The SRC Family Tyrosine Kinase HCK and the ETS Family Transcription Factors SPIB and EHF Regulate Transcytosis across a Human Follicle-associated Epithelium Model*

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A critical step in the induction of adaptive mucosal immunity is antigen transcytosis, in which luminal antigens are transported to organized lymphoid tissues across the follicle-associated epithelium (FAE) of Peyer’s patches. However, virtually nothing is known about intracellular signaling proteins and transcription factors that regulate apical-to-basolateral transcytosis. The FAE can transcytose a variety of luminal contents, including inert particles, in the absence of specific opsonins. Furthermore, it expresses receptors for secretory immunoglobulin A (SIgA), the main antibody in mucosal secretions, and uses them to efficiently transcytose SIgA-opsonized particles present in the lumen. Using a human FAE model, we show that the tyrosine kinase HCK regulates apical-to-basolateral transcytosis of non-opsonized and SIgA-opsonized particles. We also show that, in cultured intestinal epithelial cells, ectopic expression of the transcription factor SPIB or EHF is sufficient to activate HCK-dependent apical-to-basolateral transcytosis of these particles. Our results provide the first molecular insights into the intracellular mechanism of antigen sampling at mucosal surfaces.

A distinct feature of the follicle-associated epithelium (FAE) is the presence of specialized epithelial cells, called microfold cells (M cells), that can transepithelially transport pathogens and toxins from the lumen to the underlying immune cells (1–5). The mechanisms by which M cells take up luminal antigens vary, depending on the physical and chemical properties of the antigens. For example, viruses and other small adherent particles are endocytosed through clathrin-coated vesicles. In contrast, large particles (over ~0.5 μm), such as bacteria, are taken up in a clathrin-independent manner by phagocytosis or macropinocytosis, both of which require dynamic rearrangement of the plasma membrane and its associated cytoskeletal actin network. Pattern recognition receptors expressed on the apical surface of M cells can be used to internalize non-opsonized microorganisms by receptor-mediated phagocytosis (3, 6, 7). For example, M cells bind and transcytose a subset of commensal and pathogenic enterobacteria using the apical membrane receptor, glycoprotein 2, that recognizes the type 1 pilus adhesin FimH (7, 8). In addition, when luminal antigens are opsonized with secretory immunoglobulin A (SIgA), M cells can capture and transport such antigens using receptors that recognize the SIgA moiety of immune complexes (3, 5, 9–12). Receptor(s) for IgA and SIgA have been found on the apical surface of mouse Peyer’s patch M cells, although the molecular identity of any receptor remains to be determined (13). Selective binding of endogenous IgA to the apical membrane of human M cells is also shown using terminal ileum biopsy samples (13).

Despite the advances made in the understanding of M cell surface receptors, little is known about intracellular mechanisms that control apical-to-basolateral antigen transcytosis. Lack of progress is due mainly to the difficulty of isolating sufficient numbers of M cells for molecular and biochemical analyses. M cells are a minor population in the FAE (4), and neither cell lines nor primary cell culture systems have been developed for M cells. To overcome these problems, in vitro models of human FAE have been developed. Upon prolonged culture in vitro, Caco-2 human colon adenocarcinoma cells spontaneously differentiate into cells with the properties of intestinal enterocytes (14). When monolayers of these differentiated Caco-2 cells are cocultured with primary murine lymphocytes or human Raji B cells, a small fraction converts into a different type of epithelial cells that displays morphological and functional characteristics of in vivo M cells (M-like cells) (15, 16). Thus, cocultured Caco-2 monolayers display the central feature...
of the FAE and have been invaluable in quantitatively analyzing M cell-mediated transcytosis of a variety of antigens and microorganisms (15–18). However, to date, the model has not been used to identify and functionally analyze intracellular proteins that regulate antigen transcytosis across the FAE.

In mucosal immunity, transcytosis across the epithelial barrier is a key regulatory component (19). In fact, SlgA is produced by a mechanism that involves basolateral-to-apical transcytosis of polymeric IgA complexed with the polymeric Ig receptor (20, 21). Unlike antigen transcytosis that proceeds in the apical-to-basolateral direction, IgA transcytosis has been extensively studied both in vivo and in vitro, and the signaling mechanism regulating this basolateral-to-apical transcytosis is known to require the SRC family non-receptor tyrosine kinase YES (20, 21). A different SRC family kinase HCK has been shown to play a crucial role in phagocytes in the internalization of both opsonized and non-opsonized particles (22, 23). Importantly, HCK is specifically induced in vivo in mouse Peyer’s patch M cells (8). In addition to HCK, the ETS family transcription factor SPIB is selectively expressed in human and mouse M cells (8, 24, 25). Interestingly, in alveolar macrophages that internalize microbial pathogens and inhaled particulates from the lung surface (26), a close homolog of SPIB called PU.1 (SPI1) regulates phagocytosis of IgG-opsonized and non-opsonized particles (27–29). Similar to SPIB, a different ETS factor EHF is highly induced in vivo in mouse Peyer’s patch M cells (8). However, to date, it remains unclear whether these SRC family kinases or ETS transcription factors are involved in antigen transcytosis.

