Cholesterol-sensitive Modulation of Transcytosis^D

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Cholesterol-rich membrane domains (e.g., lipid rafts) are thought to act as molecular sorting machines, capable of coordinating the organization of signal transduction pathways within limited regions of the plasma membrane and organelles. The significance of these domains in polarized postendocytic sorting is currently not understood. We show that dimeric IgA stimulates the incorporation of its receptor into cholesterol-sensitive detergent-resistant membranes confined to the basolateral surface/basolateral endosomes. A fraction of human transferrin receptor was also found in basolateral detergent-resistant membranes. Disrupting these membrane domains by cholesterol depletion (using methyl- β -cyclodextrin) before ligand-receptor internalization caused depolarization of traffic from endosomes, suggesting that cholesterol in basolateral lipid rafts plays a role in polarized sorting after endocytosis. In contrast, cholesterol depletion performed after ligand internalization stimulated cargo transcytosis. It also stimulated caveolin-1 phosphorylation on tyrosine 14 and the appearance of the activated protein in dimeric IgA-containing apical organelles. We propose that cholesterol depletion stimulates the coupling of transcytotic and caveolin-1 signaling pathways, consequently prompting the membranes to shuttle from endosomes to the plasma membrane. This process may represent a unique compensatory mechanism required to maintain cholesterol balance on the cell surface of polarized epithelia.

INTRODUCTION

In humans, the major antibody that mediates immunological defense against mucosal infections is dimeric IgA (dIgA). This antibody is produced by plasma cells in the lamina propria located underneath the mucosal surface (reviewed in Lamm *et al.*, 1995; Rojas and Apodaca, 2002) and is carried from the blood to mucosal secretions by the polymeric immunoglobulin receptor (pIgR). The itinerary of pIgR and its ligand was intensively studied in the polarized Madin-Darby canine kidney (MDCK) cell line that heterologously expresses rabbit pIgR (for a recent review, see Rojas and Apodaca, 2002). The receptor is initially delivered from the *trans*-Golgi network (TGN) to the basolateral plasma membrane, where it encounters and binds dIgA. The complex is subsequently internalized and transcytosed to the apical plasma membrane of the cell. At that surface, the ectodo-

main of pIgR is proteolytically cleaved off to yield the secretory component (SC), which is released together with dIgA into mucosal secretions. In the course of transcytosis, pIgR-dIgA complexes traverse various endosomal compartments, among which is an endocytic compartment located immediately underneath the apical surface (Gibson *et al.*, 1998). This apical compartment, defined as apical recycling endosomes (AREs), is enriched in dIgA and is relatively deficient in recycling transferrin (Apodaca *et al.*, 1994; Barroso and Sztul, 1994; Brown *et al.*, 2000). AREs are thought to play a unique role in transcytosis, possibly by exploiting apical recycling mechanisms to shuttle transcytotic cargo to the apical surface.

dIgA binding to pIgR on the basolateral surface stimulates transcytosis of rabbit pIgR expressed in MDCK cells (Song *et al.*, 1994) and in rat liver *in vivo* (Giffroy *et al.*, 1998). Ligand-mediated receptor dimerization (Singer and Mostov, 1998) might be required to elicit the subsequent intracellular signaling events contributing to the regulation of transcytosis. Within 10 s after dIgA binding, several cellular proteins (but not pIgR itself) become tyrosine-phosphorylated (Luton *et al.*, 1998). This early and transient event could be mediated by the recruitment of the nonreceptor tyrosine kinase p62yes (Luton *et al.*, 1999). The most important findings suggested that stimulation of transcytosis by dIgA requires pIgR sensitization by ligand binding at the basolateral surface and signal transduction to the apical cytoplasm (Luton and Mostov, 1999). Collectively, these observations argued that signaling events originating from the basolateral surface and/or basolateral early endosomes are essential for the stimulation of dIgA transcytosis.

A growing body of evidence indicates that a significant portion of the signal transduction pathways takes place via

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Abbreviations used: dIgA, dimeric IgA; pIgR, polymeric immunoglobulin receptor; MDCK, Madin Darby canine kidney; ZO1, zonula occludens-1; DRMs, detergent-resistant membranes; m β CD, methyl- β -cyclodextrin; m β CD/chol, m β CD saturated with cholesterol; Cav1, caveolin-1; SC, secretory component; AREs, apical recycling endosomes; hTfn, human transferrin; hTfnR, human transferrin receptor; pY14-Cav1, Cav1 phosphorylated on Tyr14; IP, immunoprecipitation.

plasma membrane receptors positioned at specialized microdomains, termed lipid rafts, which are enriched in cholesterol and glycosphingolipids (Moffett *et al.*, 2000; Simons and Toomre, 2000; Waugh *et al.*, 2001; Inoue *et al.*, 2002). Here we hypothesized that dIgA binding stimulates the insertion of pIgR into cholesterol-rich domains (lipid rafts) located on the basolateral surface and/or basolateral endosomes of polarized MDCK cells. This event could play a pivotal role in the sorting of dIgA-pIgR complexes into the transcytotic pathway. However, previous experiments that attempted to address this hypothesis failed to yield consistent results (Hansen *et al.*, 1999; Sarnataro *et al.*, 2000; De Marco *et al.*, 2002). In this study, we directly investigated the functional significance of membrane cholesterol in polarized traffic after endocytosis.

MATERIALS AND METHODS

Reagents

Methyl- β -cyclodextrin (m β CD, average substitution: 10.5–14.7 methyl groups per molecule, C-4555), its inactive analog α -cyclodextrin (α CD, C-4642), and cholesterol (C-8503) were from Sigma (St. Louis, MO). Iodine-125 (125 I) and [35 S]Cys/Met (PRO-MIX) were from Amersham Biosciences (Amersham, United Kingdom).

Proteins and Antibodies

Purified human dimeric IgA (dIgA) antibodies were manufactured by J.-P. Vaerman. Human apo-transferrin (hTfn; Biological Industries, Beit Haemek, Israel) was loaded with iron as described (Podbilewicz and Mellman, 1990). Anti-human transferrin receptor (hTfnR) antibody (H68.4), sheep anti-rabbit SC antibodies, and monoclonal SC166 antibody directed against the pIgR's cytoplasmic tail have been described elsewhere (Solari *et al.*, 1985; White *et al.*, 1992; Aroeti and Mostov, 1994). Fluorescein isothiocyanate (FITC)-labeled iron-loaded hTfn and AlexaFluor 594 donkey anti-rabbit and AlexaFluor 488 goat anti-mouse antibodies were from Molecular Probes (Eugene, OR). Different antibodies have been used for detecting caveolin-1 (Cav1). Rabbit anti-Cav1 (610059, BD Transduction Laboratories, Lexington, KY) was used for immunofluorescence analyses. Mouse anti-Cav1 clone 2297 (BD Transduction Laboratories) and rabbit anti-Cav1 (C-3237, Sigma, St. Louis, MO) were used for detecting Cav1 in Western blotting. Mouse anti-Cav1 (pY14), phospho-specific (clone 56) from BD Transduction Laboratories was used for detecting Cav1 phosphorylated on tyrosine 14 (pY14-Cav1). Monoclonal anti-calnexin antibodies were from StressGen Biotechnologies (San Diego, CA). The rat anti-zonula occludens-1 (ZO1) R40.76 antibodies (Dr. D. A. Goodenough, Harvard University, Cambridge, MA) were used to label MDCK tight-junctions. FITC-conjugated affinity-purified goat antibodies to human IgA were from Sigma. AffiniPure rabbit anti-human serum IgA and α -chain-specific antibodies were from Jackson ImmunoResearch Labs (West Grove, PA).

Cell Culture

MDCK and PTR-MDCK cells (MDCK coexpressing rabbit pIgR and hTfnR; Brown *et al.*, 2000; Wang *et al.*, 2000) were cultured routinely in 10-cm dishes, in minimal essential medium (MEM, Biological Industries) containing 5% fetal calf serum (Biological Industries) and 1% antibiotics (Biological Industries). To induce polarity, cells were cultured on polycarbonate filter supports (0.4- μ m pore size, Transwell, Costar, Cambridge, MA) for 4 d before each experiment (Aroeti *et al.*, 1993). For flotation experiments, fungicides were omitted from the growth medium for at least 24 h before the experiment. Cell monolayer integrity was estimated by monitoring the transepithelial electrical resistance, using a Millicel-ERS-system (Millipore, Bedford, MA), equipped with a silver/silver-chloride electrode. The value from a blank filter (with no cells, but otherwise treated identically) was subtracted from the resistance values of filters on which cells were plated.

