The transcytosis of divalent metal transporter 1 and apo-transferrin during iron uptake in intestinal epithelium

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Caco-2 cells grown in bicameral chambers are a model system to study intestinal iron absorption. Caco-2 cells exhibit constitutive transport of iron from the apical (luminal) chamber to the basal (serosal) chamber that is enhanced by apo-transferrin in the basal chamber, with the apo-transferrin undergoing endocytosis to the apical portion of the cell. With the addition of iron to the apical surface, divalent metal transporter 1 (DMT1) on the brush-border membrane (BBM) undergoes endocytosis. These findings suggest that in Caco-2 cells DMT1 and apo-transferrin may cooperate in iron transport through transcytosis. To prove this hypothesis, we determined by confocal microscopy that, after addition of iron to the apical chamber, DMT1 from the BBM and Texas red apo-transferrin from the basal chamber colocalized in a perinuclear compartment. Colocalization was also observed by isolating endosomes from Caco-2 cells after ingestion of ultra-small paramagnetic particles from either the basal or apical chamber. The isolated endosomes contained both transferrin and DMT1 independent of the chamber from which the paramagnetic particles were endocytosed. These findings suggest that iron transport across intestinal epithelia may be mediated by transcytosis.

Caco-2 cells; intestinal iron transport

Iron transport across the intestinal epithelium is complex and is usually thought of as occurring in three phases. In the first or uptake phase, Fe(III) at the brush-border membrane (BBM) is reduced by a ferrreductase (18) and then transported into the cell via the divalent metal transporter 1 (DMT1; also known as DCT1 and NRAMP2) (10, 14). In the second or transcellular phase, iron is transported across the cell to the basolateral surface. The transport may be via vesicular trafficking, as supported by our recent demonstration that DMT1 on the BBM of rat intestinal epithelium is internalized into vesicles when the rat is fed a bolus of iron (34). Alternatively, Fe(III) may be transported across the cell on chaperones such as the calreticulin-like protein mobilferrin (32). In the third phase, iron is transported out of the cell across the basolateral membrane. Recently, two proteins, ferroportin1 (also known as MTP and IREG1) (1, 7, 19) and hephaestin (33), have been hypothesized to be involved in basolateral membrane iron transport. Ferroportin1 is highly expressed in the duodenum and when transfected into Xenopus oocytes allows for the efflux of iron transported into the oocytes by DMT1. Hence, ferroportin1 is postulated as the ferrotransporter on the basolateral surface of intestinal epithelium (7). Hepsaestin is a multicopper oxidase with homology to ceruloplasmin. In the sIab mouse, a mutation in hephaestin impedes the transport of iron out of the enterocyte, suggesting that, although iron is transported into the cell as Fe(II), transport out of the cell requires oxidation to Fe(III) (33).

Iron homeostasis is regulated primarily at the level of intestinal iron uptake. With iron deficiency, iron transport into the epithelium is increased, as is the efficiency of iron transport from the intestinal into the systemic circulation. Conversely, iron overload decreases both the uptake into the mucosa and transport out of the intestine. The regulation of intestinal iron transport is controlled by the gene HFE, as evidenced by the increased iron transport that occurs with the C282Y mutation in HFE seen in hemochromatosis (8). The mechanism by which HFE regulates iron uptake is not yet certain (11, 16). HFE interacts with the transferrin receptor to modulate transferrin binding to the receptor (9). The mutated HFE found in hemochromatosis no longer binds to the transferrin receptor, and the cells functionally act as if iron deficient, although variable effects on DMT1 expression have been reported (11, 16). Recently, hepcidin, a liver-expressed antimicrobial peptide, has been implicated in regulation of iron transport out of the intestinal epithelium (12, 20).

