Endocytic trafficking from the small intestinal brush border probed with FM dye

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Hansen GH, Rasmussen K, Niels-Christiansen LL, Danielsen EM. Endocytic trafficking from the small intestinal brush border probed with FM dye. Am J Physiol Gastrointest Liver Physiol 297: G708–G715, 2009. First published August 13, 2009; doi:10.1152/ajpgi.00192.2009.—The small intestinal brush border functions as the body’s main portal for uptake of dietary nutrients and simultaneously acts as the largest permeability barrier against pathogens. To enable this, the digestive enzymes of the brush border are organized in lipid raft microdomains stabilized by cross-linking galectins and intelectin, but little is known about the dynamic properties of this highly specialized membrane. Here, we probed the endocytic membrane trafficking from the brush border of organ-cultured pig intestinal mucosal explants by use of a fixable, lipophilic FM dye. The fluorescent dye readily incorporated into the brush border, and by 15 min faint but distinct punctae were detectable ~1 μm beneath the brush border, indicative of a constitutive endocytosis. The punctae represented a subpopulation of early endosomes confined to the actomyosin-rich terminal web region, and their number and intensity increased by 1 h, but trafficking further into the enterocyte was not observed except in immature epithelial cells of the crypts. A powerful ligand for receptor-mediated endocytosis, cholera toxin B subunit, increased apical endocytosis and caused membrane trafficking to proceed to compartments localized deeper into the cytoplasm of the enterocytes. Two major raft-associated brush border enzymes, alkaline phosphatase and aminopeptidase N, were excluded from endocytosis. We propose that the terminal web cytoskeleton, by inhibiting traffic from apical early endosomes further into the cell, contributes to the overall permeability barrier of the gut.

endocytosis; small intestine

THE MAIN FUNCTION OF THE SMALL intestinal epithelium is to act as a digestive/absorptive surface that enables efficient uptake of the dietary nutrients required by the body (20, 22, 34). At the same time, however, the brush border must act as a permeability barrier preventing luminal pathogens from gaining entry into the organism (13), and, to complicate matters, both of these tasks have to be accomplished in a harsh working environment frequently exposed to pancreatic degradatory enzymes and hepatic bile. Reflecting this dual functioning, the enterocyte brush border contains an unusually high proportion of glycolipids (>30%), mainly mono-, di-, and pentohexosylceramides (6). This membrane composition favors the formation of lipid raft microdomains that are relatively resistant to detergents (31), and most of the major digestive hydrolases of the enterocyte brush border are known to partition into the detergent-resistant membrane fraction (1, 2, 7, 25). Furthermore, a number of di- and multivalent β-galactoside recognizing lectins, including galectin-3 and -4 (10–12, 32) and intelectin (37), are deposited at the brush border. Here, they cross-link the glycolipids and glycoproteins, creating microdomains with an unusually high detergent resistance (“superrafts”) (5). This supramolecular organization helps stabilizing the membrane and prevents excessive loss of digestive enzymes to the gut lumen (8).

However, as with any cell membrane, the enterocyte brush border is capable of undergoing endocytosis. In neonatal life, receptor-mediated apical endocytic uptake of IgG from mother’s milk, followed by transcytosis to the basolateral membrane, is an important mechanism for conferring passive immunity to the newborn (29, 35). This route thus represents a potentially dangerous pathway from the gut lumen to the blood, but it abruptly stops shortly after birth. Nevertheless, intestinal absorption of locally administered intact peptides, such as insulin, has been described (38), and later in life, extensive endocytosis from the enterocyte brush border may occur in inflammatory disease states that involve transepithelial breeches in the gut barrier (13). Together, these observations underline the importance of keeping the endocytic activity at the brush border under tight control. However, little is known to what extent mature enterocytes in the healthy state engage in apical endocytosis or how this may occur without compromising the epithelial permeability barrier.