Cholesterol Depletion and Enrichment

The relevance of cholesterol-rich microdomains in cellular processes is typically examined by altering cholesterol levels in cell membranes. The drug cyclodextrin specifically disrupts lipid rafts by chelating cholesterol from cells (Kilsdonk *et al.*, 1995; Yancey *et al.*, 1996). For acute cholesterol depletion of cellular membranes, filter-cultured PTR-MDCK cells were treated for different times, not longer than 60 min at 37°C with 10 mM m β CD, dissolved in MEM containing bovine serum albumin (MEM/BSA: MEM containing HBSS, 20 mM HEPES, pH 7.4, and 0.6% BSA). The agent was applied simultaneously into the apical and basolateral chambers of the Transwell support. m β CD saturated with cholesterol (m β CD/chol) was prepared as previously described (Grimmer *et al.*, 2000). Cholesterol enrichment of cellular membranes was achieved by treating the cells for 60 min at 37°C with 10 mM m β CD/chol

dissolved in MEM/Hanks' salts lacking BSA. Cellular cholesterol levels were determined for confluent cell cultures grown in 2 $\times$ 5-cm dishes. Cells were solubilized in SDS buffer (0.1% SDS, 1 mM EDTA, 0.1 M Tris-Cl, pH 7.4) at 37°C and cholesterol levels were determined using the Infinity cholesterol reagent kit (Sigma). Cholesterol content was normalized to the lysate's protein levels (determined by the microbicinchoninic acid reagent kit; Pierce, Rockford, IL). Typically, cell treatment with m β CD at 37°C reduced the cellular cholesterol content by 30–40% of the control. Cell exposure to m β CD/chol resulted in elevated cholesterol levels for up to ~60% of the control.

Flotation of Detergent-resistant Membranes

Detergent-resistant membranes (DRMs) were isolated as previously described (Brown and Rose, 1992) with some modifications (Naslavsky *et al.*, 1997). Cells cultured on 75-mm polycarbonate filters were lysed in the cold with ice-cold buffer containing 1% Triton X-100 (10 mM Tris, pH 7.5, 10 mM EDTA, 100 mM NaCl, and 1% Triton X-100) supplemented with protease and phosphatase inhibitors. Lysates were homogenized by 10 passages through a 25-gauge needle and subsequently adjusted to 35% Nycodenz (D-2158, Sigma) dissolved in ice-cold TNE buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). The solution was loaded at the bottom of a TLS-55 ultracentrifuge tube. An 8–35% Nycodenz linear step gradient was overlaid above the lysate-containing layer, and after centrifugation for 4 h at 55,000 rpm in a TLS-55 Beckman rotor (Fullerton, CA) at 4°C, 12 180- μ l fractions were collected from the top. Proteins in each fraction were precipitated by methanol/chloroform and analyzed by SDS-PAGE and Western blotting. A third of the protein quantity in each of fractions 9–12 was analyzed. The partitioning of a given protein with DRMs and detergent-soluble fractions was based on comparing its flotation profile with the classical lipid raft protein Cav1 and the nonraft marker calnexin, respectively. Cav1 was typically distributed within fractions 1–6 [designated DRMs], whereas calnexin was distributed mainly in fractions 7–12, representing the detergent soluble lysate (see Figure 1A).

Detection of pIgR in DRMs at the Basolateral Surface

Seventy-five-mm filter-cultured PTR-MDCK cells were either exposed to 300 μ g/ml purified human dIgA introduced at the basolateral side or remained untreated. After two washings with cold MEM/BSA and subsequently with PBS to remove unbound ligand, the basolateral surface was treated with 1-mg/ml EZ-Link Sulfo-NHS-LC biotin (dissolved in ice-cold PBS; Pierce) for 1 h on ice. After quenching of residual biotin with 50 mM glycine in MEM/BSA, cells were lysed in buffer containing Triton X-100 and subjected to flotation of DRMs as previously described. The pIgR in each fraction was immunoprecipitated using monoclonal antibodies (SC166) directed against its cytoplasmic tail. SDS-PAGE and Western blotting were used to analyze the immunoprecipitated proteins. For detecting biotinylated pIgR, nitrocellulose membranes were blocked with BSA and initially probed with streptavidin-horseradish peroxidase (HRP; S-2438, Sigma; 1:1000 dilution of 1.1 mg/ml protein stock dissolved in PBS/Tween 20). Reprobing the membranes with SC166 antibodies enabled us to detect the total amount of immunoprecipitated pIgR.

Detection of hTfnRs in Basolateral DRMs

The basolateral surface of 75-mm filter-grown PTR-MDCK cells was biotinylated in the cold as described above. In some experiments, biotinylated surface proteins were allowed to internalize by incubating the cells at 37°C for various times. After quenching of residual biotin, cells were lysed in buffer containing Triton X-100. A flotation assay for isolation of DRMs was performed, and biotinylated proteins in each fraction were pulled down with streptavidin-agarose beads (S-1638, Sigma). After analysis by SDS-PAGE and Western blotting, hTfnR was detected by probing the nitrocellulose membranes with H68.4 monoclonal antibodies directed against its cytoplasmic domain.

Immunofluorescence Analysis

Cells were fixed with 4% paraformaldehyde in PBS, pH 8.0 (10 min at 4°C followed by 20 min at 22°C); the remaining active sites were quenched with 75 mM NH₄Cl + 20 mM glycine in PBS, pH 8.0, and cells were subsequently processed for immunofluorescence and confocal microscopy, as previously described (Orzech *et al.*, 1999). For immunolabeling of pY14-Cav1, cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, pH 8.0, after fixation, and subsequent steps were performed as above.

Confocal images were acquired either with a Bio-Rad MRC1024 (Richmond, CA) coupled to a Zeiss Axiovert 135M (Thornwood, NY), or on an Olympus FV1000 (Melville, NY). With either system, two excitation lasers were used sequentially: 488 nm for the FITC or Alexa488 and either 543 nm (FV1000) or 594 nm (MRC1024) for the Alexa594. A narrow-band emission filter (505–525 nm for the FV1000, 505–545 nm for the MRC1024) was used in the FITC channel. Sequential frame acquisition was used to eliminate cross-talk between the two fluorescence channels. Colocalization was characterized using the method of Lachmanovich *et al.* (2003). Briefly, the peaks were segmented using a local threshold set at half the maximum value of the local peak. Objects were considered colocalized if the center of mass of one class fell within

Figure 1. dIgA uptake stimulates the incorporation of pIgRs into DRMs. (A) Association of pIgR with DRMs: The basolateral surface of PTR-MDCK cells was exposed to human dIgA (300 μ g/ml) for the specified times or left untreated. For cholesterol depletion, cells were treated with mβCD for 30 min and subsequently allowed to bind dIgA for 1 min at 37°C. Cells were lysed in ice-cold 1% Triton X-100 and processed for DRM flotation analysis as described in *Materials and Methods*. Proteins were precipitated and analyzed by Western blotting using SC166 anti-pIgR antibodies or sheep anti-SC polyclonal antibodies. The location of fractions containing DRMs and detergent-soluble proteins is indicated. In the left panel representative gels are shown. In the right panel, the band intensity of each fraction was assessed, and the sum of fractions 1–6 (DRMs) was normalized to the sum of intensities contributed by all bands. The results represent the mean of two to four independent experiments. Individual results did not deviate more than 15% from the mean. (B) Association of pIgR with DRMs on the basolateral plasma membrane. The basolateral surface of PTR-MDCK cells was exposed to dIgA for 3 min at 37°C or left untreated. Proteins at that surface were exclusively biotinylated in the cold. Cells were then lysed and subjected to DRM flotation analysis. The entire pIgR population was immunoprecipitated (IP) from each fraction with SC166 anti-pIgR antibodies and analyzed by immunoblotting (IB) using the same antibodies. Biotinylated pIgR was detected by probing the nitrocellulose membranes with Streptavidin-HRP.

the area of the second. Results were compared with a binomial model, and randomized images were used to verify that random distributions of staining produced the expected value as predicted by the binomial distribution.