Here, we reproduced the FAE model using the Caco-2 subclone TC7 (30). Among many Caco-2 subclones tested, TC7 shows the differentiation properties (as verified by morphology and the activities of marker enzymes, such as alkaline phosphatase (AP) and cytochrome P450 3A4) and barrier functions (as assessed by transepithelial electrical resistance and the paracellular passage of mannitol) that best represent those of the small intestinal epithelium in vivo (31–33). Thus, TC7 is one of the most commonly used subclones for in vitro studies of the human FAE (17, 18, 34). We show that the SRC family kinase HCK regulates apical-to-basolateral transcytosis of non-opsonized and SlgA-opsonized particles. Importantly, expression analysis of the HCK gene resulted in the identification of the ETS family transcription factors SPIB and EHF as critical regulators of HCK-dependent transcytosis across the FAE model. We also show that one of the SPIB- and EHF-regulated genes encodes the transmembrane receptor CD300LF that can bind HCK and transcytose SlgA-opsonized particles in vitro.

EXPERIMENTAL PROCEDURES

Cell Culture and AP Enzyme Assay—Cell culture reagents were from Invitrogen unless otherwise stated. TC7 cells (a gift from Dr. M. Rousset, INSERM, France) were cultured at 37 °C under 10% CO₂ in RPMI 1640 medium supplemented with 10% FBS, Glutamax, and non-essential amino acids. All other cell lines used in this study were also obtained from ATCC and cultured according to their recommendations.

For cocultures, TC7 cells were seeded (6 × 10⁴ cells/cm²) on Transwell permeable membranes (0.33 cm² unless otherwise stated, polyester membrane, 3-μm pore size; Corning Inc.) as described by Gullberg et al. (16). Confluent monolayers of differentiated TC7 cells were generated by incubating the cells for 19–21 days with daily medium change. Raji cells (1 × 10⁵ cells monolayer) were then suspended in TC7 medium and added to the basolateral chambers. Cocultures were maintained for 2.5 days unless otherwise specified. After the addition of Raji cells, only the medium in the apical chamber of each coculture was changed daily. When indicated, doxycycline (Dox; 1 μg/ml) was added to the apical chambers. Monocultures were generated and treated identically but without Raji cells. Reproducible results were obtained when TC7 medium was changed daily, and TC7 cells were cultured on the membrane specified above.

Transepithelial electrical resistance of monocultures was measured using Millicell-ERS (Millipore), and monocultures with resistance between 200 and 300 ohms-cm²² were used for the experiments. Apical AP activities were determined as described (16), except that each monolayer was incubated with 250 μl of AP substrate.

Transfection—To obtain stably transfected cells, the expression vectors described in the supplemental Experimental Procedures were introduced into TC7 cells by calcium phosphate-mediated transfection. The amount of DNA used for transfection was 0.27 μg/cm². Cells transfected with the Dox-inducible expression vector pLNI00 or the same vector expressing p59HCKDN or p61HCKDN were selected with puromycin. Other transfectants were selected with Geneticin. Stably transfected cells were then pooled and used for the experiments. Expression levels of cloned cDNAs were determined directly by immunoblotting or indirectly by monitoring the fluorescence intensity of GFP expressed from the second cistron. In general, cells that show lower levels of cDNA expression grow faster and, over time, become a major population in pooled stably transfected cells. To avoid this problem and ensure high levels of cDNA expression, transfection was routinely repeated.

Luciferase (LUC) Reporter Assay—Cells grown in 24-well plates were transiently transfected by the calcium phosphate method with a firefly LUC reporter construct (0.05 μg of DNA/well). To normalize for transfection efficiency, cells were cotransfected with a control plasmid containing the Renilla LUC gene driven by a constitutive SV40 promoter (Promega; 0.5 ng of DNA/well). Where indicated, cells were also transfected with effector plasmids (0.5 μg DNA/well). The final DNA concentration was adjusted to an identical level using empty vectors. Unless otherwise specified, LUC activities were determined 48 h after transfection using a Dual-Luciferase assay kit as instructed by the manufacturer (Promega), and firefly LUC values were normalized to Renilla LUC values.