The Caco-2 cell line grown in bicameral chambers has been used as a system to model intestinal iron uptake (2, 3, 5). These cells form a polarized monolayer when grown on a semiporous membrane, allowing
demonstration of unidirectional iron transport from the apical to the basal chamber. Caco-2 cells have the components currently known to be required for iron transport, including DMT1, hephaestin, ferroportin1, and HFE. As in the intestine, iron deprivation of Caco-2 cells increases DMT1 expression. Iron transport is regulated similarly to that seen in the intestine, with iron transport being inversely proportional to the iron status of the cells. Although there is constitutive transport of iron across the Caco-2 cells, the transport of iron is stimulated by the presence of apo-transferrin in the basal chamber (3). Apo-transferrin can bind to the basolateral surface of the Caco-2 cells (23). By confocal microscopy, it is possible to demonstrate the endocytosis of apo-transferrin to a perinuclear location, in contrast to the basal compartment into which ferrotransferrin is transported (4).

Our laboratory has recently demonstrated (34) that on ingestion of iron DMT1 on the BBM of intestinal epithelium is internalized. That apo-transferrin is also internalized suggests that DMT1 and apo-transferrin may interact via transcytosis. Transcytosis in Caco-2 cells has been well described and in the context of iron transport could serve as a regulatory phenomenon, sequestrating DMT1 from the BBM and limiting iron uptake. Alternatively, or possibly additionally, transcytosis of DMT1 could provide a route by which iron may be transported across the cell. In this hypothesis, iron in vesicles internalized from the apical surface would interact with vesicles derived from the basal surface. The iron would be transferred to the basal surface-derived vesicles and then be transported out of the cell, presumably by ferroportin1. To substantiate that vesicles containing DMT1 do undergo transcytosis, we used confocal microscopy to demonstrate that DMT1 internalized from the BBM of Caco-2 cells localized in the same perinuclear compartment as basolateral-derived vesicles bearing apo-transferrin. Evidence of a pool of endosomes that contained both DMT1 and apo-transferrin was provided by isolation of endosomes from the Caco-2 cells by use of ultra-small paramagnetic beads (21, 26, 30). In the absence of iron in the apical chamber, the endosomes contained DMT1 as well as transferrin. The addition of iron to the apical chamber increased the DMT1 and transferrin content of the endosomes, with a marked increase in the amount of DMT1 relative to transferrin. Furthermore, when the apical chamber contained 59Fe and the basolateral chamber contained 125I-apo-transferrin, both moieties were found in endosomes containing the paramagnetic particles. Together, these observations suggest that, after internalization, DMT1 is involved in transcytosis and that this process, in cooperation with apo-transferrin, might provide a mechanism for iron passage from the apical to the basolateral membrane of Caco-2 cells.

MATERIALS AND METHODS

Cell culture. Caco-2 cells (HTB 37) from the American Type Culture Collection (Rockville, MD) were maintained in DMEM supplemented with 10% FBS (GIBCO, Gaithersburg, MD), 1% nonessential amino acids, and antibiotics/antimycotic (100 U/ml penicillin-G, 100 U/ml streptomycin, and 250 ng/ml Fungizone; GIBCO). Cells were grown in 6-mm-diameter Transwell bicameral chambers with 0.4-μm pore size membranes (Costar, Cambridge, MA) coated with collagen. The collagen film was applied to the filter as 50 μl of collagen solution (3 mg/ml, 60% ethanol, rat tail, type I; Boehringer-Mannheim, Mannheim, Germany), and then the Transwells were inverted and dried under sterile laminar airflow. Formation of a Caco-2 cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicell electrical resistance system (Millipore, Bedford, MA). Confocal microscopy experiments were performed only after the TEER had risen to a level indicating the formation of an intact monolayer (TEER at least 250 Ω·cm2) (2). Typically, the cell monolayers were used after 12–14 days in culture. The cell monolayers were depleted of serum proteins as previously described (3). Before each experiment, cell monolayers were washed with iron-free DMEM, without FBS, incubated for 20 min in DMEM three times, and transcytosis to a new well for at least 1 h before the start of the experiment.