In the present work, we studied endocytic membrane trafficking from the enterocyte brush border of organ cultured mucosal explants by use of a fixable lipophilic FM dye. After rapid incorporation into the microvillar membrane, FM was slowly internalized into early endosomes situated in the subapical terminal web region where it remained for 1 h or longer. A ligand for receptor-mediated endocytosis, cholera toxin B subunit (CTB), increased the apical membrane internalization into the terminal web early endosomes (TWEEs) and also promoted membrane trafficking further into the cell. We conclude that a sluggish constitutive endocytosis does occur at the enterocyte brush border, but that the subapical terminal web, by acting as an intracellular diffusion barrier, inhibits further trafficking. In addition, no internalization of either dextran or two major lipid raft-associated brush border enzymes was observed. This indicates that both soluble cargo and proteins residing in lipid raft microdomains are excluded from the ongoing constitutive endocytosis.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following suppliers: fixable analogs of FM lipophilic styryl dyes (FM 4-64 FX and FM 1-43 FX), Alexa-conjugated secondary antibodies, Alexa-conjugated CTB, and Cascade blue-conjugated dextran (molecular mass ~10,000) from Invitrogen (www.invitrogen.com/), a rabbit antibody to nonmucosal myosin II heavy chain A from Covance (www.CRPinc.com), a rabbit antibody to intestinal alkaline phosphatase from AbD Serotec (www.biogenesis.co.uk/), a mouse monoclonal antibody to early endosomal antigen 1 (EEA 1) from BD Biosciences (www.
bdbeurope.com), an affinity-purified goat antibody to LAMP-2 from Santa Cruz Biotechnology (www.scbt.com), and antifade mounting media from Dako (www.dako.com). A rabbit antibody to intestinal aminopeptidase N was previously described (17).

Pig small intestines were kindly provided by Letty Klarskov and Mette Olesen (Department of Experimental Medicine, the Panum Institute, Copenhagen, Denmark).

**Organ culture of mucosal explants.** Sections of jejunum were surgically obtained from adult pigs by licensed staff and rinsed in ice-cold MEM medium. Mucosal explants were then rapidly excised and cultured for periods up to 3 h as previously described (9). During culture, freshly dissolved FM dye was present in the medium at a concentration of 20 μg/ml. In some experiments, Cascade blue-conjugated dextran (1 mg/ml) or Alexa-conjugated CTB (10 μg/ml) was added to the medium.

**Fluorescence microscopy.** Immediately after culture, the mucosal explants were rinsed in fresh medium and fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (SP), pH 7.2, for 2 h at 4°C. After a rinse in SP, the tissues were frozen in precooled 2-methylbutane and mounted on a precooled cryostat table. Sections were cut in a Leica CM1850 cryostat at −20°C and collected on glass slides. For immunolabeling, the sections were incubated for 1 h at room temperature or overnight at 4°C with primary antibodies (1:50–1:2,000 dilution in 50 mM Tris·HCl, 150 mM NaCl, 0.5% ovalbumin, 0.1% gelatin, 0.05% Tween 20, 0.2% teleostean gelatin, pH 7.4), followed by incubation for 1 h at room temperature with Alexa-conjugated secondary antibodies (1:200 dilution in the above buffer). In all experiments, controls with omission of the primary antibodies were included. The labeled sections were mounted in antifade medium and examined in a Leica DM 4000 B microscope equipped with a Leica DC 300 FX digital camera. Image analysis was performed by using the image processing and analysis program ImageJ with the MBF plug-in compilation (www.macbiophotonics.ca/imagej/). The distance between apical labeled punctae and the brush border was measured with the linear measuring tool. Intensity measurements were made by applying the measurement tool on defined areas of similar size of brush border membrane and the corresponding underlying subapical punctae. The values obtained were corrected by subtracting the background labeling intensity.

**Electron microscopy.** Pieces of small intestine were fixed in 2.5% glutaraldehyde in SP, for 2 h at 4°C. After a wash in the SP, the tissue was treated with osmium tetroxide in SP for 1 h, dehydrated in graded concentrations of acetone, and finally embedded in Epon. Ultrathin sections were cut in a LKB Ultratome III ultramicrotome, stained in 1% uranyl acetate in water and lead citrate, and finally examined in a Zeiss EM 900 electron microscope equipped with a Mega View II digital camera system.

**RESULTS**

The enterocyte brush border is a permeability barrier. Endocytosis is a basal function performed by all cells, reflecting its essential role in the maintenance of cellular homeostasis (24). In the mammalian small intestine, however, endocytosis from the enterocyte brush border essentially ceases shortly after birth, a phenomenon aptly termed “closure.” At the same time, the expression of many digestive brush border enzymes and nutrient transporters sharply increases, enabling a switch from intracellular to extracellular degradation of dietary macromolecules. The poor ability of enterocytes from a mature animal to absorb macromolecules from the luminal side is illustrated in Fig. 1. Thus, after culture for 1 h in the presence of primary antibodies and secondary antibodies, the presence of dextran (Fig. 1A) and FM dye (Fig. 1B) was detected at the luminal surface of enterocytes but not in the underlying cytoplasm.