It should be stressed that the colocalization measurement is only meaningful to the extent that the result differs significantly from that which would be expected from a random distribution of the fluorescent objects.

Radio-iodination of Ligands

Iron-saturated hTfn and dIgA were radioiodinated to a specific activity of $5\text{--}9 \times 10^6$ cpm/ μ g using the iodine monochloride method (Breitfeld *et al.*, 1989).

Trafficking Assays

Analysis of Endocytosis. Endocytosis of radioiodinated ligands was measured as previously described (Okamoto *et al.*, 1992). A thiol-cleavable biotin compound (sulfo-NHS-SS-biotin, Pierce) was used as an alternative approach to determine internalization of unoccupied receptors (Okamoto *et al.*, 1994).

Analysis of ^{125}I -hTfn Recycling and Transcytosis. The fate of a single cohort of basolaterally internalized ^{125}I -hTfn was monitored principally as described (Gan *et al.*, 2002). Briefly, ^{125}I -hTfn was internalized from the basolateral side of 12-mm filters at 37°C. After several washes at 17°C with MEM/BSA, cells were treated for 30 min at 37°C with cyclodextrin. In some experiments, cholesterol depletion preceded the ligand internalization step. Surface-bound ligand was stripped off with a buffer containing 50 mM MES, pH 5.0, and 200 mM NaCl for 2 min. Cells were then incubated at 37°C with plain MEM/BSA containing 3 μ g/ml unlabeled hTfn and 100- μ M deferoxamine mesylate (an iron chelator, D-9533, Sigma). Radioactivity was measured using a γ -counter, as described (Gan *et al.*, 2002).

Analysis of ^{125}I -dIgA Recycling and Transcytosis. Transcytosis of ^{125}I -dIgA was monitored as previously described (Brown *et al.*, 2000), with some modifications. To ensure a follow-up of ^{125}I -dIgA traffic from endosomes, the ligand was internalized at 37°C. Unbound ligand was removed by washing at 17°C. Cells were then exposed to mβCD for 30 min at 37°C. Surface-associated ligand was removed by treating the cells with the MES, pH 5.0/NaCl buffer for 60 min at 4°C, and the ligand was chased in MEM/BSA at 37°C. In some cases, cell treatment with mβCD preceded the ligand internalization step.

Radioactivity was measured using a γ -counter, as described (Aroeti and Mostov, 1994).

Analysis of Biotinylated pIgR Transcytosis. Constitutive transcytosis of the empty pIgR was monitored as described (Aroeti and Mostov, 1994). Cells grown on 24-mm filters were metabolically labeled with [35 S]Cys/Met for 45 min at 37°C. Proteins at the basolateral surface were biotinylated at 37°C (0.1-mg/ml sulfo-NHS-LC biotin). In some experiments, cyclodextrin treatment (incubation for 30 min at 37°C in MEM/BSA containing m β CD) preceded the cell surface biotinylation step, whereas in other experiments cyclodextrin treatment was applied after biotinylation. Receptor traffic was promoted by further incubating the cells for 30 or 60 min at 37°C in MEM/BSA. Biotinylated pIgR and SC were immunoprecipitated (IPed) from filters and media, respectively. The IPed material was eluted by resuspending the immunobeads in 10% SDS and heating for 3 min at 95°C. Biotinylated pIgR and SC were recovered from the eluate using streptavidin-coated agarose beads. Radioactive proteins were analyzed by SDS-PAGE. Protein bands were visualized and quantified by the Fuji FLA 3000 apparatus (Tokyo, Japan).

Analysis of Biotinylated TfnR Transcytosis. Transcytosis of the empty TfnR was monitored essentially as described (Burgos *et al.*, 2004). PTR or parental MDCK cells grown on 24-mm filters were treated (or not) with m β CD for 60 min; thereafter, the basolateral surface was biotinylated in the cold using the cleavable sulfo-NHS-SS-biotin (0.1-mg/ml). After quenching of residual biotin, cells were incubated in MEM/BSA for 60 min at 37°C to promote trafficking. At the end of the incubation period, the apical surface of cells cultured on one set of filters was treated with reducing L-glutathione in the cold. After quenching of free SH-groups with 5-mg/ml iodoacetamide, cells were lysed in solution containing 1% Triton X-100. Streptavidin agarose-coated beads were used to precipitate biotinylated proteins in the cold, which were then analyzed by 10% SDS-PAGE. Biotinylated TfnRs were detected by Western blotting using the H68.4 monoclonal antibodies and the Fuji LAS 3000 machine was used to assess the protein band intensity. Augmented transcytosis results in the exposure of a larger fraction of biotinylated receptors at the apical surface, leading to their efficient stripping by the apical-selective glutathione treatment and consequently to a reduced signal contributed by biotinylated receptors.

Analysis of Cav1 Tyrosine 14 Phosphorylation

PTR-MDCK cells were grown on 24-mm filters in medium lacking fungicides. In some experiments, the cell's cholesterol levels were manipulated by treating with cyclodextrin and the levels of pY14-Cav1 in lysates were determined by quantitative Western blotting. In other experiments (see Figure 7B for details), pY14-Cav1 levels were quantified in cells exposed to m β CD and internalizing ligands. At the end of each manipulation, cells were quickly washed in ice-cold phosphate-buffered saline (PBS) pH 7.4, and subsequently lysed in the cold in 100 μ l of buffer containing 20 mM HEPES, pH 7.4, 125 mM NaCl, and 1% NP-40 supplemented with protease and phosphatase inhibitors. After a 10-min top speed centrifugation in the cold, the protein in each sample was quantified using the Bradford Reagent (Sigma). Equal protein levels (typically 30- μ g) were loaded onto 13% SDS-PAGE, and Cav1 phosphorylated on tyrosine 14 was detected on Western blots, using the anti-pY14 Cav1 monoclonal antibodies.

Coimmunoprecipitation of Cav1 with pIgR

The protocol was basically adopted from other studies that established the coimmunoprecipitation (co-IP) conditions for Cav1 (Lu *et al.*, 2001). To ensure the identification of interacting Cav1, we used a two-step IP protocol. In the first step, pIgR was IPed from two parallel samples under native conditions. Confluent PTR-MDCK cells cultured on two 10-cm dishes, in the absence of fungicides for 24 h, were washed with cold PBS, pH 7.4, and subsequently lysed in the cold in 1-ml RIPA buffer (50 mM Tris, pH 7.4, 135 mM NaCl, 1% [vol/vol] Triton X-100, and 60 mM octylglucoside) supplemented with protease and phosphatase inhibitors. Lysates were homogenized by 10 passages through a 21-gauge needle, and after a 30-min 12,000 $\times$ g centrifugation in the cold, the supernatants were divided into two identical fractions containing ~1 mg protein each. Equal amounts of sheep anti-SC antibodies bound to protein A-Sepharose beads were introduced into the two lysates, and the mixtures were incubated for 60 min at 4°C, with end-to-end rotation. At the end of the incubation period, proteins bound to the beads were washed five times with RIPA buffer and released by boiling in 50 μ l of 10% SDS. Nine hundred fifty microliters of 2.5% Triton dilution buffer (100 mM triethanolamine chloride, pH 8.6, 100 mM NaCl, 5 mM EDTA, 0.025% NaN₃, 2.5% [wt/vol] Triton X-100) was added to the SDS solution. Cav1 was IPed for 16 h at 4°C from the released proteins, using specific anti-Cav1 antibodies, or irrelevant IgG coupled to protein A-Sepharose. IPed pIgR and co-IPed Cav1 were detected using SC166 and rabbit anti-Cav1 antibodies, respectively.