To analyze promoter activation by Raji cell-conditioned medium, TC7 cells grown on Transwell permeable membranes (4.67 cm²) were transiently cotransfected by the calcium phosphate method with a firefly LUC reporter construct (1 μg DNA/
membrane) and a Renilla LUC control plasmid (1 ng of DNA/membrane). Transfected cells became confluent about 24 h after transfection. At 60 h after transfection, the medium in the basolateral chambers was replaced with Raji cell-conditioned medium or fresh Raji cell medium, and the monolayers were incubated for 12 h before the LUC assay. LUC activities were determined as described above.

**Particle Transcytosis Assay**—Carboxylated fluorescent polystyrene microspheres (0.5 μm in diameter) from Molecular Probes (F-8812) were used as non-opsonized particles. To generate antibody-coated particles, the microspheres were conjugated to human colostrom SlgA (Sigma) or a recombinant chimeric anti-CD20 IgG1 monoclonal antibody (Rituximab, Genentech) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) as instructed by the manufacturer. Our pilot experiments showed that there is no difference in the binding to and transcytosis by TC7 cocultures between the recombinant antibody and human IgG isolated from normal serum. The efficiency of antibody coating was determined by flow cytometry using an anti-human κ light chain antibody conjugated to fluorescein isothiocyanate.

Transcytosis was analyzed at 37 and 4 °C, and the solutions used for the following procedures were all pre-equilibrated to 37 or 4 °C. Monolayers were first washed twice with Hanks’ balanced salt solution (HBSS) supplemented with 2% FBS buffered to pH 7.4 with 25 mM Hepes (16) (HBSS/FBS) and preincubated in HBSS/FBS for 20 min at 37 or 4 °C. After preincubation, the apical and basolateral solutions were replaced with 100 μl of HBSS/FBS containing fluorescent particles (1 × 10⁸ particles/ml unless otherwise specified) and 400 μl of HBSS/FBS, respectively. Transcytosis was carried out in the dark for 2 h, and the number of particles transported to the basolateral membrane was counted by a FACS Calibur flow cytometer (BD Biosciences). Transepithelial electrical resistance of monolayers was always measured before and after transcytosis. Furthermore, when monolayers were used for transcytosis at 37 °C, their integrity was occasionally verified by repeating the transcytosis assay at 4 °C and confirming the lack of particle transport. When indicated, the apical surface of monolayers was thoroughly washed with ice-cold HBSS, and cells were lysed to determine cDNA expression levels by immunoblotting.

**Antibody Binding Assay**—The apical surface of monolayers was washed with HBSS/FBS and incubated for 90 min at 37 °C with SlgA or IgG (200 μg/ml in HBSS/FBS). After incubation, monolayers were quickly washed four times with cold HBSS/FBS, fixed, and blocked with 3% bovine serum albumin in HBSS. Monolayers were then incubated with an anti-human κ light chain antibody conjugated to horseradish peroxidase (HRP), washed, and incubated with a chemiluminescence substrate (SuperSignal West Pico, Pierce). Generated light was detected as described above. A DNA fragment specific for CD20 was amplified using intron-spanning primers 5′-GAAAAACTCCCATCTACCCCAATTCT-3′ (forward) and 5′-TTCCATGCAAAAAAGCCAGATGAGT-3′ (reverse). Amplification cycles were 45 and 35 for TC7 and HL-60 DNA samples, respectively. A DNA fragment specific for CD20 was amplified using intron-spanning primers 5′-GGG-GGGGCTTCCTGAGAATGTCA-3′ (forward) and 5′-GGTCCACTGGGTCTTTCAACCA-3′ (reverse). Amplification cycles were 45 and 30 for TC7 and Raji cell cDNA samples, respectively. A DNA fragment specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified (30 cycles) using the previously published primers (36) 5′-GGATATTTCGCGTCTTCC-3′ (forward) and 5′-CTGCAATGAGTGGCATGGACTGCA-3′ (reverse). PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide.

**Particle Internalization Assay**—Monolayers generated on permeable membranes (4.67 cm²) were incubated at 37 °C in the absence or presence of fluorescent particles added to the apical surface. After incubation, both sides of monolayers were thoroughly washed four times with HBSS and once with trypsin/EDTA (0.05% trypsin, 0.5 mM EDTA), pre-equilibrated to 37 °C, and then incubated at 37 °C for 30 min with trypsin/EDTA. After trypsinization, cells were removed from the membranes by vigorous pipetting, collected by centrifugation, washed twice with HBSS/FBS, and resuspended in the same buffer. Aggregated cells were removed by filtering through cell strainers. To quench any remaining extracellular fluorescence, cells were treated with trypan blue as described previously (37, 38). Fluorescence intensity was determined by a flow cytometer.

