Iron Imports.

V. Transport of iron through the intestinal epithelium

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Iron Imports. V. Transport of iron through the intestinal epithelium. Am J Physiol Gastrointest Liver Physiol 290: G417–G422, 2006; doi:10.1152/ajpgi.00489.2005.—Iron absorption across the brush-border membrane requires divalent metal transporter 1 (DMT1), whereas ferroportin (FPN) and hephaestin are required for exit across the basolateral membrane. However, how iron passes across the enterocyte is poorly understood. Both chaperones and transcytosis have been postulated to account for intracellular iron transport. With iron feeding, DMT1 undergoes endocytosis and FPN translocates from the apical cytosol to the basolateral membrane. The fluorescent metalloprotein calcein offered to the basolateral surface of enterocytes is found in endosomes in the apical compartment, and its fluorescence is quenched when iron is offered to the apical surface. These experiments are consistent with vesicular iron transport as a possible pathway for intracellular iron transport.

Caco-2 cells

THE PHYSIOLOGY OF MANY ASPECTS of the transport of iron across the intestinal epithelium has been defined for some time: absorption and transport into the systemic circulation occurs only in the duodenum and upper jejunum, acidification of the intestinal lumen appears essential, and both absorption and transport are inversely proportional to body iron stores (5). The effects of exogenous factors on absorption have also been well defined. That the composition of food can have salutary or deleterious effects on iron absorption has been important in determining the causes of iron deficiency, which, to this day, is a world-wide problem. Likewise, that the presence of hemolysis can affect (increase) iron absorption is a factor in the pathophysiology of several common diseases such as sickle cell disease and the thalassemias. Despite the efforts of a number of investigators, the actual molecular mechanisms underpinning the physiology of iron absorption have escaped definition until recently. Many aspects of the molecular mechanisms of intestinal iron uptake are well reviewed in other contributions to this series, and, as might well be expected, the more the mechanisms that were defined, the greater the complexity of the iron uptake system. For example, HFE, a MHC class I-like protein encoded on chromosome 6, was defined as the gene mutated in classic hemochromatosis and that led to the increased iron absorption characteristic of this disease. Yet, although iron is taken up by every cell and HFE is ubiquitously expressed throughout the body, dysregulation of iron transport is seen in only two cell types: enterocytes and macrophages. This enigma, that the HFE mutation affects iron uptake only in the intestine and macrophages, has been partially explained by the discovery that hepcidin (Hepc) is the iron stores regulator. Expressed in hepatocytes, hepcidin expression is regulated by iron flux into hepatocytes, which, in turn, is regulated by the interaction of transferrin receptor and transferrin, an interaction modulated by the interaction of HFE with transferrin receptor (19). Hepc then affects the expression of ferroportin 1 (FPN) (SLC40A1; also known as IREG1 and MTP1), the protein necessary for iron efflux from the intestine and macrophages. In this scenario, Hepc binds to FPN causing the internalization of FPN and its degradation in lysosomes (20). It is also clear that neither DMT1 nor FPN operates alone as a transporter of iron. Duodenal cytochrome d (DcytB) is a ferric reductase located on the brush-border membrane that reduces Fe(III) to Fe(II) and provides the substrate for iron transport into the cell by DMT1 (SLC11A2) (15). At the basolateral surface, export of iron requires oxidation of the ferrous ion via hephaestin (Heph), a multicopper oxidase (27). Mutation of Heph in sex-linked anemia (Slal) mice leads to iron accumulation in the epithelia and results in Slal mice having a systemic iron deficiency. Lost in this complex transport and regulatory system is the need for iron to traverse the intestinal epithelium. Absorbed iron gains entry through DMT1 and exits via FPN, but how iron passes from the first transporter system to the second needs to be defined.