RESULTS

Because cholesterol is an important constituent of cell membranes and lipid rafts, it was important to establish condi-

tions whereby acute cholesterol depletion or enrichment did not compromise the integrity of the epithelial monolayer. Cholesterol depletion or enrichment did not alter the distribution of the tight junctional ZO1 protein (Supplementary Figure S1A), nor did they affect the transepithelial electrical resistance values of MDCK monolayers (Supplementary Figure S1B). Because a major focus of our studies was to examine the effects of cholesterol depletion on membrane trafficking after endocytosis, it was important to examine whether m β CD treatment affects the structure of endosomes. Confocal imaging of hTfnR, a common marker of endosomes, revealed no apparent structural changes in the shape and/or intracellular distribution of endosomes in m β CD-treated MDCK cells (Supplementary Figure S1C). These measurements suggest that the integrity of MDCK cell monolayers and endosomal morphology subjected to manipulations in membrane cholesterol levels remained largely intact.

dIgA Binding Stimulates the Incorporation of pIgR into DRMs

We hypothesized that dIgA binding to pIgR increases the receptor's abundance in rafts. To investigate this hypothesis, we used filter cultured PTR-MDCK cells exposed to dIgA from the basolateral surface and assessed the presence of pIgR in floating DRMs, which biochemically may define cholesterol-sensitive membrane domains enriched with signaling factors (Foster *et al.*, 2003). Western blots and their quantification are presented in Figure 1A, left and right panels, respectively. Although minimal levels of pIgR existed in DRMs before dIgA binding (Figure 1Aa), a greater quantity of the receptor shifted into DRMs upon 1 min of basolateral uptake of the ligand (Figure 1Ab). The shift to fractions with lighter buoyancy was further increased, reaching fractions 1 and 2, when dIgA was first internalized for 1 min and subsequently chased for 3 min in the absence of ligand (Figure 1Ac). However, after 1 min of ligand uptake and a 15-min chase (conditions leading to dIgA accumulation in AREs), the pIgR signal in the floating fractions diminished, but not to basal levels of untreated cells (Figure 1Ad). Cell treatment with m β CD before dIgA uptake (for 1 min) eliminated the ability of dIgA to relocate its receptor into the lower density fractions (Figure 1Ae).

It was previously shown that a pIgR mutant lacking the carboxy-terminal 30 amino acids, pIgR-725t, is defective in dIgA transcytosis, and consequently most of the ligand is recycled. This region of pIgR's cytoplasmic tail was also shown to be required for dIgA-induced tyrosine kinase activation (Luton *et al.*, 1998). In MDCK cells expressing pIgR-725t, low levels of mutant receptors occurred in DRMs, comparable to those observed for pIgR alone (Figure 1Af). Challenging these cells with dIgA for 1 min resulted in only a slight increase in DRM association of mutant receptors (Figure 1Ag). In these experiments, the flotation profiles of Cav1 and calnexin (Figure 1A, h-j) served as controls for DRM-associated and detergent-soluble proteins, respectively. Notably, consistent with previous reports (Foster *et al.*, 2003), the distribution of Cav1 in DRMs was not susceptible to cholesterol depletion (Figure 1A, compare lanes h and i). This phenomenon may be attributed to structural properties that affect Cav1 interactions with cholesterol and DRMs.

Interestingly, the observation that pIgR is incorporated into DRMs after a brief exposure to the ligand suggests that these DRMs are located on the basolateral surface and juxtaposed endosomes. To further address this point, we investigated whether dIgA-pIgR complexes are associated with DRMs originating from the basolateral plasma membrane. To this end, dIgA was taken up continuously for 3 min from

the basolateral side, and proteins at the basolateral surface were selectively biotinylated in the cold. Cells were solubilized in Triton X-100 and processed for flotation analysis, as before. A fraction of biotinylated pIgR associated with DRMs could be observed only in cells exposed to dIgA (Figure 1B). Taken together, the results so far indicate that dIgA binding to pIgR on the basolateral surface prompts receptor insertion into DRMs located on that surface. Association with DRMs may be pursued, but to a lesser extent in endosomes. We also concluded that the receptor's last 30 amino acids are required for dIgA-pIgR to be associated with DRMs.

Acute Cholesterol Depletion before Ligand Uptake Depolarizes the Postendocytic Transport of dIgA

The notion that dIgA-pIgR complexes enter DRMs at the basolateral surface/early endosomes suggests that cholesterol-sensitive mechanisms located at these compartments play a role in their trafficking after endocytosis. To address this hypothesis, we analyzed the endocytic and polarized postendocytic traffic of dIgA in cells whose plasma membrane cholesterol levels had been reduced. In these experiments, PTR-MDCK cells were treated for 15, 30, and 60 min at 37°C with m β CD. The agent was washed out, and ¹²⁵I-dIgA was internalized from the basolateral surface. Cell surface-associated ligands were removed and trafficking from endosomes to the basolateral and apical poles of the cell was monitored over time. m β CD treatment for 15 min slightly facilitated the kinetics of dIgA transcytosis (Figure 2A, top panel), whereas basolateral recycling of the ligand remained unaffected (Figure 2A, bottom panel). In contrast, m β CD treatment for 30 or 60 min significantly diminished dIgA transcytosis, which was accompanied by a corresponding increase in basolateral recycling. Treatment with α CD had only a minor effect on transcytosis. When cholesterol was first depleted and subsequently replenished (+m β CD+m β CD/chol), dIgA transcytosis and recycling were restored almost completely to levels initially observed for untreated cells.

Acute cholesterol depletion perturbs the formation and function of clathrin-coated pits (Rodal *et al.*, 1999; Subtil *et al.*, 1999), shown to mediate pIgR endocytosis (Okamoto *et al.*, 1992). Thus, it was reasonable to assume that cholesterol depletion inhibits dIgA-pIgR internalization. Indeed, m β CD treatment conferred partial inhibition of dIgA internalization, but the inhibitory effect was instantly restored upon cholesterol replenishment (Figure 2B). Notably, whereas treatment with m β CD for 15 min diminished dIgA endocytosis, the same treatment did not decrease the transcytosis of dIgA (Figure 2A), suggesting that the two processes are not necessarily coupled. Concurrent with this notion are findings indicating that transcytosis of biotinylated empty receptors internalized via a cholesterol-depleted plasma membrane was similar to that of untreated cells (Figure 2C, left panel), despite the profound inhibitory effect that m β CD has on biotinylated pIgR internalization (Figure 2C, right panel). These findings were surprising because inhibition of endocytosis should have reduced the levels of subsequent transcytosis. However, as shown below, the kinetics of receptor transcytosis from endosomes is stimulated in response to cholesterol depletion, a phenomenon that may compensate for the reduced endocytic activity.

A Larger Fraction of the Human, Rather Than the Canine, TfnRs Occupies DRMs

Although TfnRs are canonically thought to be excluded from DRMs, recent evidence seems to support a possible link between TfnRs and lipid rafts: 1) recycling endosomes en-

riched with Tfn immunisolated from MDCK cells contained caveolins and other raft components (Gagescu *et al.*, 2000); 2) the folate receptor (a glycosylphosphatidylinositol-linked protein), which typically clusters in caveolae, has been found in endosomes containing Tfn (Mayor *et al.*, 1998); 3) recycling endosomes rich in TfnRs were localized in proximity to centrosome-associated caveosomes (Maxfield and Yamashiro, 1987; Mundy *et al.*, 2002); and 4) a fraction of TfnRs recruited under certain conditions into immunological synapses was reported to be associated with DRMs (Batista *et al.*, 2004).

Prompted by these observations, we took a closer look into the possible link between hTfnRs and DRMs in polarized PTR-MDCK cells. Flotation analyses clearly revealed the association of a fraction of hTfnRs with DRMs (Figure 3A, top panel). In contrast with pIgR and dIgA, uptake of the ligand did not significantly alter the flotation profile of the receptor, whereas cell treatment with m β CD considerably shifted its distribution toward denser fractions.

PTR-MDCK cells express hTfnR at about a 5–7-fold higher level than the endogenous canine receptor (cTfnR) in parental MDCK cells. Increased receptor concentrations on the cell surface can augment receptor association with rafts, either directly, or indirectly via interactions with accessory proteins recruited into rafts. In agreement with this prediction are quantitative analyses basically showing that although ~15% of the total hTfnR population was associated with DRMs, only ~4% of the cTfnR occupied these fractions (Figure 3A, bottom panel).