**Immunoprecipitation and Immunoblotting**—To cross-link CD300LF, transiently transfected cells were incubated at 37 °C for 15 min with a monoclonal anti-CD300LF antibody (rat IgG, 1 μg/ml; R&D Systems) and, after washing, incubated at 37 °C for 10 min with polyclonal anti-rat IgG (5 μg/ml). Control cells were incubated with an isotype-matched control antibody instead of anti-CD300LF. CD300LF was also cross-linked by incubating with SlgA-opsonized particles (1 × 10¹⁰ particles/ml). Whole-cell extracts were then obtained using a co-immunoprecipitation kit (Active Motif) as instructed by the manufacturer, and equal amounts of extracts were subjected to immunoprecipitation with a polyclonal anti-CD300LF antibody (goat IgG, 0.5 μg; R&D Systems) and protein G beads. The beads containing immune complexes were pelleted, washed extensively, and resuspended in gel loading buffer. Precipitated proteins or equal amounts of cell extracts were resolved by SDS-PAGE. Proteins were electroblotted on polyvinylidene difluoride membranes that were subsequently blocked in StartingBlock buffer (Pierce). Membranes were then probed with various antibodies, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed by SuperSignal West Pico (Pierce). Chemiluminescence was detected by a CCD camera.
Statistical Analysis—Differences between two groups of unpaired data were analyzed using the two-tailed nonparametric Mann-Whitney test. \( p < 0.05 \) was considered significant.

RESULTS

TC7 Cocultures Transcytose Particles in both Opsonin-independent and SlgA-dependent Manners—We first verified that, similar to previous studies (15, 16, 39), TC7 cocultures, but not monocultures, transcytose non-opsonized inert particles \( (1 \times 10^{10} \text{ particles/ml}) \) in a temperature-dependent manner (Fig. 1A) and that only a fraction (~10%) of cocultured TC7 cells internalize the particles (Fig. 1B). We then determined whether TC7 cocultures can be used to analyze SlgA-dependent transcytosis. Animal studies show that the apical surface of M cells specifically binds SlgA but not IgG (13) and that M cells internalize and transcytose SlgA-opsonized particles more efficiently than particles opsonized with an irrelevant protein (10, 11). We found that SlgA strongly binds the apical surface of cocultures but not monocultures and that IgG shows only background levels of binding to both cocultures and monocultures (Fig. 1C and D). We also found that cocultures transcytose, at \( 37^\circ \text{C} \), SlgA-opsonized particles \( (1 \times 10^8 \text{ particles/ml}) \) more efficiently than non-opsonized or IgG-opsonized particles (Fig. 1E). Very few particles were transcytosed by cocultures at \( 4^\circ \text{C} \) or by monocultures at either temperature in this experiment (Fig. 1E) and the one shown in Fig. 1A, where a higher concentration \( (1 \times 10^{10} \text{ particles/ml}) \) of non-opsonized particles was used. Thus, TC7 cocultures transport particles in both opsonin-independent and SlgA-dependent manners using the transcellular, but not paracellular, pathway, successfully reproducing a key feature of the \textit{in vivo} FAE.

\( p59\text{HCK} \) Regulates both Opsonin-independent and SlgA-dependent Transcytosis in Vitro—Using RT-PCR, we analyzed expression of SRC family tyrosine kinases and found that HCK is specifically induced in TC7 cocultures (Fig. 2). The low level of HCK expression was expected because only ~10% of cocultured TC7 cells internalized particles (Fig. 1B). HCK is expressed as two isoforms, p59HCK and p61HCK, from a single mRNA, and these isoforms exhibit distinct subcellular localization and functions (22, 40). p59HCK is mainly associated with the plasma membrane, and its constitutively active mutant (p59HCK\textsuperscript{CA}) induces actin reorganization and plasma membrane protrusions important for phagocytosis. p61HCK, which contains an additional 21-amino acid sequence at the N terminus, cofractionates with lysosomes, and constitutively active p61HCK (p61HCK\textsuperscript{CA}) induces podosomes that are involved in cell adhesion and migration. As shown in Fig. 3A, TC7 monocultures constitutively expressing p59HCK\textsuperscript{CA} or p61HCK\textsuperscript{CA} did not transcytose non-opsonized particles at \( 37^\circ \text{C} \), suggesting that HCK activation alone is not sufficient to convert TC7 cells into transcytotic cells. However, when cocultured, monocultures expressing p59HCK\textsuperscript{CA} but not p61HCK\textsuperscript{CA}, exhibited enhanced transcytosis of non-opsonized particles at \( 37^\circ \text{C} \) as compared with untransfected monocultures or monocultures transfected with an empty vector (Fig. 3A). At \( 4^\circ \text{C} \), non-opsonized
p59HCK, but not p61HCK, plays an important role in opsonin-independent transcytosis.