MOVEMENT OF IRON TRANSPORTERS

The principles of iron transport are dictated, in part, by the chemistry of iron. Fe(III) is extremely insoluble at physiological pH, and both Fe(III) and Fe(II) are well known to be extremely reactive, generating reactive oxygen species through the Haber-Weiss reaction. The properties of transferrin, the plasma iron transporter, and ferritin, the intracellular iron storage protein, have been thought to have evolved to maintain iron solubility and to prevent iron reactivity. The transport of iron across the intestinal cell must similarly maintain iron solubility and prevent iron reactivity. There could be two mechanisms for iron transport across the cell that could allow the principles of maintenance of solubility and minimization of reactivity to pertain: iron could be bound to a chaperone that would shuttle the iron from the apical surface to the basolateral membrane or iron could pass between the two surfaces by transcytosis. To date, no iron chaperone has been described except for the possible exception of a complex of β2-integrin and mobilferrin (26), which has been postulated to be a transporter of ferric iron in human and rat enterocytes and in K562 cells, a cell line derived from chronic myelogenous leukemia that can be caused to undergo erythroid differentiation. Mobilferrin is highly homologous with calretilcin (6). Calretilcin is a Ca2+-binding protein that resides in the endoplasmic reticulum and is involved in calcium homeostasis and in the folding of newly synthesized glycoproteins via the calretilcin-calnexin cycle. Iron binding can be demonstrated...
to occur to calretilcinc in vitro. There are no data showing that iron binds to calretilcinc in vivo or that calretilcinc interacts with DMT1 to accept iron or with FPN to donate iron for export. Furthermore, a demonstration of vectorial movement of calretilcinc across the enterocyte is lacking. Nonetheless, iron binding to mobilferrin has been described, and pulse-chase experiments with $^{59}$Fe infused into gut sacs suggests that mobilferrin is an intermediary in the transport of iron across the rat enterocyte. Because mobilferrin can be isolated in association with $\beta_1$-integrin, flavin monoxygenase, and $\beta_2$-microglobulin, it is speculated that this large-molecular-weight protein complex termed paraferritin transports iron across enterocytes (26).

To evoke transcytosis as a method for iron movement across the intestinal epithelia, it is first necessary to demonstrate that the two transporters exhibit vectorial movement when cells are offered iron. DMT1 functions as an apical plasma membrane iron transporter in intestinal enterocytes and as an endosomal iron transporter in the transferrin-transferrin receptor mechanism for iron uptake in erythroid cells and other peripheral tissues. In a variety of studies examining subcellular localization of transfected DMT1, the protein has been localized in intracellular vesicular compartments (4, 24). The precise compartment, early versus late endosomes, appears to depend on the DMT1 isoform that is expressed. DMT1 isoforms are the result of splice variations that lead to two different NH$_2$-terminal and two different COOH-terminal amino acid sequences. The net result of these studies, though, is to demonstrate that DMT1, which is expressed initially on the plasma membrane, does localize into endosomes to allow for iron transport into the cytosol. In enterocytes, DMT1 also has been visualized on the apical brush-border membrane, on the basolateral membrane, and in cytoplasmic vesicles. DMT1 on the brush-border membrane is presumably the isoform derived from the IRE containing mRNA, because it is detected by an antibody specific for the unique COOH-terminal amino acid sequence encoded by that RNA (4, 8). In the iron-starved state, DMT1 can be found primarily in the brush-border membrane both in rats and in Caco-2 cells, a cell line commonly used to study iron absorption and transport. With the use of confocal microscopy to determine the location of DMT1 in Caco-2 cells fed iron, it is possible to demonstrate that with iron feeding, DMT1 undergoes endocytosis and can be detected in the apical cytoplasm above the nucleus (Fig. 1) (14). The phenomenon occurs rapidly, and within 10 min of exposure of the brush-border membrane of Caco-2 cells to iron, $>30\%$ of the DMT1 are internalized. With the continued presence of iron in the apical chamber that bathes the brush-border membrane, internalization continues for $\sim$40 min, after which equilibrium appears to be reached with some return of DMT1 to the brush-border membrane. FPN also can be demonstrated to translocate within the enterocytes in response to iron feeding. In the iron-starved rat enterocyte, FPN is seen in vesicles in the apical half of the cell primarily above the nucleus, and with iron feeding, FPN translocates to the basolateral surface (Fig. 1); similar movement of FPN1 can also be observed in Caco-2 cells (data not shown). The movement of DMT1 and FPN1 with iron feeding is also seen in the Belgrade rat. Both the Belgrade rat and the microcytic anemia mouse have a common missense mutation, G185R, in DMT1. Animals that are homozygous for the mutation (b/b animals) exhibit decreased iron uptake across the intestine and decreased iron uptake into erythroid cells and hence have a severe systemic iron deficiency. When b/b rats are starved of iron, the mutated protein decorates the brush-border membrane of the small intestine, and with feeding, DMT1 and FPN movement is observed. These observations suggest either that the defect of iron transport is not complete or that alternate iron transport mechanisms exist. In any case, entry of iron into the cell triggers a signal for internalization of DMT1 and for translocation of FPN. To be determined is the nature of the signal and the mechanism(s) for the movement.