Fluorochromes and transferrins. Texas red-labeled human transferrin and TO-PRO-3 were obtained from Molecular Probes (Eugene, OR). The labeled transferrin was reconstituted in water and rendered to the iron-free, apo form by lowering the solution pH to 4.5 with Na citrate in the presence of Chelex resin (30% vol/vol) under constant stirring. The pH of the solution was raised to 7.0 by dialysis against 0.15 M NaCl-10 mM HEPES (pH 7.0) in the presence of Chelex. The concentration of apo-transferrin was adjusted to 100 μM and made 100 μM in desferoxamine, frozen, and used within 6 mo. For experiments utilizing 125I-apo-transferrin, ferri-transferrin was labeled with 125I as previously described before preparation of apo-transferrin (17).

Laser scanning confocal microscopy and image analysis. The Caco-2 cell monolayers were observed under a laser scanning confocal microscope (Bio-Rad MRC 1024 scan head; Nikon Diaphot microscope). Images were collected utilizing a ×60 Nikon (apo-planar DIC) oil objective. Images for analysis were collected at a 512 × 512-pixel resolution. The Caco-2 cell monolayer was optically sectioned in the z-axis with a step size of 2 μm to give ~12 sections per imaged field. The images were analyzed by Lasersharp software (Bio-Rad), and pictures and graphs were generated in Adobe Photoshop and Excel (v. 6.0; Microsoft), respectively.

Antibodies to DMT1. The production of an antibody against the DMT1 protein has been described previously (34). In brief, we synthesized a polypeptide with amino acid sequences deduced from the iron-responsive element (IRE) isoform of rat DMT1 cDNA (14) consisting of amino acids 540 to 553 (CGRSVSISKVILSE) near the COOH terminus of the protein. The polypeptide was covalently linked to keyhole limpet hemocyanin, and the conjugate was used to inoculate New Zealand White rabbits. The antiserum, designated anti-540, has been previously shown to be specific to DMT1 (34).

Immunohistochemistry. Caco-2 cells grown to confluence on semipermeable membranes were washed extensively with PBS for 15 min, fixed with 2% paraformaldehyde in PBS for 20 min at 4°C, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then washed extensively with PBS and blocked with 5% BSA and 1% goat serum in PBS for 1 h at room temperature. The cells were then incubated with anti-540 antibody or preimmune rabbit serum at a 1:500 dilution in PBS for 1 h at room temperature. Following extensive washing with PBS, the cell layers were incubated with Alexa 488-labeled goat anti-
rabbit IgG (Molecular Probes) at a 1:500 dilution and with a 1:1,000 dilution of TO-PRO-3 for 1 h at room temperature. The cell monolayers were then washed extensively with PBS and mounted with ProLong Antifade kit (Molecular Probes).

**Isolation of endosomes with superparamagnetic colloidal particles.** Superparamagnetic colloidal iron particles coated with dextran with an average diameter of 8 nm were prepared by the procedure described by Rodriguez-Paris and colleagues (21, 26, 30). Caco-2 cells were incubated with the paramagnetic particles either in the basal or apical chamber for 20 min. The cells were washed extensively with Hanks’ balanced saline-1 mM EDTA, scraped from the membrane, and disrupted by two passages in a stainless steel homogenizer (clearance 0.12 mm) in homogenization buffer [250 mM sucrose, 10 mM Tris (pH 7.4), 2 mM EDTA, 1 mM PMSF].

The homogenate was centrifuged at 1,000 g for 5 min, and the postnuclear supernatant was passed over a stainless steel wire mesh column surrounded by a 1.2-T magnet. The column was washed extensively, and endosomes containing the paramagnetic particles were collected by turning off the magnet, rinsing the column with homogenization buffer, and pelleting the endosomes at 45,000 g for 1 h at 4°C.