FIGURE 3. p59HCK regulates opsonin-independent and SIgA-dependent transcytosis across the FAE model. A, monolayers were generated using TC7 cells stably transfected with an empty vector (U) or the same vector constitutively expressing p59HCKCA (59) or p61HCKCA (61). As a control, monolayers were also generated using untransfected (U) TC7 cells. Monolayers were then cultured in the presence (+) or absence (−) of Raji cells and used to analyze transcytosis of non-opsonized particles. The number of particles transcytosed at the indicated temperatures is presented as in Fig. 1A. Expression levels of p59HCKCA and p61HCKCA were determined by immunoblotting and are shown to the left.

B, monolayers were generated using TC7 cells stably transfected with an empty vector (Vector) or the same vector constitutively expressing p59HCKDN (p59DN) or p61HCKDN (p61DN). Monolayers were then cocultured (Raji +) and used to analyze, at 37 °C, transcytosis of non-opsonized (N), IgG-opsonized (G), and SIgA-opsonized (A) particles. The number of particles transcytosed is presented as in Fig. 1A. None of these particles were significantly transcytosed by cocultures at 4 °C or by monocultures (data not shown). Expression levels of p59HCKDN and p61HCKDN were determined by immunoblotting and are shown to the right. C, untransfected and p59HCKDN-expressing monolayers were cultured in the presence (+) or absence (−) of Raji cells. Apical AP activities were determined as described under “Experimental Procedures” and expressed as the absorbance at 405 nm (A405). Each point represents a value from a single monoculture or coculture. The mean values (longer horizontal lines) and S.D. (error bars) are also shown. D and E, differentiated TC7 monolayers stably expressing p59HCKDN (p59DN) from a Dox-inducible promoter were cocultured (Raji +) for 2 days without Dox (−Dox). At day 2 of coculture, some of the monolayers were used to analyze, at 37 °C, transcytosis of non-opsonized (N) and SIgA-opsonized (A) particles. The rest of the monolayers were cocultured for an additional 2 days in the absence or presence (+Dox) of Dox and used for the same transcytosis analysis at day 4. The number of particles transcytosed was determined and presented as in Fig. 1A.

particles were not significantly transcytosed by cocultures expressing p59HCKCA or p61HCKCA (Fig. 3A). These results suggest that p59HCK, but not p61HCK, plays an important role in opsonin-independent transcytosis across the FAE model.

Although SIgA-opsonized particles were also transcytosed more efficiently by cocultures expressing p59HCKCA but not p61HCKCA (supplemental Fig. S1A), it remained unclear whether p59HCK also controls SIgA-dependent transcytosis because SIgA-opsonized particles are probably transported by both SIgA-dependent and -independent mechanisms. To address this issue, we next expressed dominant negative mutants of p59HCK and p61HCK (p59HCKDN and p61HCKDN) independently in TC7 monolayers. As shown in Fig. 3B, p61HCKDN did not affect transcytosis of non-opsonized, IgG-opsonized, or SIgA-opsonized particles. In contrast, p59HCKDN greatly reduced transcytosis of non-opsonized and IgG-opsonized particles (Fig. 3B). Importantly, transcytosis of SIgA-opsonized particles was also almost completely eliminated by p59HCKDN (Fig. 3B), suggesting that p59HCK also regulates SIgA-dependent transcytosis.

Although unlikely, it is possible that p59HCKDN inhibited transcytosis by blocking the development of M-like cells. It has been shown that, similar to in vivo M cells (41), in vitro-generated M-like cells exhibit reduced levels of AP activity in the apical membrane, providing a quantitative tool for estimating the relative number of M-like cells in Caco-2 cocultures (16, 42). We found that untransfected and p59HCKDN-expressing cocultures show similar levels of reduction in AP activity (Fig. 3C), suggesting that comparable numbers of M-like cells are induced. Furthermore, p59HCKDN inhibited transcytosis even if its expression was induced after M-like cells were established in cocultures (Fig. 3, D and E). When TC7 cells stably trans-
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affected with a vector expressing p59HCK\textsuperscript{DN} from a Dox-inducible promoter were cocultured in the absence of Dox, particle transcytosis was detected 2 and 4 days after the start of coculture, with SlgA-opsonized particles transported more efficiently than non-opsonized particles (Fig. 3, \(D\) and \(E\)). These results indicate that, in the absence of Dox, M-like cells are normally induced by day 2 of coculture and remain active until day 4. In contrast, when Dox was added to the cells at day 2, transcytosis of both non-opsonized and SlgA-opsonized particles was greatly reduced by day 4 (Fig. 3, \(D\) and \(E\)). The addition of Dox did not affect transcytosis across control monolayers transfected with an empty vector (supplemental Fig. S1B). Thus, p59HCK regulates both opsonin-independent and SlgA-dependent transcytosis \textit{in vitro}. We speculate that \textit{in vivo} transcytosis is also controlled by this tyrosine kinase because its expression is specifically induced in mouse Peyer’s patch M cells (8).