Movement of metal transporters has been described for copper transporters. Copper, similar to iron, exists in two redox
states, Cu(I) and Cu(II), allowing copper-containing enzymes to catalyze electron-transfer reactions. The ease of copper oxidation and reduction between the two redox states also makes copper toxic at high levels within cells with the generation of hydroxyl radicals. To control intracellular copper levels, translocation of various copper transporters has been defined. For example, the Menkes and Wilson disease genes, ATP7A and ATP7B, respectively, are located in the trans-Golgi network (21, 23). With increased intracellular copper levels, ATP7A moves to the plasma membrane to transport copper out of the cell and ATP7B moves to post-Golgi vesicles to compartmentalize copper before excretion. The human copper transporter (hCtr1) is also subject to posttranslational regulation (10). Ctr1 is essential for copper uptake in mammalian cells with deletion of Ctrl in mice resulting in an embryonic lethal phenotype. Ctrl is located on the plasma membrane, and its expression is increased with low copper levels. However, when cells are placed in media with elevated levels of copper, hCtr1 undergoes endocytosis resulting in decreased availability of Ctrl on the plasma membrane for copper uptake.

**EVIDENCE FOR TRANSCYTOSIS IN ENTEROCYTES**

The movement of DMT1 and FPN1 in the enterocyte could serve either to regulate iron uptake or to facilitate iron transport across the cell. In analogy to the movement of Ctrl, the internalization of DMT1 could be a regulatory phenomenon designed to limit the absorption of iron by decreasing the number of transporters on the brush-border membrane. It is not clear whether the movements observed for DMT1 and FPN1 are required for regulation of iron uptake or are involved in the transport process. If the movements of DMT1 were involved in regulation, then presumably iron uptake would decrease with DMT1 internalization. In fact, in Caco-2 cells, iron uptake increases linearly for at least 6 h (1, 17), whereas internalization of DMT1 is a rapid event and is observed within 10 min (14).

Two well-studied systems of transcytosis in enterocytes are the delivery of IgA from intestinal associated lymphoid tissue to the intestinal lumen in a basal to apical direction and the uptake of maternal IgG across the intestinal epithelial in an apical to basal direction (12, 22). Transcytosis of transferrin has been described in Caco-2 cells (11, 13). In these studies, apical uptake either of a particulate or soluble marker could be demonstrated to interact with endocytic vesicles derived from the basal surface and containing the transferrin receptor. Mixing of the apical and basal labels took place in the apical portion of the cells. These experiments demonstrate that endocytic vesicles from the brush-border membrane and from the basolateral surface can reach common endosomal elements in the apical cytoplasm and the Golgi. Very interestingly, markers taken up from the basolateral surface were visualized in the apical compartment without an obvious dwell time in the basal portions of the cells and with a large percentage of transferrin receptors from the basolateral surface colocalizing with a marker derived by endocytosis from brush-border membrane. The endosomes both from the basolateral surface and the brush-border membrane appear to enter a compartment of multivesicular bodies in the apical cytoplasm where sorting and fusion takes place, allowing for recycling of proteins to the appropriate surface and for transfer of endosomal contents.