Next, we analyzed the possibility that hTfnRs in DRMs originate at the basolateral surface. Briefly, the basolateral cell surface was biotinylated in the cold, solubilized in cold detergent, and immediately subjected to membrane flotation analysis. Probing with anti-hTfnR antibodies revealed a fraction of biotinylated receptors in DRMs (Figure 3B). Association with DRMs was slightly diminished upon receptor internalization for 5 min, and no detectable signal was observed when receptor endocytosis was extended for 15 min. These results suggest that, like in the case of dIgA bound to pIgR, a selected population of hTfnRs occupies DRMs on the basolateral surface, and their capacity to reside in DRMs is rapidly reduced upon internalization.

Cholesterol Depletion before Receptor/Ligand Internalization Depolarizes the Postendocytic Traffic of the Human But Not the Canine TfnR

Our next goal was to investigate whether these differences in DRM occupancy correlate with a differential response of TfnR's postendocytic traffic to acute cholesterol depletion. As expected from previous work (Rodal *et al.*, 1999; Subtil *et al.*, 1999), acute cholesterol depletion inhibited the endocytosis of both receptor types (Figure 4A, top panel). Internalization of ¹²⁵I-hTfn was also inhibited by m β CD treatment and was partially rescued upon subsequent cholesterol enrichment (Figure 4A, bottom panel). TfnR's transcytosis was examined by a biotinylation-based assay, schematically depicted in Figure 4B, top panel. About a 30% reduction in the signal contributed by biotinylated hTfnRs in m β CD-treated cells was observed, indicating that 30% of the biotinylated receptor population transcytosed to the apical surface. Cholesterol enrichment (+m β CD/chol) of membranes had no effect on hTfnR transcytosis. Under the same experimental conditions, biotinylated canine receptors were not missorted to the apical surface of m β CD-treated cells.

Augmented transcytosis of hTfnR was also detected by following the postendocytic fate of its radiolabeled ligand. In untreated cells, the majority (~90%) of internalized ligand

Figure 2. The postendocytic traffic of dIgA depolarizes in cells subjected to mβCD treatment before ligand internalization. (A) Fate of ¹²⁵I-dIgA. Filter-cultured PTR-MDCK cells were treated with mβCD, or with αCD for the indicated times, or treated with mβCD for 60 min and subsequently exposed to mβCD/chol for 60 min at 37°C (+mβCD+mβCD/chol). Untreated cells were incubated with plain MEM/BSA. ¹²⁵I-dIgA (4 μg/ml) was internalized from the basolateral surface for 15 min, surface bound ligand was removed and its transport to the basolateral (recycling) and apical (transcytosis) poles of the cell was monitored over time. Results represent the mean ± SE of three independent experiments, each of which was performed in duplicate. (B) Endocytosis of ¹²⁵I-dIgA. Cells were first treated with cyclodextrin for the indicated times and then exposed basolaterally to ¹²⁵I-dIgA (4 μg/ml) for 60 min at 4°C. Ligand internalization was determined after 5 min of uptake at 37°C, as described in *Materials and Methods*. Results represent the mean ± SE of three independent measurements. (C) Constitutive transcytosis of empty pIgR. Left, a biotinylation-based assay was applied for monitoring the constitutive transcytotic pathway of the pIgR, as described in *Materials and Methods*. For measuring the fate of empty pIgR internalized through a cholesterol-depleted plasma membrane, mβCD treatment preceded the cell surface biotinylation step. The results are expressed as the percentage of biotinylated SC released to the apical (Ap) or basolateral (Bl) media, and represent the mean of three independent experiments. Right, internalization of pIgR biotinylated at the basolateral surface, after 5 min of incubation at 37°C, was measured in untreated versus mβCD-treated cells as described in *Materials and Methods*.

is recycled to the basolateral surface, whereas ~5% is transcytosed to the apical domain. Clearly, acute cholesterol depletion resulted in an increase in basolateral-to-apical transcytosis and a parallel decrease in basolateral recycling of ¹²⁵I-hTfn (Figure 4C). The effect was entirely reversible,

because transcytosis and recycling of the ligand were restored to nearly the control values upon cholesterol replenishment. Cell treatment with αCD had slightly attenuated only the kinetics of receptor recycling. These data principally support the possibility that disrupting the structure

Figure 3. DRM association of canine versus human Tfns. (A) Top, flotation of total Tfns. PTR-MDCK cells overexpressing the hTfnR were either left untreated, exposed to hTfn (300-μg/ml) from their basolateral side for 1 min, or treated with mβCD. Parental MDCK cells expressing the endogenous cTfnR were left untreated. Cells were solubilized in an ice-cold 1% Triton X-100 containing buffer and lysates were subjected to DRM flotation analysis, as in Figure 1A. Bottom, quantitative analysis of DRM association. Band intensity contributed by Tfns in pooled fractions 1–6 (DRM) and 7–12 (soluble lysate) was determined by quantitative immunoblotting. The percentage of receptor associated with the DRM fractions is presented. (B) Flotation of biotinylated (cell surface) hTfnRs. The basolateral surface of PTR-MDCK cells was biotinylated at 4°C. Cells were either left untreated or were incubated for 5 or 15 min at 37°C to allow internalization of biotinylated proteins. Next, cells were washed with ice-cold buffer, solubilized in 1% Triton X-100, and subjected to DRM flotation, as before. Biotinylated proteins in each fraction were precipitated using streptavidin-agarose beads and then subjected to SDS-PAGE followed by Western blotting analysis. Finally, biotinylated hTfnRs were detected with anti-hTfnR antibodies.

and function of rafts disturbs cholesterol-sensitive mechanisms that mediate polarized postendocytic trafficking.

Acute Cholesterol Depletion after Ligand Uptake Stimulates the Kinetics of Receptor Transcytosis

The observation that DRM association is diminished (but not eliminated) upon internalization suggested that cargo molecules might encounter a different cholesterol-sensitive environment in endosomes than in the plasma membrane. The next set of experiments was designed to examine the effects of cholesterol depletion on polarized postendocytic trafficking of cargo molecules whose internalization occurred via intact plasma membrane rafts. For that purpose, ligands were first internalized, and immediately thereafter, cells were subjected to acute cholesterol depletion. Surface-bound ligand was stripped off and polarized postendocytic transport was assessed. We observed that the rate of ^{125}I -dIgA

transcytosis was considerably enhanced in the cholesterol-depleted cells (Figure 5A, top). The final levels of dIgA recycling, although doubled in mβCD-treated cells (Figure 5A, bottom), remained sufficiently low enough to preserve polarized transport of the ligand to the apical domain. A similar stimulatory effect on the kinetics of transcytosis was observed for the constitutive pathway of biotinylated pIgR (Figure 5B). As in the case of ^{125}I -dIgA, the amount of biotinylated SC released to the basolateral medium was considerably higher in the cholesterol-depleted than in untreated cells (Figure 5B).

Our analysis of pIgR transcytosis and recycling is based on an assay that measures SC release. A concern was raised about the ability of cholesterol depletion to stimulate the activity of cellular proteinases, including those that mediate pIgR proteolysis to SC, rather than having just a direct effect on pIgR traffic. In an attempt to address this potential difficulty, we performed a pulse-chase experiment that relies on SC release for monitoring the biosynthetic transport of pIgR mutant whose basolateral-sorting motif has been deleted in frame (pIgR-ΔR655-Y668; Casanova *et al.*, 1991). The results presented in Figure 5C indicate that compared with untreated cells, the extent of metabolically labeled SC released to the apical medium (contributed by biosynthetic delivery of pIgR to the apical surface) was not significantly different in mβCD-treated cells. In contrast, the release of radiolabeled SC to the basolateral medium was markedly augmented in these cells. These results suggest that cholesterol depletion does not have a broad stimulatory effect on the apical release of SC. However, they do not preclude the possible effect of cholesterol depletion on the efficiency of pIgR cleavage to SC on the basolateral plasma membrane.