**SPIB Regulates the Conversion of Differentiated TC7 Cells into M-like Cells**—We found that the promoter of the human HCK gene contains a binding site for SPIB (Fig. 4A) and that the HCK promoter fused to the LUC reporter gene can be transactivated in TC7 cells by ectopically expressed SPIB (Fig. 4B). We also found that the human \(S\)PIB promoter fused to the reporter gene is induced in TC7 monolayers incubated with Raji cell-conditioned medium (Fig. 4C). Thus, we hypothesized that SPIB controls expression of genes involved in transcytosis across the FAE.

To test this hypothesis, we first expressed the DNA-binding domain of SPIB (SPIB\textsuperscript{DBD}) in TC7 cells. Because SPIB\textsuperscript{DBD} lacks the transactivation domain of SPIB, it acts as a dominant negative inhibitor and blocks SPIB-mediated induction of the HCK promoter (Fig. 4B). When expression of SPIB\textsuperscript{DBD} was induced in TC7 cocultures after M-like cells were established (2 days after the start of coculture), it did not affect transcytosis of non-opsonized or SlgA-opsonized particles (Fig. 5, \(A\) and \(B\)). This indicates that, unlike p59HCK\textsuperscript{DN}, SPIB\textsuperscript{DBD} is unable to block transcytosis in established M-like cells. In contrast, when SPIB\textsuperscript{DBD} was induced in TC7 monolayers before the start of coculture (but after enterocyte differentiation), it dramatically reduced transcytosis of both non-opsonized and SlgA-opsonized particles (Fig. 5, \(C\) and \(D\)). This reduction was not observed with a mutant form of SPIB\textsuperscript{DBD} (mSPIB\textsuperscript{DBD}) carrying two amino acid substitutions that eliminate DNA binding activity (43) (Fig. 5, \(C\) and \(E\)). However, we also found that SPIB\textsuperscript{DBD}, but not mSPIB\textsuperscript{DBD}, induced before the start of coculture inhibits down-regulation of apical AP activity (Fig. 5F). SPIB\textsuperscript{DBD} induced after the start of coculture did not block AP down-regulation (supplemental Fig. S2A). Taken together, these results suggest that SPIB regulates the conversion of differentiated TC7 cells into M-like cells and that, when induced before the start of coculture, SPIB\textsuperscript{DBD} blocks transcytosis indirectly by preventing the formation of M-like cells.

SPIB and EHF Can Coordinate Induce Transcytosis-related Genes \textit{in Vitro}—Although SPIB\textsuperscript{DBD} was useful in identifying an important function of SPIB, the dominant negative inhibitor was not an ideal tool for analyzing the role of SPIB in the regulation of transcytosis-related genes. Thus, we next transfected TC7 cells with a vector expressing full-length SPIB from a Dox-inducible promoter. When monolayers of transfected cells were differentiated and then treated with Dox, they transcytosed, without coculture with Raji cells, non-opsonized particles at 37 °C (Fig. 6, \(A\) and \(B\)). A flow cytometric analysis of these cells indicated that, similar to cocultured TC7 cells, only a fraction (~15%) of cells in Dox-treated monolayers efficiently internalize particles (supplemental Fig. S3). Transcytosis of non-opsonized particles was not observed at 4 °C with SPIB-expressing monocultures (Fig. 6, \(A\) and \(B\)) or at 37 °C with monocultures expressing mutant SPIB deficient in DNA binding (data not shown). Importantly, SPIB-expressing monocultures transcytosed SlgA-opsonized particles more efficiently than non-opsonized particles (Fig. 6B). Furthermore, SPIB-induced transcytosis of non-opsonized and SlgA-opsonized particles was greatly reduced by p59HCK\textsuperscript{DN} but not p61HCK\textsuperscript{DN} (Fig. 6C). Thus, SPIB can directly control p59HCK-dependent transcytosis by regulating gene expression.
In addition to SPIB, the ETS transcription factor EHF is specifically expressed in Peyer’s patch M cells (8). We found that, similar to SPIB, ectopically expressed EHF can transactivate the promoter of the human HCK gene in TC7 cells (Fig. 4D). Furthermore, the human EHF promoter fused to the LUC reporter gene was induced in TC7 monolayers incubated with Raji cell-conditioned medium (Fig. 4C). Importantly, we found that ectopically expressed EHF can also induce p59HCK-dependent transcytosis of non-opsonized and S IgA-opsonized particles in TC7 monolayers at 37 °C but not at 4 °C (Fig. 6D). Coexpression of SPIB and EHF did not enhance transcytosis (data not shown), suggesting that these transcription factors do not act cooperatively in TC7 cells. By contrast, the ETS transcription factors SPDEF and ETS2, which are implicated in the differentiation of Paneth and goblet cells (44, 45), failed to induce transcytosis in TC7 monolayers (Fig. 6D). Apical AP activity was not down-regulated in monolayers expressing SPIB or EHF (supplemental Fig. S2B), suggesting that overexpression of these ETS proteins alone is not sufficient to fully convert differentiated TC7 cells into M-like cells. Nonetheless, the results described above clearly indicate that, in differentiated TC7 cells, ectopically expressed SPIB or EHF can partially substitute for Raji cell-stimulated signals and coordinately induce expression of genes involved in both opsonin-independent and S IgA-dependent transcytosis.

