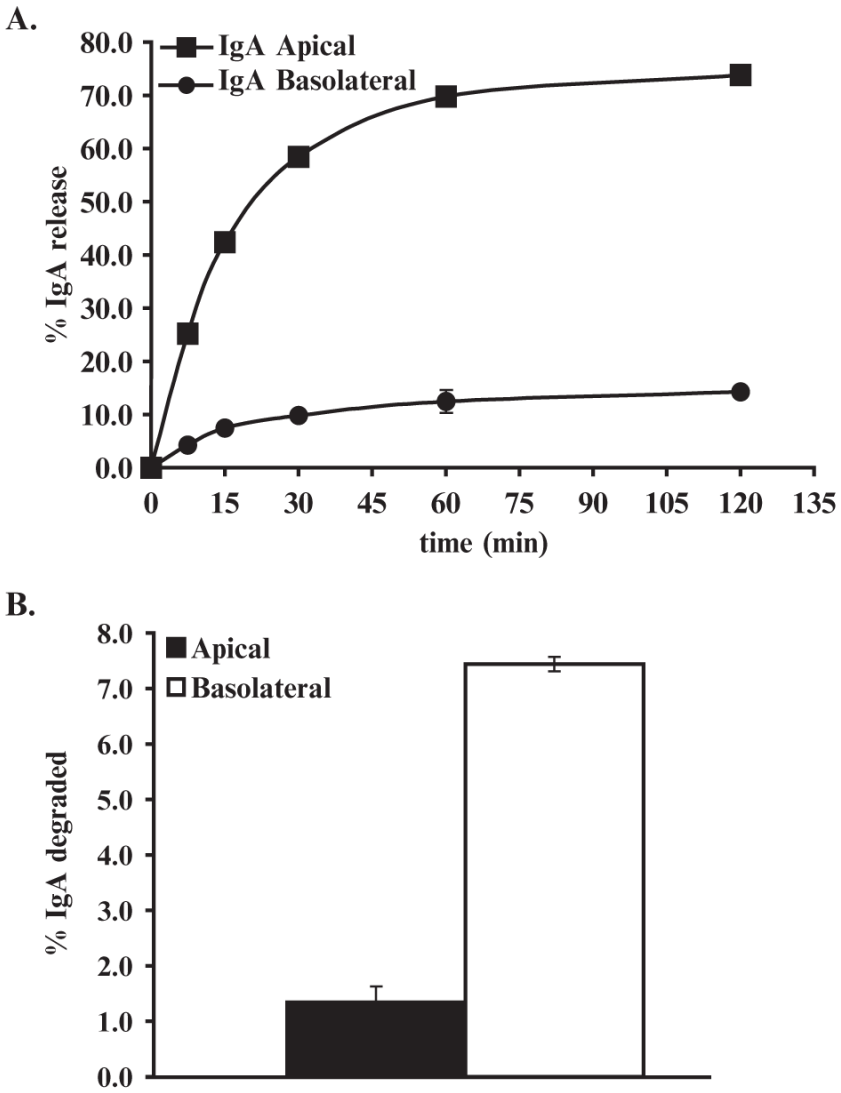

Fig. 4.

Postendocytic fate of basolaterally internalized IgA in polarized MDCK cells expressing pIgR. ^{125}I-IgA was internalized from the basolateral surface of the cells for 10 min at 37 °C, the cells were washed, and the cells were chased in the absence of ligand for 120 min. **(A)** The percentages of total ligand released basolaterally (recycled) and apically (transcytosed) is shown. **(B)** The percentage of degraded ligand released apically or basolaterally is shown.

Table 1

Representative Calculation for Quantifying the Fate of Basolaterally Internalized ^{125}I-IgA in a Single Transwell of Polarized MDCK Cells Expressing the pIgR

	Sample	Count	Percentage	Running Percentage
	7.5 min	2039.0	24.6	24.6
	15 min	1525.0	18.4	43.0
Apical Pellet	30 min	1221.0	14.7	57.7
	60 min	859.0	10.3	68.0
	120 min	341.0	4.1	72.1
	7.5 min	363.0	4.4	4.4
	15 min	273.0	4.4	4.4
Basolaternal Pellet	30 min	227.0	2.7	10.4
	60 min	375.0	4.5	14.9
	120 min	93.0	1.1	16.1
Filter		249.0	3.0	(Cell Associated)
Apical Supernatant		131.0	1.6	(Degraded, Apical)
Basal Supernatant		605.0	7.6	(Degraded, Basal)
Total		8301.0		