***Fig. 1.*** Impermeability of the enterocyte brush border to dextran. **A:** fluorescent image of a mucosal explant cultured at 37°C for 1 h in the presence of Cascade blue-conjugated dextran. The fluid phase marker lined the villus surface but was not taken up by enterocytes. **B:** light microscopy of the same image. Bar, 20 μm.

***Fig. 2.*** FM dye rapidly incorporates into the enterocyte brush border. Fluorescent image of a mucosal explant cultured at 4°C for 1 h in the presence of FM dye. The entire brush border was homogeneously labeled, but no intracellular or intercellular staining was detectable (shown in higher magnification in inset). Bar, 20 μm.
of fluorescent dextran (molecular weight ~10 kDa), no internalization of the fluid phase marker was detectable.

**FM dyes as fluorescent probes of apical endocytic membrane trafficking in enterocytes.** FM dyes are water-soluble lipophilic styryl compounds that are nontoxic to cells and only become fluorescent when incorporated into cell membranes. When added to the medium of living cells, they are thought to be membrane impermeable and to insert spontaneously into the outer leaflet of the cell membrane bilayer without compromising the membrane integrity. These properties have enabled FM dyes to be widely used for studying vesicle trafficking in living cells (4), and in the present work we used fixable FM dyes to monitor endocytic trafficking from the brush border of enterocytes by subsequent microscopic analysis of mucosal cryosections.

As shown in Fig. 2, FM was incorporated into the enterocyte brush border of mucosal explants, cultured for 1 h at 4°C. At this temperature, which is nonpermissive for endocytosis, FM labeled the brush border homogeneously, but the dye was not detectable intracellularly. Likewise, no intercellular FM was observed, indicating that the tight junctions remain intact during organ culture. A similar staining was seen already by 15 min, indicating that the dye is rapidly incorporated into the brush border membrane (results not shown). FM dyes therefore seem well suited as probes for endocytic trafficking from the enterocyte brush border membrane in the mucosal culture system.

**Constitutive apical endocytosis to a terminal web-localized compartment.** After culture at 37°C for 15 min in the presence of FM, the labeling was much like that observed after labeling at 4°C, i.e., a dense and homogeneous staining of the enterocyte brush border. However, in some but not all areas along the villi a faint and sparse punctate labeling was observed in the apical terminal web region ~1 μm from the brush border, indicating the formation of apical endocytic vesicles (Fig. 3A). In addition, a massive uptake of dye could be seen in a few of fluorescent dextran (molecular weight ~10 kDa), no internalization of the fluid phase marker was detectable.

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scattered epithelial cells. These cells could be identified morphologically as goblet cells where FM accumulated basally in the cell below the secretory granules (Fig. 3A).

The pentameric toxin CTB binds to ganglioside GM1 at the cell surface (14) and has previously been shown to rapidly induce clathrin-dependent endocytosis from the brush border of cultured mucosal explants (15). In the presence of CTB, both the number and intensity of the terminal web-localized punctae were markedly increased after labeling for 15 min (Fig. 3B).

By 1 h of labeling with FM, most of the dye was still at the enterocyte brush border, but the subapical punctae in the terminal web region were more dense and intense than by 15 min of labeling (Fig. 4A). However, still no labeling was observed to penetrate much deeper than ~1 μm into the cytoplasm. In contrast, in the presence of CTB, most of the FM labeling was seen in numerous intracellular punctae by 1 h, and these were distributed in a broad zone, extending up to ~5 μm into the apical cytoplasm (Fig. 4B).

A quantitative analysis of the uptake of FM dye from the brush border into the supapical punctae is given in Table 1. Collectively, the FM labeling experiments show that endocytic membrane trafficking occurs constitutively from the enterocyte brush border. Moreover, the dye accumulated in a distinct compartment localized ~1 μm below the brush border in the terminal web region of the enterocyte and it largely remained there for as long as 1 h. The presence in the medium of CTB, a ligand for receptor-mediated endocytosis, markedly increased the endocytic trafficking to this terminal web-localized compartment, and it also stimulated further trafficking to compartments localized deeper into the apical cytoplasm.