Polarized epithelia contain early endosomes in the apical and basolateral compartments. These compartments are distinct populations that may or may not interact via a vesicular apical compartment variously termed the apical recycling endosome (ARE) or common endosome (CE) (3, 18). We (14) have shown by confocal microscopy in Caco-2 cells that ferritransferrin offered from the basal chamber remains in the basolateral compartment, whereas basolateral-derived vesicles marked either with apotransferrin or with dextran go directly to the apical compartment. In this compartment, the basolateral vesicles carrying apotransferrin colocalize with apical-derived vesicles marked by DMT1 but not the basolateral vesicles marked with dextran. These results indicate that there is selective endosomal trafficking and a recognition system that distinguishes between vesicles allowing in the ARE the fusion of apotransferrin-marked basolateral vesicles, but not dextran-marked basolateral vesicles, with DMT1-containing vesicles. These observations are consistent with extensive studies on the transcytosis of IgA that demonstrate that the ARE is not uniform but has subcompartments and that targeting of vesicles for transport to the plasma membrane or to another vesicle for fusion includes accessory proteins such as the integral membrane proteins present on vesicles (v-SNAREs) or target membranes (t-SNAREs), SNARE activation, the Sec1/Munc18 proteins, and the small-molecular-weight GTP-binding rab proteins.

**EVIDENCE THAT TRANSCYTOSIS IS INVOLVED IN IRON TRANSPORT ACROSS ENTEROCYTES**

Does the movement of DMT1 and FPN allow for their participation in transcytosis and perhaps the transport of iron across the enterocyte? It is unlikely that DMT1 or FPN1 are iron-carrier proteins, because neither possesses typical iron-binding motifs. The stoichiometry of iron uptake would also preclude DMT1 as an iron carrier unless there was an extremely high turnover time. The hypothesis that we entertain is that internalized vesicles containing DMT1 could “hold” onto iron until fusion occurs with basolateral-derived vesicles, and DMT1 from the apical vesicles could then transport iron into the basolateral vesicles.

As noted above, we and others have demonstrated DMT1 in vesicles in polarized intestinal epithelium. In nonpolarized cells, DMT1 has also been demonstrated in vesicles including endosomes carrying the transferrin-transferrin receptor complex. The status of the localization of FPN in enterocytes is not entirely clear, because a recent study suggests that FPN may be expressed on the brush-border membrane (25). Basolateral FPN membrane expression was seen in the studies demonstrating that hepcidin binds to FPN resulting in endocytosis and degradation of FPN, but these studies used transient expression of FPN in HEK-293 cells, which are not enterocytes (20). Our data demonstrate FPN in the cytosol of both enterocytes and Caco-2 cells. In addition, transient transfection of both DMT1 and FPN into Caco-2 cells results in cytoplasmic as well as plasma membrane expression and both DMT1 and FPN could be seen to interact with other proteins (Fig. 2, A and B). After iron feeding, DMT1 colocalized with Rab5 and EEA1 (Fig. 2, A and B). In the absence of iron, no colocalization was observed nor was colocalization observed after iron feeding with markers of late endosomes or lysosomes such lysosomal-
Fig. 2. Colocalization of EEA1 and Rab5 with DMT1 in Caco-2 cells transiently transfected with GFP-DMT1 after iron feeding and colocalization of Fe and apotransferrin with a metalosensor. A and B: Caco-2 cells were transfected with pCDNA-EGFP-DMT1, and, after 48 h, the cells were fixed and incubated with antibodies to EEA1 (A) and Rab5 (B), followed by incubation with Alexa 594-labeled anti-mouse IgG secondary antibody and examination by confocal microscopy. Cellular localization of GFP-DMT1 was in both the membrane and punctuated vesicles distributed throughout the cytoplasm. After iron feeding of the cells for 30 min, there was increased colocalization of the GFP-DMT1 with EEA1 and with Rab5, as seen by merging the images of the green EGFP-DMT1 with the red staining of EEA1 and Rab5 as indicated by yellow. C1: metalosensor calcein (green fluorescence) was offered to Caco-2 cells from the basal chamber along with Alexa 594 apotransferrin (red fluorescence) for 20 min. The cells were fixed and observed by confocal microscopy with the appearance of some colocalization (yellow). C2: calcein and Alexa-594 apotransferrin were offered from the basal chamber, and Fe-ascorbate was offered in the apical chamber for 20 min with total quenching of calcein fluorescence observed. C3: to cells treated as in C2, the apical contents were replaced with medium containing the iron chelator o-‘-dipryridal for 5 min with restoration of calcein fluorescence and colocalization of calcein with apotransferrin.

associated membrane protein (LAMP) (data not shown). Similarly, after transient transfection of FPN into Caco-2 cells with iron feeding, FPN was observed to interact with various partners including hem oxygenase 1 (data not shown). These results suggest that Fe transport into the enterocyte generates a signal that causes DMT1 to interact with proteins that are required for endocytosis and are characteristic of the proteins that associate with early endosomes.