Acute cholesterol depletion after ligand uptake had no detectable effect on basolateral recycling of ^{125}I -hTfn (Figure 5D, bottom). However, the same treatment caused a twofold increase in hTfn transcytosis (Figure 5D, top), reinforcing the hypothesis that acute cholesterol depletion facilitates transcytosis. The fact that transcytosis was augmented kinetically in one case (pIgR) and elevated its final levels in another case (hTfn) suggests that cholesterol-sensitive pathways utilized by these receptors may share common mechanisms that differ in at least one mechanistic step.

Cholesterol Enrichment Selectively Inhibits Transcytosis of dIgA

Because cholesterol depletion stimulates transcytosis of receptors internalized via undisturbed rafts, we reasoned that cholesterol enrichment would produce the opposite effect. Cholesterol enrichment by cell treatment with the mβCD/chol complex neither affected the ability of dIgA to stimulate the association of pIgR with DRMs (Figure 6A), nor the capacity of ^{125}I -dIgA to undergo endocytosis (Figure 6B, inset) and recycling (Figure 6B, bottom panel). It did, however, have a significant inhibitory effect on basolateral-to-apical transcytosis of ^{125}I -dIgA (Figure 6B, top panel). Interestingly, the same treatment neither affected the trafficking of ^{125}I -hTfn (Figure 6C), nor the transcytotic pathway of biotinylated pIgR (data not shown). The selective inhibitory effect may further signify cholesterol as a negative regulator of dIgA-pIgR transcytosis.

Ligand Uptake before mβCD Treatment Stimulates Cav1 Phosphorylation on Tyrosine 14

To investigate further the stimulation of transcytosis in cholesterol-depleted cells, it was reasonable to assume that signaling mechanisms sensing the rapid reduction in plasma

Figure 4. Acute cholesterol depletion augments final transcytosis levels of human but not of canine TfR. (A) Endocytosis of TfRs. Top, PTR or parental MDCK cells were treated, or not, with mβCD for 60 min at 37°C. Internalization of unoccupied human and canine TfRs for 5 min was determined by a biotinylation-based procedure, as described in *Materials and Methods*. Bottom, internalization of the ligand ¹²⁵I-hTfR was measured as described for dIgA in Figure 2B. (B) Transcytosis of biotinylated TfRs. A biotinylation-based assay was used to monitor transcytosis of unoccupied human and canine TfRs. Top, the outline of the assay; middle, immunoblots of a representative experiment; bottom, the quantitative analysis of three independent experiments. The cholesterol-depleted basolateral surface of parental or PTR-MDCK cells was biotinylated with the cleavable sulfo-NHS-SS-biotin at 4°C. The temperature was shifted to 37°C for 60 min to allow internalization and subsequent trafficking into endocytic compartments. Then, biotin-labeled receptors arriving at the apical surface (via transcytosis) were subjected to stripping by exposure to reducing glutathione. The level of biotin-TfRs recovered from the glutathione-treated cells (TfR-biotin in glutathione-treated cells) was normalized to the protein level contributed by the entire biotinylated receptor population pulled down from parallel cell cultures whose surface biotin had not been removed (TfR-biotin-total). The signal in cyclodextrin-treated cells was further normalized to that of untreated cells. (C) ¹²⁵I-hTfR transcytosis and recycling. Cells were treated first with cyclodextrin for the indicated

times, and thereafter ¹²⁵I-hTfR (4 μg/ml) was internalized for 15 min at 37°C from the basolateral side. Plasma membrane-bound ligand was removed, and its polarized fate from endosomes was monitored as described in *Materials and Methods*. Results represent the mean ± SE of three independent experiments, each of which was performed in duplicates.

membrane cholesterol were activated. Previous reports have linked fluctuation of membrane cholesterol levels to the activation of signaling pathways associated with caveolins (Ikonen and Parton, 2000). They also demonstrated that Cav1 is phosphorylated on tyrosine 14 (pY14-Cav1) by the oncogenic v-Src kinase (Li *et al.*, 1996) and by normal cellular Src kinases (Lee *et al.*, 2001; Kim *et al.*, 2002), including c-Yes (Lee *et al.*, 2001), a kinase previously shown to be involved in the stimulation of dIgA transcytosis (Luton *et al.*, 1999). The proximity between intracellular caveolar structures and re-

cycling endosomes (Gagescu *et al.*, 2000; Mundy *et al.*, 2002) led us to assume that a cross-talk between postendocytic membrane traffic pathways and intracellular Cav1 may be established in cholesterol-depleted cells. The result of this process could be the overall facilitation of signal transduction pathways relevant for stimulation of transcytosis.

Using specific anti-pY14-Cav1 antibodies, combined with quantitative Western blotting analysis, we demonstrated that Cav1 phosphorylation on tyrosine 14 is markedly augmented in mβCD-treated cells, whereas subsequent cholesterol replen-

Figure 5. Transcytosis is facilitated in cells subjected to ligand internalization before cyclodextrin treatment. (A) ^{125}I -dIgA transcytosis and recycling. ^{125}I -dIgA was internalized from the basolateral surface for 30 min at 37°C . Cells were rapidly washed with MEM/BSA and then treated with $\text{m}\beta\text{CD}$ for 30 min at 37°C . Surface-bound ligand was removed and its polarized postendocytic transport was determined as in *Materials and Methods*. Results represent the mean $\pm$ SE of four independent measurements. (B) Transcytosis and recycling of biotinylated pIgR. A biotinylation-based assay was applied for monitoring the constitutive transcytotic pathway of pIgR, as described in *Materials and Methods*. In these experiments, $\text{m}\beta\text{CD}$ treatment followed the cell surface biotinylation step, as outlined in the top panel. pIgR and SC were immunoprecipitated from filters and media, respectively. Results are expressed as the percentage of total biotinylated SC released into the apical (due to transcytosis) or basolateral (due to recycling) media. Results represent the mean $\pm$ SE of three independent experiments. (C) Biosynthetic delivery of pIgR- $\Delta\text{R655-Y668}$. Filter-cultured cells expressing the pIgR mutant were subjected to 15-min pulse with ^{35}S Cys/Met, $\text{m}\beta\text{CD}$ treatment (or left untreated) and were then chased for 30 min in medium lacking radioactive amino acids. SC and pIgR were IPed from media and cells, respectively, as previously described (Aroeti *et al.*, 1993). The fraction of SC released to the apical and basolateral media was calculated after band quantification by phosphorimaging. (D) ^{125}I -hTfn transcytosis and recycling. ^{125}I -hTfn was internalized for 30 min from the basolateral surface and cells were subsequently treated with $\text{m}\beta\text{CD}$ as described for dIgA. The postendocytic transport of ^{125}I -hTfn was determined as in *Materials and Methods*. Results represent the mean $\pm$ SE of three independent measurements.

ishment reduced the phosphorylation of Cav1 to nearly baseline levels. Treatment with αCD had no effect, however (Figure 7A). These results indicate that Cav1 phosphorylation on tyrosine 14 responds reversibly to acute alterations in cellular cholesterol levels. Interestingly, phosphorylation levels of this residue were further elevated when cells had first been allowed to internalize dIgA and were subsequently treated with $\text{m}\beta\text{CD}$

(Figure 7B; +dIgA + $\text{m}\beta\text{CD}$). When $\text{m}\beta\text{CD}$ treatment preceded ligand internalization (+ $\text{m}\beta\text{CD}$ + dIgA), phosphorylation levels of Cav1 resembled those observed in cells treated only with $\text{m}\beta\text{CD}$. Internalization of hTfn and subsequent cholesterol depletion did not appreciably stimulate Cav1 phosphorylation to levels that are higher than those observed for $\text{m}\beta\text{CD}$ treatment alone (+hTfn + $\text{m}\beta\text{CD}$). Ligands internalized into untreated

Figure 6. Cholesterol enrichment selectively inhibits transcytosis of dIgA. (A) pIgR association with DRMs. Cells were first treated with mβCD/chol for 60 min and then exposed to dIgA for 1 min at 37°C. Flotation analysis was performed as in Figure 1A. (B) ¹²⁵I-dIgA transcytosis and recycling. Cells were treated with mβCD/chol for 60 min and then subjected to ligand internalization for 15 min at 37°C. Polarized postendocytic fate, or 5 min endocytosis (inset) of ¹²⁵I-dIgA was monitored as described. (C) ¹²⁵I-hTfn transcytosis and recycling. Cells were treated with mβCD/chol and

cells (+dIgA or +hTfn) had only a minor stimulatory effect on Cav1 phosphorylation.