**Statistical analysis.** Where indicated, statistical comparisons were made by using the unpaired Student’s t-test.

**RESULTS**

**Anti-540 detects DMT1 in Caco-2 cells.** The anti-540 antiserum is directed to the unique COOH terminus of the IRE-containing isoform of rat DMT1. The antiserum has been shown by us (34) to interact specifically with DMT1 from rat tissues. Although the amino acid sequences in DMT1 are highly conserved among species, the unique COOH-terminal amino acid homology between rat and human is ~70%. It was important then to demonstrate that anti-540 did interact specifically with DMT1 from Caco-2 cells, a cell line derived from human intestine. Figure 1 shows the Western blot of Caco-2 lysates compared with rat duodenal lysates separated by SDS-PAGE. In both the Caco-2 cells (lane 1) and the rat lysate (lane 2), anti-540 detected a band of ~43 kDa that was eliminated by the presence of the immunizing peptide (lanes 3 and 4).

**DMT1 is expressed on the BBM of Caco-2 cells and colocalizes with Texas red apo-transferrin after the addition of ferrous ascorbate.** DMT1 has been demonstrated on the BBM of rat (14), mouse (6), and human intestinal epithelium (31). To demonstrate that DMT1 is on the BBM of Caco-2 cells, cells starved of iron overnight were exposed to ferrous ascorbate in the apical chamber and Texas red apo-transferrin in the basal chamber. After 20 min, the cells were washed extensively and the membrane holding the cell layer was embedded in JB-4 embedding solution (Polyscience, Warrington, PA), sectioned, and stained with anti-540 antisera. As seen by immunofluorescence microscopy (Fig. 2A), DMT1 is clearly expressed on the apical surface of the cells, with some vesicles apparent in the upper portion of the cell. Texas red apo-transferrin can also be seen to have been transported well into the cells. (Under the conditions of immunofluorescence microscopy, the TO-PRO-3-stained nuclei also appear red, giving the red “block” appearance in some cells).

In subsequent experiments, the distribution of DMT1 was examined by confocal microscopy at various times after the addition of 1.0 μM Fe(II) to the apical chamber and 1 μM Texas red-apo-transferrin to the basal chamber. After being fixed and stained with anti-540, the Caco-2 cells were then examined by confocal microscopy, with the cell layer being optically sectioned in the z-axis in 2-μm sections. At t = 0 min (Fig. 2, B-1 and B-2), DMT1 was detected only in the two uppermost sections, a depth of 4 μm from the apical surface. For brevity, the remaining sections are not shown. In these views along the z-axis before the addition of iron, DMT1 was above the level of the nuclei, which were detected by staining with TO-PRO-3. At t = 60 min, DMT1 had advanced into the cell and could be detected in perinuclear sections, as indicated by the blue fluorescence of the nuclei (Fig. 2, C-1, -2, and -3). These sections were at a depth of ~6–12 μm from the apical surface. At the same time, Texas red apo-transferrin could now also be seen in perinuclear sections (Fig. 2, D-1, -2, and -3). These sections are also at a depth ~6–12 μm from the apical surface. By superimposition of Fig. 2, C (red) and D (green), it was possible to demonstrate that DMT1 and apo-transferrin colocalized, as shown by the yellow in a perinuclear section (Fig. 2E-1). The colocalization can also be visualized by reconstructing the cell layer as if viewed from a lateral position (Fig. 2E-2). In this view, it is possible to see DMT1 (green) in the apical portion of the cells, Texas red apo-transferrin (red) in a more...
basal position, with the yellow representing the colocalized compartment of DMT1 and apo-transferrin situated between the two in a perinuclear position. With the addition of ascorbate alone to the apical chamber, we observed neither internalization of DMT1 nor colocalization of DMT1 with Texas red apo-transferrin (data not shown).

After the addition of iron to the apical chamber, the progression with time of DMT1 and apo-transferrin into the Caco-2 cells was quantified. For each time point, the total pixels were summed separately for each of the two entities, DMT1 and Texas red apo-transferrin. The pixels per section for each entity were then expressed as a percentage of the total pixels for that entity in the cells being observed at that time point. As seen in Fig. 3, after only 10 min of exposure to iron DMT1 had internalized into the cells. Maximum internalization occurred by 40–60 min. Intriguingly, with additional time of exposure to iron, DMT1 began to shift to more apical sections. Texas red apo-transferrin rapidly appeared in apical sections, and then between 60 and 120 min the distribution shifted to more basal areas. The change in distribution of Texas red apo-transferrin reflects the continued presence of Texas red apo-transferrin in the basal chamber with continued endocytosis followed eventually by exocytosis.

