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PHYSIOLOGY AND MOLECULAR BIOLOGY OF DIETARY IRON ABSORPTION

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INTRODUCTION AND SCOPE OF THE REVIEW

Remarkable progress has been made in identifying the proteins involved in the absorption of intestinal nonheme iron and several recent reviews have been published on the subject of iron transport related to genetic hemochromatosis (28, 38, 47, 59). Advances have also been made in understanding the regulation of iron absorption with the identification of several candidate regulatory molecules. Work based on more traditional approaches has continued, resulting in a significant advance in our understanding of iron absorption in the last decade. Interestingly the mechanism of heme iron absorption remains a mystery despite its importance in nutritional

terms and the fact that it is far more efficiently absorbed than nonheme iron. This review brings together recent findings on the physiology, molecular biology, and biochemistry of iron absorption. For a review of recent advances in the related area of iron bioavailability, see Wienk et al. (91).

OVERVIEW OF IRON METABOLISM IN MAMMALS

In man the normal diet should contain 13–18 mg of iron per day of which only 1 mg is absorbed. Even in iron deficiency, absorption is only 2–4 mg and in iron overload it is reduced to 0.5 mg.

McCance & Widdowson were first to suggest that body iron stores were determined by regulation of intestinal iron absorption and that there was no significant excretion of iron by the kidneys (45). They found that when various metals were intravenously injected into the body, iron was the only metal not rapidly excreted in the urine. The formation of red blood cells requires about 30 mg of iron daily and this is balanced by an equal flux of iron from the breakdown of senescent red blood cells by the reticuloendothelial (RE) cells in the spleen and Kupffer cells. Body iron losses are small in comparison, consisting of losses of iron with epithelial cells (skin, gastrointestinal cells, urinary tract cells) and fluids (e.g., tears, sweat, and in menstruating women, blood). This accounts for the loss of 1 mg per day (or 2 mg per day for women) of iron. In man dietary iron intake consists of two components: heme iron (e.g., as present in red meat) and nonheme or inorganic iron (e.g., as in vegetables, cereals, etc.). The absorption of both is discussed below.

Nonheme Iron Absorption

Nonheme (or inorganic) iron is present in the diet as either the reduced ferrous (FeII) form or the oxidized ferric (FeIII) form. Under normal physiological conditions (i.e., neutral pH and in the presence of oxygen) ferrous iron is rapidly oxidized to ferric that can then precipitate as iron hydroxide. In the luminal contents of the gut, iron is likely to be in the ferric form and therefore poorly bioavailable. Several luminal factors in the diet can have marked effects on absorption of dietary iron (see below). Nonheme iron is absorbed early in digestion mainly in the duodenum, where the low pH favors solubility of iron. Further down the intestine it is likely that formation of insoluble ferric complexes reduces bioavailability. The transport of nonheme iron across the duodenal mucosa has been studied intensively over the years and is highly adaptive to changes in iron status (stores, erythropoesis, hypoxia). Much progress has been made in the last few years in identifying the proteins involved in this process and will be dealt with later in this review.

Heme Iron Absorption

Although this review will focus mainly on nonheme iron transport, it is noteworthy that heme iron derived from meat is an important source of iron and, unlike inorganic iron, is highly bioavailable. It is believed that the metal porphyrin ring

is split from the globin in the lumen and that the intact Fe porphyrin is transported across the brush border membrane rather than being split further into free iron and the porphyrin (12, 85, 87). Once inside the cell the heme may be split further into ferrous iron and bilirubin by heme oxygenase (65) or transported as the intact porphyrin ring (12). The released ferrous iron is then likely to enter into the low molecular weight pool of iron in the enterocyte and exit the enterocyte via a common pathway along with iron absorbed as nonheme iron. Canine species appear to have the highest and mice the lowest ability to utilize heme iron (dog>guinea pig>rat>mouse) (2). As with nonheme iron, the duodenum is the main site of heme iron absorption and there is gradient of mucosal heme uptake along the length of the intestine similar to that seen for nonheme uptake [duodenum>jejunum>ileum (87)]. Opinion is divided on whether heme iron absorption is regulated by iron status to the same extent as absorption of inorganic iron. Several studies have shown that ^{59}Fe-heme absorption was unaffected in phlebotomized or iron-deficient rats while nonheme iron absorption was increased several fold (12, 85). In another study, rats fed hemoglobin as the only source of iron became iron-deficient, showing a poorly developed heme-absorptive pathway (85). However, other studies in iron-deficient rats demonstrated 10- and 3-fold increases in absorption of labeled heme when compared to controls (2, 87).