We have examined the time course of the interaction of DMT1 with EEA1 and Rab5. In these experiments, cells were starved of iron overnight, and the localization of DMT1 was determined by immunohistochemistry and confocal microscopy at various times after the addition of Fe-ascorbate to the apical chamber. Before iron feeding, ~78.2 ± 19.9% (means ± SD of 3 experiments for this value and all following values) of DMT1 was in the brush border, and by colocalization analysis ~58.1 ± 21.4% of the DMT1 was colocalized with EEA1. By 10 min after feeding iron, the DMT1 content in the brush border decreased to 42.6 ± 4.2%, of which colocalization with EEA1 occurred with only 28.3 ± 10.1% of the DMT1. Before iron feeding in a 2-μm slice through the ARE, little DMT1 was detected and the colocalization of EEA1 to this compartment of DMT1 was only 10.4 ± 3.2%. However, 10 min after iron feeding, the amount of DMT1 in this same section increased to ~34.5 ± 9.2%, of which 36.2 ± 8.4% was colocalized with EEA1. Because only a portion of the DMT1 on the brush border was associated with EEA1, this suggests that DMT1 needs to be modified before EEA1 can bind and the complex can be internalized.

Do the vesicles carrying both DMT1 and EEA1 and Rab5 interact with vesicles derived from the basolateral membrane? When Caco-2 cells are grown as polarized monolayers in bicameral chambers, the addition of apotransferrin to the basolateral membrane stimulated apical iron uptake and total iron transport through the cells (2, 17). These experiments suggest communication between the two surfaces of the Caco-2 cells. We have subsequently incubated Caco-2 cells with paramagnetic particles either to the brush-border membrane or to the basolateral membrane, disrupted the cells, and isolated vesicles containing the paramagnetic particles by trapping the vesicles with a magnet (14). The composition of the vesicles was then examined by fractionation on SDS-PAGE with subsequent Western blot analysis. Whether the paramagnetic particles were offered in the apical or basolateral chamber, the presence of iron in the apical chamber increased the amount of DMT1 detected in the trapped vesicles. If apotransferrin were in the basolateral chamber, the trapped vesicles contained DMT1 and apotransferrin. In addition, by using 59Fe in the apical chamber, it was possible to detect 59Fe in the vesicles. If 125I-labeled apotransferrin was offered simultaneously in the basolateral chamber, then 59Fe- and 125I-labeled apotransferrin were found in approximately equal molar amounts in the vesicles. The presence of apotransferrin is certainly not required for transcytosis, because iron transport across the enterocyte occurs in the
absence of apotransferrin. However, as shown in our confocal microscopy studies, apotransferrin is a good marker for endosomes formed at the basolateral surface and that can gain access to the apical compartment compared with endosomes containing ferritin. Which stay relegated to the basolateral portion of the cell. The hypothesis that transcytosis is involved in iron transport across the enterocyte has gained support by recent elegant studies in Caco-2 cells using a variety of inhibitors of vesicular trafficking (17). These studies support that at least half of the iron transported across the cell uses a vesicular pathway and that a significant portion of the vesicular pathway involves vesicles marked with apotransferrin.

The transcytosis of iron can be demonstrated by use of fluorescent metallosensors in which the chelation of iron quenches fluorescence. These metallosensors have been used to define cellular compartments accessible to iron chelators (9). By presenting the sensors, e.g., calcein, as either the free chelator or as the chelator-iron complex, endosomes can be labeled and used to monitor the flux of iron into endosomes with iron feeding or the efflux of iron induced by iron chelators. We have used this approach in Caco-2 cells to observe whether iron offered from the apical surface communicated with vesicles derived from the basolateral surface loaded with calcein (Fig. 2C). In these experiments, calcein and apotransferrin were offered to Caco2 cells from the basal chamber and were colocalized in the apical portion of the cells. After 20 min, ion was offered from the apical chamber with considerable quenching of calcein. The addition of the Fe(II) chelator αα′-dipyridyl restored calcein fluorescence and colocalization of calcein and apotransferrin was once again observed. When calcein-iron was offered from the apical compartment and apotransferrin from the basal chamber, initially little fluorescence was observed. However, with time, fluorescence was restored and colocalization of calcein and apotransferrin was observed in vesicles in the apical portion of the cells.