**Does the internalization of DMT1 result in increased DMT1 catabolism?** The apparent shift of DMT1 back toward the apical portion of the cell seen in Fig. 3A could result from DMT1 undergoing exocytosis and returning to the BBM. Alternatively, with internalization DMT1 might undergo degradation and be replaced by newly synthesized DMT1 targeted to the apical surface. To distinguish between these two possibilities, the synthetic and degradation rates of DMT1 were analyzed. After overnight incubation without iron in the apical chamber, the Caco-2 cells were incubated with $[^{35}\text{S}]$methionine in methionine-free medium in the apical chamber in the presence or absence of apical iron. Cells were lysed at various times up to 3 h, DMT1 was isolated by immunoprecipitation with anti-540, and the $[^{35}\text{S}]$methionine radioactivity was determined by autoradiography of SDS-PAGE separation of the immunoprecipitated DMT1 (Fig. 4A). The relative rates of synthesis in arbitrary units from scanned autoradiographs were 223.7 ± 47.6 and 216.9 ± 28.2 for cells exposed or not exposed to apical iron, respectively (means ± SE of 3 experiments). The failure of a
bolus of iron to stimulate synthesis of DMT1 in Caco-2 cells has been demonstrated previously (27).

To study the degradation of DMT1, Caco-2 cells were labeled with [35S]methionine for 1 h. The apical medium was replaced with buffer containing nonradioactive methionine in the presence or absence of apical iron, and the [35S]methionine radioactivity in DMT1 was determined after 3 h. The radioactivity in DMT1 was determined from scanned autoradiographs (Fig. 4A). The relative rates of DMT1 synthesis and degradation in Caco-2 cells has been demonstrated previously (27).

Fig. 3. The rate of internalization of DMT1 and Texas red apo-transferrin. Caco-2 cells grown to confluence were examined by confocal microscopy at various times after the addition of 1 μM ferrous ascorbate to the apical chamber and 1 μM Texas red apo-transferrin to the basal chamber. The cell layers were sectioned in 2-μm sections in the z-axis. At each time point, the total numbers of pixels were summed for DMT1 and apo-transferrin. A: percentage of DMT1 present in each section for each time point.

B: percentage of Texas red apo-transferrin. Section 1 is the most basal section and section 12 the most apical. The perinuclear sections, as defined by nuclear staining with TO-PRO-3, extend from about section 6 to 10.

Fig. 4. The relative rates of DMT1 synthesis and degradation in Caco-2 cells. With the use of the techniques detailed in MATERIALS AND METHODS, Caco-2 cells were grown to confluence on 24-mm semiporous membranes. Before the synthetic and degradative rates of DMT1 were determined, the cells were grown overnight in the absence of iron. A: DMT1 synthetic rate was determined by incubating the Caco-2 cells for 3 h with 100 μCi/ml [35S]methionine in medium without methionine in the presence (+ Fe) or absence (- Fe) of 1 μM ferrous ascorbate in the apical chamber. B: to determine the degradation of DMT1, the Caco-2 cells were labeled with [35S]methionine for 1 h (t = 0). The apical medium was replaced with nonradioactive methionine in the presence or absence of apical 1 μM ferrous ascorbate for 3 h. The radioactivity of DMT1 was determined by immunoprecipitation of DMT1 with anti-540, followed by SDS-PAGE separation of the immunoprecipitate and detection of the [35S]methionine radioactivity by autoradiography. The radioactivity was quantitated by scanning a PhosphorImager and quantitated with the ImageQuant software. Representative autoradiograph of 3 experiments is shown.
ferri-transferrin 20 min after the addition of iron to the apical chamber and ferri-transferrin to the basal chamber. Ferri-transferrin remained in the basal portions of the cells, and no colocalization was observed with DMT1 at 20 min, nor was colocalization observed with longer incubation times (data not shown). Figure 6A is the reconstruction 20 min after the addition of iron to the apical chamber and FITC-dextran to the basal chamber. Although FITC-dextran migrated to a perinuclear region, only very slight colocalization was seen with DMT1. These experiments support that Texas red apo-transferrin is marking a unique population of vesicles that interact with vesicles derived from the apical surface and that contain DMT1.