Studies on the subcellular route of heme iron absorption in the duodenum have suggested that heme transport involves endocytosis with heme being collected in tubulovesicular structures in the apical cytoplasm (57). The presence of a brush border heme receptor has also been reported (33), and more recently it was shown that the intestinal cell line Caco-2 transports heme (92), but the identity of the protein(s) involved in these processes have not been reported.

LUMENAL ASPECTS OF INTESTINAL IRON ABSORPTION AND ITS REGULATION

At present, it is possible to describe various lumenal factors that affect iron absorption, but a complete, quantitative model of iron interactions in the intestinal lumen remains some way off. The rate of iron absorption is partly determined by the rate of delivery of suitable chemical forms of iron to the duodenal surface (5).

Iron Speciation in the Intestinal Lumen

The exact nature of iron species "suitable" for absorption remains to be clearly defined, but much work suggests that soluble, low molecular weight iron complexes and Fe^{2+} ions are well absorbed. Food iron is in high molecular weight species, hence digestive release of iron is important for iron absorption. There has been little support for suggestions that specific high-affinity iron chelators act as accessories to iron absorption. Instead, ascorbate seems to be the most important lumenal accessory for iron—by acting as a reductant at acid pH and a low-affinity ligand at neutral pH, it helps to release iron from food and maintains it in a soluble, low

molecular weight form (5). Iron speciation in the intestinal lumen is determined by the digestion and release of iron and potential ligands and reductants from food, together with mixing of these with ligands, acids, bases, and reductants secreted by the gastrointestinal tract. Parts of this complex process have been biochemically analyzed in animal models (see below), but only a very limited analysis has been attempted in man (40).

pH and Ligands for Iron

In the stomach, the low pH is important in releasing iron from ligands in food, as is the action of various digestive enzymes, which destroy high molecular weight ligands and create new ligands. Also present are mucin and reductants such as ascorbate, which can be present in the meal, or, in many animals, secreted in the gastric juice. Early work showed that stomach acid is important (5) and more recent work in experimental animals has confirmed this (32). The widespread medical use of inhibitors of gastric acid secretion does not, however, seem to be likely to lead to iron deficiency (76), as they are not prescribed at levels which block stomach acid levels sufficiently to induce deficiency in adults.

The complicated mixture that is expelled from the stomach into the duodenal lumen undergoes a rapid increase in pH, is mixed with additional digestive enzymes, more iron ligands, and HCO_3, and is further digested. The residence time of material in the duodenum is comparatively short, with thermodynamic equilibrium not likely to be reached, as ligands are being further degraded and new ones created. Attempts have been made to analyze potential ligands for metal ions in rodent duodenum and these analyses revealed such compounds as ascorbate (9), glutathione and cysteine (88), and lactate, pyruvate, and histidine (64), although no regulatory role for these in iron absorption has been confirmed (90). These compounds can be secreted by the gastrointestinal tract (13) or derived from food (e.g., 50).

Role of Mucus

Mucin (gastroferrin) has also been implicated as an important iron ligand in the duodenum (5, 63). Iron must traverse the mucus layer and acid microclimate at the mucosal surface. The former will tend to block entry of large molecular mass species and can bind metal ions such as Fe^{2+} and Fe^{3+}. Work done in the 1960s suggested that a stomach glycoprotein, termed gastroferrin, might be an important regulator of iron absorption [reviewed by Powell (63)], and later work in the 1970s confirmed that gastroferrin was a mucin-Fe complex formed when iron-containing gastric juice is neutralized. Further investigations by Conrad and coworkers (10) reemphasized that mucin does indeed bind iron during Fe absorption. Investigations of lumenal ligands for aluminium (64) have shown the importance of mucin as a ligand for metals, including iron. One study by Wien & Van Campen (89) has favored an inhibitory role for mucus. Mucin may, however, donate iron to the duodenal iron uptake system (63). As pointed out by the latter study, both outcomes

remain possible and further work is required to elucidate the role of mucin and other ligands in iron uptake.