CD300LF Is a SPIB- and EHF-inducible Transcytotic S IgA Receptor—The result that SPIB- and EHF-expressing monolayers transcytose S IgA-opsonized particles more efficiently than non-opsonized particles (Fig. 6) suggested that one of the genes induced by these transcription factors is a S IgA receptor. Thus, to better understand the transcriptional regulation of S IgA-dependent transcytosis, we searched for a SPIB- and EHF-inducible S IgA receptor. CD300LF is a member of the Ig superfamily and a close homolog of CD300LG that can bind and internalize IgA but not IgG (46). The extracellular Ig-like domain of CD300LF also shows significant homologies to the Ig domains of the IgA receptors polymeric Ig receptor and Fcα/γ receptor (47). We found that CD300LF can bind S IgA, but not IgG, when expressed on the apical surface of TC7 monolayers (Fig. 7A). Interestingly, the receptor failed to bind S IgA when expressed on the surface of 293T or HeLa cells (data not shown). This may indicate that, similar to the Fcα receptor (48), CD300LF requires a specific accessory protein to interact with S IgA. We also found that the promoter of the human CD300LF gene contains a conserved sequence that is highly similar to the SPIB binding site (27) (Fig. 7B). Using a LUC reporter assay, we showed that this conserved sequence alone can mediate the induction of the CD300LF promoter by ectopically expressed SPIB (Fig. 7C, compare the middle and bottom constructs). However, for the efficient induction of the promoter, the DNA sequence located upstream of the conserved sequence was also required (Fig. 7C, compare the top and middle constructs). Similarly, ectopically expressed EHF transactivated the CD300LF promoter (Fig. 7C, the top construct), although EHF was unable to induce the CD300LF promoter by recognizing only the conserved sequence (Fig. 7C, compare the middle and bottom constructs). Consistent with the observation that the CD300LF promoter can be transactivated by ectopically expressed SPIB or EHF, TC7 monolayers ectopically expressing SPIB or EHF induced CD300LF on their
apical surface (Fig. 7D). Furthermore, cocultured TC7 monolayers also showed apical expression of this receptor (Fig. 7E). Thus, CD300LF is a SPIB- and EHF-inducible SIgA receptor expressed in the FAE model.

SPIB- and EHF-induced transcytosis of SlgA-opsonized particles requires p59HCK (Fig. 6, C and D). We coexpressed CD300LF and p59HCK in TC7 cells and showed that these proteins are communoprecipitated when the receptor is cross-linked by anti-CD300LF antibodies (Fig. 8A). Under the same conditions, we did not detect the interaction between CD300LF and YES (Fig. 8A), demonstrating functional specificity between HCK and YES in transcytosis. Importantly, the interaction between CD300LF and p59HCK was also induced by SlgA-opsonized particles (Fig. 8A) but not IgG-opsonized particles (data not shown). Finally, we showed that TC7 monolayers ectopically expressing CD300LF can transcytose SlgA-opsonized, but not IgG-opsonized, particles at 37 °C without coculture with Raji cells (Fig. 8B). This CD300LF-mediated transcytosis was enhanced when the receptor was coexpressed with p59HCKCA but not p61HCKCA (Fig. 8C), supporting the idea that CD300LF functionally interacts with p59HCK. This is similar to the finding that p59HCK, but not p61HCK, regulates FcyRIIa-mediated phagocytosis of IgG-opsonized particles (40).

**DISCUSSION**

ETS family transcription factors are often expressed in specific cell lineages and control lineage commitment and cell functions by regulating gene expression (49). For example, SPDEF is expressed in cells of the goblet and Paneth cell lineages, and the deletion or transgenic expression of this transcription factor in mice leads to major defects in the maturation of both goblet and Paneth cells (45). Recent reports indicate that SPIB is specifically expressed in M cells and that M cell differentiation is prevented in Spib−/− mice, leading to impaired transcytosis of non-opsonized luminal antigens (24, 25). These results are consistent with our observations (Figs. 5 and 6D) and greatly substantiate the validity and usefulness of the in vitro model. Unlike Spib−/− mice, our in vitro model allowed us to demonstrate that SPIB plays an important role in the functional maturation of the FAE by regulating expression of genes, such as HCK and
CD300LF, that are involved in antigen transcytosis. Our in vitro analysis also revealed that the ETS protein EHF, which is highly induced in M cells (8), can substitute for SPIB in the regulation of transcytosis. Because M cell differentiation is blocked in Spib-/- mice (24, 25), EHF is unable to substitute for SPIB in M cell lineage commitment. Unlike SPIB, EHF may regulate only functional maturation of the FAE.