DMT1 and iron are present in endosomes derived from the basolateral membrane. To further demonstrate that DMT1 undergoes endocytosis and that the endosomes carrying DMT1 interact with endosomes derived from the basolateral membrane, we made use of the ability to separate endosomes with ultra-small paramagnetic colloidal particles. In these experiments, the ultra-small particles are trapped within the endosomal space and serve as a marker for the endosomes, as previously described both in Dictyostelium (21, 26) and mammalian cells (30). In the first set of experiments (Fig. 7A), endosomes were isolated from Caco-2 cells that had been incubated without apical iron and with (lane 3) or without (lanes 1 and 2) apo-transferrin and with magnetic particles added either to the basal chamber (lanes 1 and 3) or the apical chamber (lane 2). The isolated endosomes were subjected to SDS-PAGE and transferred to nylon membranes, and DMT1 and transferrin were detected by Western blotting. Protein concentrations in all three lanes were similar, as judged by staining the membranes with Ponceau red before Western blotting. Similar amounts of DMT1 were present whether magnetic beads were placed in the basal (lane 1) or apical (lane 2) chamber. Since by confocal microscopy we never observe endocytosis of apical DMT1 unless iron is present in the apical chamber.

Fig. 6. Subcellular localization of DMT1 and Texas red ferri-transferrin and FITC-dextran in Caco-2 monolayers. As detailed in MATERIALS AND METHODS, 1 μM ferrous ascorbate was added to the apical chamber of Caco-2 cells grown to confluence on semiporous membranes and either 1 μM FITC-dextran (molecular weight 70,000; A) or 1 μM Texas red ferri-transferrin (B) was added to the basal chamber. At 20 min after the additions, cells were examined by confocal microscopy with anti-540 antiserum to detect DMT1 and direct immunofluorescence to detect Texas red ferri-transferrin and FITC-dextran. In A, DMT1 was detected using Alexa-594-labeled goat anti-rabbit IgG, and hence the red fluorescence for DMT1 in contrast to the green fluorescence in B.
ber, these findings suggest that there is constitutive endocytosis of the small amounts of DMT1 present on the basolateral surface of the cells and that vesicles containing this DMT1 meet with endosomes from the apical surface. When apo-transferrin was added to the basal chamber (lane 3) and magnetic beads were in the apical chamber, the isolated endosomes contained apo-transferrin and amounts of DMT1 similar to that observed in lanes 1 and 2. These experiments support the observation of constitutive endocytosis of apo-transferrin and the interaction of these endosomes with apical-derived endosomes, as identified by the endocytosis of beads from the apical chamber.

In the experiments represented in Fig. 7B, we studied the effect of adding iron to the apical chamber on the relative amounts of DMT1 and transferrin in the isolated endosomes. In all three lanes, similar amounts of total protein were present, yet in the absence of iron (lane 1) the amount of DMT1 and transferrin was always less then when iron was present in the apical chamber (lanes 2 and 3). Furthermore, in the absence of iron (lane 1), the ratio of DMT1 to transferrin in arbitrary units was 0.38 ± 0.07 (mean ± SD of 4 experiments). With iron present in the apical chamber (lanes 2 and 3) the ratios were 0.91 ± 0.18 and 0.79 ± 0.12 (means ± SD of 4 experiments) for paramagnetic particles in the basal and apical chambers, respectively. These differences were highly significant for no iron vs. apical iron whether the magnetic particles were in the basal chamber (lane 2; \( P = 0.0003 \)) or the apical chamber (lane 3; \( P = 0.0012 \)). There was no significant difference in the relative proportions of DMT1 to transferrin whether the paramagnetic beads were added to the basal or apical chamber. In similar experiments with \(^{59}\)Fe added to the apical chamber and \(^{125}\)I-apo-transferrin to the basal chamber, \( \sim 3 \times 10^{-14} \) mol of each moiety were found in the endosomes.