Unexplored as yet is the role of hepcidin in the regulation of transcytosis by altering the compartmentalization of DMT1 and FPN or by altering the rates of movement. The so-called crypt hypothesis suggests that regulation of iron uptake requires reprogramming of villous crypt cells, which requires a lag of several days for the crypt cells to mature and migrate to the villous tips where iron absorption occurs. Hence, physiological events that alter iron absorption would do so only after a lag of several days. A recent study (7) has suggested that the switch from an iron-replete to an iron-deficient diet rapidly increases iron absorption and that therefore a regulatory mechanism other than crypt cell reprogramming must exist. We have shown in enterocytes that hepcidin decreases FPN protein expression within 6 h (28). Yet to be studied, though, is whether the altered FPN expression induced by hepcidin affects 1) the rate of DMT1 internalization, 2) the fusion of apically derived vesicles with vesicles derived from the basolateral surface, and/or 3) the translocation of FPN to the basolateral surface.

THE MUCOSAL BLOCK HYPOTHESIS: THE CASE AGAINST TRANSCYTOSIS

Early studies of iron absorption recognized that only a fraction of presented iron was absorbed and transported into the animal. That fraction increased in the iron-deficient animal and decreased with increased iron stores. The term “mucosal block” was proposed to describe a diminished absorption of Fe following an orally administered Fe dose, and mucosal ferritin synthesized as a result of a bolus of iron was postulated to serve as a block to the absorption of excess Fe. Under the mucosal block hypothesis in iron-replete or overloaded animals, the iron that entered into ferritin was not available for transport into the systemic circulation and would be shed into the lumen when the enterocytes were exfoliated. Numerous studies have demonstrated that absorbed iron does go to ferritin, that a bolus of iron even in an iron-depleted animal can increase ferritin expression in the enterocytes, and that eventually a bolus of iron will decrease iron absorption. Hence, even if DMT1 containing vesicles conduct iron to meet a putative partner from the basolateral membrane, some iron must pass into the cytosol. The mucosal block hypothesis has been reexamined in light of the transport proteins DMT1 and FPN and the other players, Dcytb and Heph, required for iron transport to determine whether regulation of these proteins can affect iron absorption and transport (7). These studies show that a large bolus of iron downregulates DMT1 and Dcytb but not FPN or Heph, strongly suggesting that the mucosal block is at the uptake step. We have shown that at 1.5 h after a bolus of iron, in both iron-replete and iron-deficient animals, the activity of iron-responsive proteins (IRP)1 and -2 is decreased (28). Correlating with the decrease of IRP activity was a decrease of DMT1 mRNA. However, DMT1 protein levels were found to increase initially, suggesting that, at least with regard to DMT1, a complex regulation by iron involving changes in RNA and protein levels. Taken together, these studies support the kinetic model that the rate-limiting step in iron uptake is at the uptake and/or intracellular steps of Fe processing and not in the transport into the plasma (16). In addition, these studies suggest that iron must gain access to the cytosol to bind to the IRP and, in the case of the translational control of DMT1, to affect the activity of the heme-controlled repressor. Hence, there must be pathways for iron to reach the cytosol and gain access to IRP1 and -2 while also being transported by transcytosis to vesicles from the basolateral membrane. As noted earlier, with a bolus of iron, not all of the brush-border membrane DMT1 undergoes endocytosis. Hence, some iron may be transported directly across the brush-border membrane into the cytosol. Alternatively, in the ARE, some apical-derived vesicles instead of fusing with basolateral-derived vesicles might transport iron directly into the cytosol or to iron carriers. Hence, whereas our studies and those of others (17) suggest that vesicular transport is involved in the transport of iron across the enterocyte, nonvesicular transport may also occur, and perhaps it is the iron conducted by the nonvesicular pathway that is involved in the regulatory events.

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Iron Transport via Transcytosis in Enterocytes