Effective induction of antigen-specific mucosal immune responses is ensured by the ability of M cells to bind and transcytose a variety of luminal contents in the absence of any specific opsonins (1–7). However, SlgA-dependent transcytosis would also play a major role in the induction of adaptive mucosal immunity because SlgA can bind a wide range of antigens and microorganisms both specifically and nonspecifically through its Fab and glycan moieties (5, 9, 12, 50, 51). Animal studies provide compelling evidence that M cell-targeted delivery of vaccine antigens is more effective in inducing both mucosal and systemic immune responses than non-targeted vaccines (2, 4). Because SlgA is extremely stable in the harsh gastrointestinal environment (52), this antibody is a promising candidate for a safe and efficient delivery vehicle of mucosal vaccines. Expression of a SlgA receptor in dendritic cells also assures efficient processing and presentation of vaccine antigens transcytosed across the FAE (53). However, it has been difficult to develop a SlgA-based vaccine delivery vehicle because there has been very little knowledge of human SlgA receptors. Here, we identified CD300LF as a potential SlgA receptor expressed on the human FAE.

Previous studies showed that the cholera toxin receptor ganglioside GM1 is also expressed on the apical surface of a human FAE model and that the receptor can bind and transcytose SlgA and SlgA-opsonized particles (54). Thus, CD300LF and GM1 appear to have overlapping functions. Because SlgA is a complex glycoprotein (2, 9, 12), it is possible that the human FAE expresses multiple SlgA receptors, each of which recognizes a different protein or glycan motif. We speculate that GM1-mediated SlgA transcytosis also requires p59HCK because p59HCKDN almost completely eliminated transcytosis of SlgA-opsonized particles across TC7 cocultures (Fig. 3B). A specific deletion of each SlgA receptor would be necessary to determine their roles in SlgA-dependent transcytosis and evaluate whether they can serve as effective targets for SlgA-based mucosal vaccines in humans.

M cell studies have traditionally used morphological and immunohistochemical techniques and focused mainly on proteins and glycans expressed on the apical plasma membrane (1, 3–7). Here, we combined molecular biological approaches with a human FAE model and identified a tyrosine kinase and transcription factors that regulate antigen transcytosis. This type of information could never be obtained by conventional microscopic examinations. In the future, the human FAE model can be used to identify the proteins that interact with SPIB, EHF, and HCK as well as the genes regulated by SPIB and EHF. The model can also be used to determine the signaling pathway(s) that activate these transcription factors. Finally, results obtained with the in vitro model can be verified in vivo using...
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A

Transfection

|        | CD300LF | HCK-FLAG | YES-FLAG |
|--------|---------|----------|----------|
| NO crosslink | +       | -        | -        |
| Anti-CD300LF | +       | -        | -        |
| S IgA | +       | -        | -        |

B

IP: CD300LF

C

FIGURE 8. CD300LF is a transcytotic receptor that can interact with p59HCK. A, TC7 cells were transiently transfected with expression vectors encoding CD300LF, FLAG-tagged p59HCK (HCK-FLAG), and FLAG-tagged YES (YES-FLAG), as indicated. Cells were then incubated with anti-CD300LF (rat monoclonal IgG) and, after washing, treated with polyclonal anti-rat IgG to cross-link CD300LF. Control cells (No crosslink) were incubated with an iso-type-matched control antibody instead of anti-CD300LF. CD300LF was also cross-linked by incubating with SiaG-opsinized particles (SiaG). Cells were lysed and subjected to immunoprecipitation (IP) with goat polyclonal anti-CD300LF. The precipitates were immunoblotted with rabbit polyclonal anti-CD300LF and anti-FLAG. Cell lysates were also immunoblotted with anti-CD300LF. The precipitates were immunoblotted with rabbit polyclonal anti-CD300LF and anti-FLAG. Cell lysates were also immunoblotted with anti-CD300LF to verify the expression of p59HCK and YES. B, TC7 monolayers constitutively expressing CD300LF were differentiated, and transcytosis of IgG-opsonized (Gp) with goat polyclonal anti-CD300LF. The precipitates were immunoblotted with rabbit polyclonal anti-CD300LF and anti-FLAG. Cell lysates were also immunoblotted with anti-CD300LF to verify the expression of p59HCK and YES.

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