**DISCUSSION**

The transport of iron across the intestinal epithelium can be thought of as proceeding through three phases: transport across the BBM, intracellular transport from the apical portion of the cell to the basolateral compartment, and transport across the basolateral membrane and into the systemic circulation. In addition, the process of iron transport is regulated to
allow for maintenance of iron homeostasis. A number of the components necessary for iron transport and regulation of the transport have been defined, especially those required for the uptake of iron into the cell and transport of the iron out of the cell. In the first phase, a ferrireductase located on the BBM is required (18, 22, 25). Then DMT1 located on the brush border of the intestinal epithelium transports Fe(II) into the cell (14, 31). For transport of iron out of the cell, ferroportin1 (also known as IREG1 and MTP) (1, 7, 19) and hephaestin (33) have been implicated. How iron is transported from the apical transporter to the basolateral transporter is as yet unknown. Separate pathways have been postulated for ferric and ferrous iron (32), and chaperones have been proposed to transport iron across the cell (32). The data presented in this report support a model of vesicular transport of the apical transporter DMT1 that may involve transcytosis with apical-derived vesicles interacting with basolateral-derived vesicles.

DMT1 has been identified on the BBM of intestinal epithelium (6, 30, 34). In mouse intestine, DMT1 was visualized in the brush border but also with a punctate appearance in the apical half of the cells, suggesting that DMT1 was present in vesicles (6). In other cell types, DMT1 has been identified both in early and late endosomes (13, 29, 31). In cells, such as erythroid precursors, in which ferri-transferrin is endocytosed into the cells bound to the transferrin receptor, it is not surprising that DMT1 would be located in endosomes because it is only after endocytosis of the transferrin-transferrin receptor complex that iron transport occurs. In K-562 cells, DMT1 is found in late endosomes or lysosomes, colocalizing with lysosomal-associated membrane protein 1 but not with transferrin receptor (29). This observation suggests that endosomes undergo fission, with vesicles containing the transferrin-transferrin receptor complex recycling to the membrane while a portion of the vesicles carrying iron and DMT1 fuse with lysosomes.

In intestinal epithelium, the question arises as to why DMT1 should undergo endocytosis. Is the internalization of DMT1 required for the transport of iron into the intestinal mucosa? That is, are iron and DMT1 located in the same internalized vesicle? Or does the internalization of DMT1 in some way regulate the uptake of iron? A regulatory role is suggested by the behavior of another metal transporter, the Menkes protein (ATP7A; MNK) that is involved in copper transport (24). The protein is normally found in the trans-Golgi network. Under elevated copper conditions, the Menkes protein is transported to the plasma membrane via clathrin-coated endosomes. During the exocytosis, the protein pumps copper from the cytosol into the vesicles (24). However, in Caco-2 cells the uptake of iron is linear for at least 3 h (Refs. 2 and 3 and unpublished studies), indicating that the internalization of DMT1 does not acutely decrease iron accumulation. In addition, in the current studies within the range of iron concentrations tested (0.1–10 μM), there was no difference in the extent of DMT1 internalized (data not shown). Hence, it seems more likely that DMT1 is involved in the vesicular trafficking of iron. Perhaps the internalization directs iron in apical vesicles to the components of the basolateral membrane required for iron transport of the cell.