Conceivably, the observed slow rate of FM internalization could be due to a rapid compensatory membrane recycling, resulting in a small steady-state accumulation of dye in intracellular punctae. To test whether membrane recycling to the brush border occurs in the enterocyte, mucosal explants were labeled for 1 h with FM followed by a chase incubation for 2 h in the absence of dye. Only little dye was left in the brush border by the end of the chase whereas punctae in the terminal web were still prominently labeled, and occasionally punctae were located deeper into the cytoplasm (Fig. 5A). In the presence of CTB, the brush border was now devoid of any labeling, and most of the punctae were relatively large and had

Fig. 5. Endocytosed membrane does not recycle to the apical surface. Fluorescent images of mucosal explants labeled with FM dye for 1 h at 37°C, followed by a chase incubation for 2 h at the same temperature in the absence of dye: A: in the absence of CTB the labeling was still mainly seen as terminal web-associated punctae, although deeper lying punctae were occasionally observed (arrow). The brush border was essentially free of labeling, whereas some diffuse labeling was present in the cytoplasm. B: in the presence of CTB, most of the labeled punctae were relatively large and were scattered deep in the cytoplasm. No brush border labeling was detectable, but a diffuse cytoplasmic labeling was prominent. C: image of epithelial cells in the crypts. Labeled punctae were not present in the apical area but distributed over the entire cytoplasm. N, nuclei. Bars, 10 μm.
progressed deeper into the cytoplasm (Fig. 5B). Together, these experiments indicate that the brush border is not to any significant extent replenished by membrane recycling.

Unlike villus enterocytes studied in the above experiments, immature absorptive cells in the crypts efficiently endocytosed FM dye. Thus, as shown in Fig. 5C, labeled punctae were scattered all over the cytoplasm after 1 h of exposure to FM dye followed by a chase for 2 h. The striking difference between crypt and villus cells indicates that the confinement of endocytosed membrane to the terminal web region depends on the maturation state of the cells.

The terminal web is an apical diffusion barrier. The terminal web is a dense filamentous, myosin-rich structure where the actin cytoskeleton of the microvilli is rooted (34). As shown in Fig. 6A, immunolabeling of nonmuscle myosin II visualized the terminal web as a narrow band localized in the subapical cytoplasm just below the brush border. In addition to providing the anchorage of microvillar rootlets, the terminal web also functions as a barrier, effectively preventing larger organelles, such as mitochondria, from reaching the apical-most region of the enterocyte cytoplasm (Fig. 6B).

TWEEs, a subclass of early endosomes in the enterocyte. As shown in Fig. 7, the terminal web-localized compartments revealed by the FM labeling for 1 h presented above were also positive for EEA-1, thus identifying them as early endosomes. However, in addition, EEA-1 visualized a host of punctae that localized deeper in the apical cytoplasm and were unlabeled by FM. This indicates that those endosomes generated by trafficking from the brush border represent a distinct subclass of early endosomes, narrowly confined to the terminal web region of the enterocytes (“TWEEs”).

The lysosomal marker LAMP-2 revealed the presence of lysosomes that were all localized deeper into the cytoplasm of the enterocytes (Fig. 7). In contrast to EEA-1, LAMP-2 showed no colocalization with FM after 1 h of labeling, indicating that trafficking and maturation of TWEEs to lysosomes must be a slow process.

Constitutive endocytic trafficking to TWEEs is devoid of cargo. Aminopeptidase N and alkaline phosphatase are two of the major lipid raft-associated membrane proteins of the enterocyte brush border (30). As shown in Fig. 8, neither of the two enzymes colocalized with FM in the TWEEs after 1 h of labeling with the dye, indicating that resident proteins of the brush border are excluded from this constitutive endocytic trafficking. This observation thus parallels the failure of dextran to be taken up by the enterocytes (Fig. 1). In the absence of a powerful endocytosis receptor ligand, such as CTB, it therefore seems that the endocytic membrane trafficking to the TWEEs visualized by FM occurs by a mechanism that largely excludes both membrane-associated and soluble cargo molecules.

**DISCUSSION**

The maintenance of the intestinal barrier against pathogens is essential for survival of the organism and is accomplished by a concerted effort involving several independent strategies (13). Thus tight junctions between neighboring epithelial cells prevent entry from the gut lumen into the blood by paracellular diffusion (28), and holes that arise in the epithelium during the rapidly ongoing extrusion of cells are rapidly filled by sealing with an impermeable substance (36). To prevent transepithelial penetration, mucus deposition on the epithelial surface with a thickness of up to several hundred micrometers creates a stable, unstirred layer that acts as a physical barrier (3). Finally, pathogens that get into close contact with the microvillus membrane of the brush border will not be endocytosed because the actin cytoskeleton of the microvillus sterically prevents membrane invagination and formation of endocytic vesicles. Endocytosis is therefore only feasible at the small “microcrypt” areas between neighboring microvilli. Nevertheless, apical endocytosis from these sites represents a possible means for...
pathogens to invade the organism, as exemplified by the entry of CTB (15).