Cav1 Codistributes with dIgA-pIgR and Rab25-positive Clusters in Apical Endosomes

The possible cross-communication between dIgA and Cav1 was investigated at the morphological level. dIgA or hTfn was internalized from the basolateral surface of the PTR-MDCK cells and subsequently chased at 37°C. Ligands and Cav1 were then subjected to an indirect double immunostaining procedure. The confocal images (Figure 8A) unambiguously show that anti-Cav1 antibodies labeled apical cap-like structures, some of which were concentrated in dense foci that reside in proximity with the internalized dIgA (marked with arrows), and are reminiscent of the previously described pericentriolar AREs (Apodaca *et al.*, 1994). These morphological data might be supported by the flotation analysis indicating that when dIgA was internalized and subsequently chased for 15 min, receptors' flotation was reduced to levels that are higher than those observed for untreated cells (see Figure 1A). Conceivably, the residual flotation quantity of pIgR could be contributed by its interactions with Cav1 in apical endosomes.

As described, AREs are enriched with transcytotic dIgA (Apodaca *et al.*, 1994) and Rab 25 (Casanova *et al.*, 1999), but are relatively depleted of Tfn (Apodaca *et al.*, 1994). Results presented in Figure 8, A and B, fulfilled these criteria, hence suggesting that Cav1-positive organelles are either linked or reside in proximity to AREs (Bush *et al.*, 2006). Cav1 could be specifically coimmunoprecipitated (co-IPed) with anti-pIgR antibodies from cell lysates (Figure 8C), providing further biochemical support for a possible link between pIgR and Cav1 in polarized cells. Interestingly, Cav1 in the apical regions could also be localized adjacent to transcytotic dIgA in mβCD-treated cells (Figure 8D), suggesting that the morphological identity of these organelles was not impaired by cholesterol depletion.

mβCD Treatment Stimulates the Apical Localization of pY14-Cav1

We next analyzed the intracellular distribution of phosphorylated Cav1. In untreated cells, confocal image analysis revealed pY14-Cav1 labeling mostly in optical sections encompassing the basal and lateral regions of the cells. pY14-Cav1 was detected as diffuse staining patterns occupying the cell apex of only 10% of cells visualized in five arbitrary chosen optical sections (a representative image is shown in Figure 9A, top panel). In contrast, similar analysis of pY14-Cav1 in mβCD-treated cells strikingly unraveled significant labeling of the phosphorylated protein in condensed foci located in the most apical region of ~30% of the cell population (Figure 9A, bottom panel). Immunostaining of dIgA and pY14-Cav1 showed partial overlap in the apical regions of the polarized cells (Figure 9B, top panel). More specifically, for the cells presented in Figure 9B, top panel, the number of pY14-Cav1 objects whose center of mass falls within the area labeled by dIgA is an average of 1.5 SDs above the expected value for a random binomial distribution.

In contrast to the apical pole, there was no statistically significant overlap of dIgA and pY14-Cav1 in the basolateral sections (Figure 9B, bottom panel). These data suggest that

¹²⁵I-hTfn transcytosis, recycling, and endocytosis (inset) were measured as described. Results represent the mean ± SE of three independent measurements.

Figure 7. Cav1 phosphorylation on tyrosine 14 is modulated by acute cholesterol depletion and dIgA uptake. (A) pY14-Cav1 phosphorylation levels are altered in response to altered cholesterol levels. Filter-cultured PTR-MDCK cells were either left untreated or were treated with mβCD (+mβCD), or treated with mβCD and subsequently exposed to cholesterol enrichment by mβCD/chol treatment (+mβCD+mβCD/chol), or else treated with αCD as a control. Equal protein quantities of cell lysates were subjected to quantitative Western blotting analysis and probed with anti-pY14 Cav1, or anti-Cav1 antibodies. Top, a representative result; bottom, the quantitative analysis of three experiments (B) pY14-Cav1 phosphorylation level is elevated in cells challenged with dIgA before cholesterol depletion. Ligands were internalized from the basolateral surface of PTR-MDCK cells for 10 s at 37°C, after which the cells were treated with mβCD for 15 min at 37°C (+dIgA, or hTfn, + mβCD). In other experiments, cells were first treated with mβCD and subsequently exposed to dIgA (+mβCD+dIgA). In these experiments, data on cells exposed to ligands and mβCD were routinely compared with data on cells treated identically with plain MEM/BSA supplemented with (+mβCD) or lacking (untreated) mβCD. Cells were lysed as specified in *Materials and Methods*, and equal protein quantities were analyzed by quantitative Western blotting for the presence of pY14 Cav1 and for total Cav1. Top, a representative gel; bottom, the average $\pm$ SE of three such experiments.

acute cholesterol depletion activates Cav1 primarily in apical endosomes that contain dIgA and possibly represent AREs.

DISCUSSION

Studies of mouse enterocytes suggested the involvement of lipid rafts in pIgR transcytosis (Hansen *et al.*, 1999). Hansen *et al.* showed that a fraction of dIgA and pIgR in membranes prepared from mouse small intestinal explants is present in DRMs. However, other studies on MDCK cells expressing pIgR reached the opposite conclusion (Sarnataro *et al.*, 2000). Consistent with our data, Sarnataro *et al.* indeed showed that pIgR alone, heterologously expressed in MDCK cells, did not float to the top of density gradients. However, in contrast to data presented herein (Figure 1), they demonstrated that basolateral application of dIgA did not increase the insolubility of pIgR in cold buffer containing detergent. The discrepancy between our results and those of Sarnataro *et al.* (2000) most likely lies in the different experimental conditions used. First, Sarnataro *et al.* (2000) did not specify the filter dimensions used in their flotation analyses. We found that, owing to the relatively low abundance of pIgR in DRMs, floating pIgR was detected reproducibly only when cells were cultured on the large (75 mm) filters. Second, Sarnataro *et al.* (2000) applied dIgA at 1 μg/ml for 2 h before performing the assay. We found that pIgR flotation was optimal when cells were allowed to internalize 300 μg/ml dIgA for up to 3 min at 37°C. Ligand uptake followed by prolonged chase times (e.g., 15 min) led to a decrease of pIgR in DRMs (Figure 1Ad). Hence, a significant finding of our work suggests that the stimulation of pIgR flotation is an early and transient event. Lengthened incubation times with the ligand, for example, such as those applied by Sarnataro *et al.*, may have shifted the steady-state distribution of pIgR into detergent soluble membrane compartments.

Our data are consistent with the view that cholesterol-sensitive DRMs on the basolateral surface (and/or basolateral early endosomes) harbor signaling platforms that control polarized trafficking of dIgA after endocytosis. These observations may not be entirely surprising in light of previous findings showing that DRMs are enriched with a variety of signaling molecules, including regulators of dIgA transcytosis such as the p62Yes tyrosine kinase (Luton *et al.*, 1999; Foster *et al.*, 2003). The possible link between DRMs and dIgA-mediated stimulation of signal transduction and transcytosis may be further emphasized by the inability of dIgA bound to pIgR-725t to induce mutant receptors to be incorporated into DRMs (Figure 1A) as well as to undergo transcytosis (Luton *et al.*, 1998). In addition, the empty pIgR is excluded from DRMs, and its transcytosis is largely unaffected by cholesterol depletion (Figure 2C). Surprisingly, these principles were not limited to the transcytotic pIgR because a subset of the basolaterally recycled hTfnR was also found in basolateral DRMs (Figure 3), and the fidelity of its polarized postendocytic traffic was susceptible to cholesterol depletion (Figure 4). An intriguing possibility is that the hTfnR communicates with signaling molecules in rafts that facilitate its polarized trafficking to the basolateral surface after endocytosis.