It was with this reasoning that, having observed the vesicular uptake of DMT1 into Caco-2 cells, we examined whether DMT1 colocalized with vesicles derived from the basolateral surface. We used two markers for vesicles derived from the basolateral surface: apo-transferrin and ultra-small paramagnetic colloidal particles. We have previously shown that, when Caco-2 cells were grown in bicameral chambers, apo-transferrin offered from the basal chamber increased the constitutive transport of iron by increasing the efficiency of transport of iron out of the cell (3). Also, by confocal microscopy apo-transferrin could be visualized reaching a perinuclear compartment in the apical portion of the cells, whereas internalized ferri-transferrin remained in a more basal compartment (4). In the current studies, we show that DMT1 and apo-transferrin colocalize and then separate with the apo-transferrin signal decreasing, suggesting its exit from the cells. The colocalization was specific, because no colocalization was observed with ferri-transferrin, which remained in the basal portion of the cells. Also, FITC-labeled dextran, which was internalized to the perinuclear region (as well as to the more basal portions of the cells), did not colocalize with DMT1. These studies illustrate that there is specificity to vesicle trafficking and subsets of vesicles. Those whose intravesicular space is marked with FITC-dextran distribute throughout the cell but do not colocalize with apical-derived vesicles carrying DMT1 in the membrane. Likewise, those vesicles with ferri-transferrin bound to the transferrin receptor have a different behavior than those bound with apo-transferrin. These results also support the observation of Nunez et al. (23) that, in contrast to other cell types, apo-transferrin has significant interactions with Caco-2 cells.

The paramagnetic particles offered in the basal chamber also served as a marker for vesicles derived from the basolateral membrane. In the absence of iron in the apical chamber, DMT1 was present in the endosomes isolated by the magnetic column with a ratio of DMT1 to transferrin significantly less than when iron was present. Both other laboratories (6) and ours (34) have shown that some DMT1 is present in the basolateral membrane and therefore might very well be present in vesicles undergoing endocytosis from the basolateral surface. With the addition of iron to the apical chamber, a maneuver that increases DMT1 endocytosis from the apical surface, the amount of DMT1 increased markedly relative to transferrin in vesicles isolated with the simultaneous exposure of the cells to magnetic particles in the basal chamber. This finding is consistent with apical-derived vesicles containing DMT1 fusing with basolateral-derived vesicles, which contained the magnetic particles. That the same relative amounts of DMT1 and transferrin were in endosomes isolated after the addition of paramagnetic par-
ticles to the apical chamber is again consistent with apical-derived endosomes containing DMT1 fusing with basolateral-derived vesicles containing transferrin. Furthermore, that the endosomes contained roughly equivalent amounts of $^{59}$Fe and $^{125}$I-apo-transferrin suggest that vesicles that contain DMT1 and transferrin also contain iron. Together, the colocalization of apo-transferrin with DMT1 observed by confocal microscopy and the results of vesicle isolation by paramagnetic beads suggest that at least some of the BBM DMT1 fuses with vesicles from the basolateral surface, as identified by containing either apo-transferrin or ultra-small paramagnetic particles. Further studies involving vesicle isolation will be required to quantify the fraction of iron that is transported across the Caco-2 cells by transcytosis.

The process of transcytosis involving transferrin has been previously described as occurring in Caco-2 cells (15). In these studies, apical uptake either of a particulate or a soluble marker could be demonstrated to interact with endocytic vesicles derived from the basolateral surface and containing transferrin. Mixing of the apical and basal labels took place in the apical portion of the cells. That DMT1 and apo-transferrin colocalize raises many questions. What cytosolic proteins mediate the endocytosis of DMT1 and the interaction of apical-derived vesicles with basolateral vesicles containing apo-transferrin? How is iron passed from apical vesicles to basolateral vesicles? Are there roles for ferroportin1 and hephaestin in the process of transcytosis? One can hypothesize that ferroportin1, with a structure of multiple transmembrane domains, may serve both as a dock for vesicles containing iron and to transport iron out of the cells from the vesicles. Hepsheastin as an oxidase may reside in the basal vesicles and be required for oxidation of iron, either to allow binding to transferrin or for export from the cell in the absence of apo-transferrin.

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