In previous reports, apical endocytosis has been assessed by monitoring the luminal uptake of various macromolecules. Use of fixable FM dyes in the present work enabled us to visualize directly the endocytic membrane trafficking and it demonstrated the existence of a constitutive apical internalization pathway in enterocytes. The fact that internalized FM was only barely detectable by 15 min testifies to the modest endocytic activity at the brush border; by comparison, fibroblasts reportedly internalize more than 50% of their cell surface per hour (33). The FM dye also showed that endocytosed membrane is deposited in early endosomes situated narrowly beneath the brush border. This so-called terminal web region is the part of the enterocyte that harbors the cytoskeletal apparatus of the brush border that is capable of undergoing Ca²⁺- and ATP-dependent contractions (23, 26). The unique morphology of the terminal web has been most impressively revealed by electron micrographs using a quick-freeze, deep-etch visualization technique (18), and, interestingly, such images also revealed the presence of numerous smooth-surfaced vesicles embedded in the dense cytoskeletal meshwork (27). In the absence of a receptor ligand such as CTB, the FM dye-positive TWEEs remained stationary for periods up to at least 1 h. Accordingly, we propose that the terminal web acts as an intracellular diffusion barrier that inhibits endosomal mobility and trafficking further into the cell. The terminal web is an integrated part of the brush border that develops morphologically during the

Fig. 7. A subclass of early endosomes in the terminal web region of enterocytes (TWEEs). Fluorescent images of mucosal explants labeled with FM dye (red) for 1 h at 37°C. After culture and fixation, cryosections were labeled with antibodies (green) to EEA-1 or LAMP-2. Early endosomes are widely distributed in the cytoplasm of the enterocytes, but only those located in the terminal web region (TWEEs) were FM positive. Lysosomes are also seen scattered in the cytoplasm but showed no colocalization with FM-positive punctae in the terminal web region. Bars, 20 μm.

Fig. 8. Major brush border enzymes are not endocytosed. Fluorescent images of mucosal explants labeled with FM dye (yellow) for 1 h at 37°C. After culture and fixation, cryosections were labeled with antibodies (red) to aminopeptidase N (ApN) or alkaline phosphatase (AP). Neither of the 2 brush border enzymes was detectable in the FM-positive TWEEs. Bars, 20 μm.
crypt-villus cellular migration and maturation, and the significance of this barrier was revealed by the lack of TWEEs in the epithelial cells of the crypts. That the barrier represented by the cytoskeletal brush border and terminal web architecture may indeed contribute to the overall mucosal defense is exemplified by antigen transport studies in inflammatory bowel disease. Here it was revealed that luminal antigens are preferentially taken up by a subpopulation of enterocytes termed “RACE” cells (21). These cells, which may represent senescent enterocytes, displayed increased and rapid antigen transport to late endosomes and were morphologically characterized by a disassembled apical cytoskeleton. In an in vivo study in Crohn’s ileitis by the same group, it was observed that luminal antigens accumulated in multivesicular late endosomes where they encountered major histocompatibility complexes I and II, indicating that enterocytes might contribute to the local antigen presentation to T cells in this disease (19).

In addition to being stationary for long periods of time, the TWEEs visualized by the FM dye were devoid of alkaline phosphatase and aminopeptidase N, indicating that resident brush border proteins escape constitutive endocytosis. Like several other major brush border enzymes they both reside in lipid rafts, and in the intestine these membrane microdomains are stabilized by lectins that cross-link glycolipids and glycoproteins (8). Conceivably, the resulting supermolecular complexes may resist inclusion into areas that have the strong negative membrane curvature required to form an invagination. Interestingly, we recently observed a transient and selective apical endocytosis of alkaline phosphatase during fat absorption (16), suggesting that this physiological process somehow disturbs the local membrane organization. The underlying mechanism is not fully understood, but altered membrane properties caused by an observed increase in the microvillar contents of free fatty acids might explain this phenomenon.

In conclusion, the results obtained by using the FM dye in the present work have revealed some novel properties of endocytic membrane trafficking from the brush border. Firstly, albeit constitutive, endocytosis occurs at a slow rate compared with other cell types. Secondly, once internalized into a sub-apical population of TWEEs, trafficking further into the cell is arrested, probably because of steric inhibition by the actomyosin-based terminal web. Together, these properties minimize the access of pathogens from the gut lumen into the blood and thus contribute to the overall maintenance of the permeability barrier operating in the gut. Finally, use of the FM dye should be a promising tool for a further and more detailed characterization of apical endocytosis, for instance in different regions of the gut and in the presence of agents such as physiological ligands or endocytosis inhibitors.

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GRANTS

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