It is possible that the association of pIgR (bound to dIgA) or hTfnR with DRMs is accomplished by transmembrane domains interacting with certain classes of lipids enriched in lipid rafts (Scheiffele *et al.*, 1997; Shvartsman *et al.*, 2003). The size of these domains could be increased in response to protein oligomerization (Harder *et al.*, 1998). On the basis of these presumptions, dIgA-mediated dimerization of pIgR (Singer and Mostov, 1998) could have increased interactions

Figure 8. Cav1 resides in proximity with protein markers of ARES. (A) Localization of Cav1 with respect to dIgA and hTfn. The basolateral surface of filter-grown PTR-MDCK cells was exposed to dIgA (top) or FITC-hTfn (bottom) for 30 s, and later chased for an additional 30 min at 37°C. Under these conditions, dIgA mostly accumulates in ARES (Apodaca *et al.*, 1994). Cells were fixed and processed for immunofluorescence microscopy, whereby dIgA was labeled with FITC anti-dIgA antibodies, and Cav1 was stained with rabbit antibodies followed by AlexaFluor 594 secondary antibodies. Images taken from the most apical region of the cells were acquired by confocal microscopy. Arrows indicate foci of dense dIgA and Cav1 coresidence. Bar, 10 μ m. (B) Localization of Cav1 with respect to GFP-Rab25. Cells were transfected with GFP-Rab25 cDNA (kindly provided by James E. Casanova, University of Virginia). Twenty-four hours after transfection, cells were seeded on filter supports and processed for immunofluorescence after an additional 48 h. Cav1 was immunostained, and images were acquired by confocal microscopy, as above. Arrows point toward areas of colocalization. Bar, 1 μ m. (C) Coimmunoprecipitation analysis. Cav1 was co-IPed with anti-pIgR antibodies from PTR-MDCK cell lysates using a two-step protocol. In the first step, cells were lysed with RIPA buffer and the lysate was divided into two equal samples, 1 and 2. Each sample was subjected to IP with anti-SC antibodies. The IPed proteins were released from the beads, and a fifth of the immunoprecipitated material was analyzed for the presence of pIgR by immunoblotting (top). In the second step, sample 1 was incubated with anti-Cav1 antibodies, whereas sample 2 was incubated with an irrelevant IgG. The re-IPed proteins were analyzed for the presence of Cav1 by immunoblotting (bottom). The amount of Cav1 in 10 μ g of cell lysate is shown in the bottom panel. The experiment was repeated two times, and a typical result is shown. (D) Cav1 coresides with dIgA in m β CD-treated cells. Cells were treated exactly as in A, except during the chase time where cells were treated with m β CD. Bar, 10 μ m.

shown in the bottom panel. The experiment was repeated two times, and a typical result is shown. (D) Cav1 coresides with dIgA in m β CD-treated cells. Cells were treated exactly as in A, except during the chase time where cells were treated with m β CD. Bar, 10 μ m.

of pIgR with DRMs. hTfnR is a homodimeric transmembrane protein (Lawrence *et al.*, 1999), acylated on cytoplasmic cysteines (Adam *et al.*, 1984; Jing and Trowbridge, 1987; Nadler *et al.*, 1994). Protein acylation could play a pivotal role in targeting to DRMs (Melkonian *et al.*, 1999); hence, in the case of the hTfnR, dimerization and acylation combined could facilitate its association with basolateral DRMs.

When cholesterol depletion was carried out after dIgA internalization, transcytosis was usually augmented without affecting the general vectorial transport. Hence, different cholesterol-dependent mechanisms may regulate the post-endocytic membrane transport of cargo molecules internalized via intact basolateral lipid rafts. Caveolae are thought to play a pivotal role in maintaining the cellular cholesterol balance (Ikonen and Parton, 2000; Martin and Parton, 2005). Previous studies have shown that Cav1 is able to recognize, recruit into caveolae, and regulate the activity of proteins involved in signal transduction, among which are heterotrimeric G-proteins and Src kinases (Okamoto *et al.*, 1998). Because some of these signaling molecules regulate transcytosis of pIgR (Bomsel and Mostov, 1992; Luton *et al.*, 1999), we reasoned that m β CD-treatment augments transcytosis by activating Cav1-associated signaling pathways. Particu-

larly interesting in this context is the observation that phosphorylation of Cav1 on tyrosine 14 is markedly augmented in cholesterol-depleted cells (see Figure 7 and Kim *et al.*, 2002). This signaling event, initially shown to be activated by Src kinases, can induce aggregation, fusion, and even the biogenesis of caveolar transport vesicles (Orlichenko *et al.*, 2006). Therefore, phosphorylated Cav1 in cholesterol-depleted cells could potentially facilitate multiple membrane transport processes, among which are those that regulate transcytosis.

We suggest that m β CD treatment in some way enhances cross-communication between membrane platforms containing activated Cav1 and dIgA-pIgR in a subapical endosomal compartment that possibly represent ARES. Two sets of data could agree with this interpretation. First, the abundance of pY14-Cav1 in subapical organelles increased in response to m β CD treatment (Figure 9A). Second, partial colocalization between pY14-Cav1 and dIgA was evident only in optical sections taken from the very apical cytoplasm (Figure 9B). Ligand transcytosis could be facilitated by stimulation of Cav-1 dependent cargo shuttling from ARES to the apical surface. M β CD-activated processes could also facilitate shuttling of dIgA from basolateral endosomes to pY14-

Figure 9. Cholesterol depletion stimulates apical localization of pY14-Cav1. (A) pY14-Cav1 distribution in polarized cells. Filter-grown PTR-MDCK cells were either treated with mβCD or left untreated. Immunolabeling of pY14-Cav1 was accomplished using mono-specific first antibodies and AlexaFluor 488 secondary antibodies. Representative confocal images taken from the apical, lateral, and basal regions of the polarized cells are presented. Bar, 5 μm. (B) pY14-Cav1 partially colocalizes with internalized dIgA. PTR-MDCK cells allowed to internalize dIgA for 30 s were subsequently treated with mβCD for 15 min. pY14-Cav1 (green) and dIgA (red) were subjected to double immunostaining, and images were analyzed by confocal microscopy. Representative apical and basal planes are shown. Arrows indicate areas in which the two proteins colocalize. Bar, 5 μm.

Cav1 decorated AREs. The latter model may agree with a recent suggestion that cholesterol and glycosphingolipids are required for the delivery of transcytotic cargo from basolateral early endosomes to the subapical endosomal compartment in hepatocytes (Nyasaie *et al.*, 2003). Either event will increase the coalescence of Cav1 and dIgA-pIgR associated signaling platforms, contributing extra signaling cascades that would eventually augment both, kinase-dependent Cav1 phosphorylation and transcytosis. Clearly, the validity of these predictions warrants further investigation.

In summary, our studies suggest a bipartite model for describing the involvement of cholesterol in polarized post-endocytic trafficking. One level of regulation may involve cholesterol-sensitive sorting machineries associated with DRMs at the basolateral surface and a second level of regulation may occur after endocytosis into Cav1 endosomes that modulate the efficiency of membrane transcytosis. We also provide initial evidence suggesting that the efficiency of transcytosis is fine-tuned by changes in plasma membrane cholesterol levels. Acute cholesterol depletion may have a

universal impact on the stimulation of transcytosis, exemplified here for pIgR and hTfR, and previously for the raft-Cav1-associated (Graf *et al.*, 1999) scavenger receptor class B type I in MDCK cells (Burgos *et al.*, 2004). Acute cholesterol enrichment yields the opposite effect.

Finally, it is well recognized that polarized epithelial cells of multicellular organisms tackle the external environment with a highly specialized apical cell membrane that differs in composition and function from the basolateral surface facing the internal milieu (Mostov *et al.*, 1992). The former surface is highly enriched with cholesterol. Cholesterol is mostly delivered to the plasma membrane, including the apical membranes, by nonvesicular pathways that do not require passage through the Golgi apparatus (Hao *et al.*, 2002; Maxfield and Wustner, 2002; Wustner *et al.*, 2002). However, a significant portion of intracellular cholesterol is localized in endocytic recycling compartments, and this pool might be important for maintaining cellular cholesterol homeostasis in the plasma membrane and communicating organelles (Hao *et al.*, 2002). We propose that the mobility of some of these cholesterol pools toward the plasma membrane could be mediated primarily by facilitating transcytosis, thereby compensating for the loss of cholesterol on the cell surface. This compensatory activity could be essential for rescuing some of the specialized epithelial-specific functions associated mostly with the apical domain.

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