Lumenal Redox Reactions

In addition to binding to ligands, redox chemistry is important for iron. Much food iron is ferric and the rising pH of the duodenum favors oxidation of ferrous formed in the stomach to ferric (73). Analysis by Wein & Van Campen (88) has shown that reductants (either secreted into the duodenal lumen or derived from food) can influence iron absorption, presumably by favoring mobile, ferrous ion, which can penetrate the mucus layer. They found no evidence for active regulation of this luminal reduction (90) in rats. Iron arriving at the surface of the intestinal epithelial cells will therefore be a mixture of ferrous ions, low molecular weight ferric complexes, and perhaps Fe-mucin. The acid microclimate of the duodenal surface [pH approx. 6–6.5 (68)] will help maintain significant levels of ferrous ion, as will cell surface reductase activity (see below). This microclimate will also provide a proton gradient directed toward the cell interior which, together with the inwardly directed (i.e., inside negative) membrane potential of the brush border membrane (53), will provide the necessary driving force for iron uptake.

Intestinal Transit

The length of time that iron species are in contact with the duodenal surface is clearly important in determining iron absorption rates, but relatively few attempts have been made to analyze this in any quantitative way. Fairweather-Tait & Wright (21) found no effect of compounds that alter small intestinal transit, while Schade et al. (69) reached the opposite conclusion. One study showed a proportionally small effect of a small change in gastric emptying rate (74) on iron absorption. This has been interpreted both for and against an effect of gastric emptying (5, 21) on iron absorption. The situation is further complicated by the fact that duodenal (as opposed to intestinal) lumenal iron content and transit time are likely the most important transit parameters to determine iron absorption and no measurements of these parameters have been made in any published study on iron absorption known to the authors. A thorough study of the importance of duodenal transit in iron absorption, though technically difficult, needs to be done.

IDENTIFICATION OF THE IRON TRANSPORT GENES

Uptake is defined as transport of dietary iron across the apical membrane of the enterocyte into the intestinal mucosa; *transfer* is the movement of iron from the enterocyte across the basolateral membrane to the circulation (44, 86). The elegant studies of Manis & Schachter and Wheby et al. defined the key physiological characteristics of iron absorption in the mammalian intestine and established several important points. The maximal rate of iron absorption was seen in the duodenum

with the lowest rate seen in the ileum. In iron deficiency the duodenal mucosa showed much more adaptation to changes in iron status compared to other regions of the gut. It is noteworthy that some of these characteristics of iron absorption were used to identify iron transport genes almost 40 years later.

Proteins Involved in Mucosal Uptake of Iron

APICAL TRANSPORTER NRAMP2/DCT1/DMT1 Nramp2 was the first mammalian iron transporter to be identified as a divalent cation or metal transporter (DCT1 or DMT1) by two groups working independently. One group used the Xenopus oocyte expression cloning system—Gunshin et al. (36) showed that in oocytes injected with DMT1 cRNA there was a rapid inward current produced in the presence of ferrous iron in the external medium in comparison with a water-injected control. The transport of iron into the oocyte was highly dependent on an inward-directed proton gradient. The mechanism of iron transport is therefore likely to be proton coupled and requires a pH gradient (77). The mRNA was highly expressed in the duodenum and strongly upregulated in iron deficiency. The mRNA contains a functional 3' iron responsive element (IRE) (35). In the other study, a group working on the genetic basis of microcytic anemia (mk) identified the putative causal gene as Nramp2—this anemia is characterized by defective iron uptake (26). The same group later showed an identical mutation was present in DMT1 in the Belgrade rat, which has a similar microcytic anemia (25). Thus the expression pattern along with transport data and powerful genetic evidence are consistent with DMT1 being responsible for the regulated step of duodenal iron uptake.

The lack of specificity of DMT1 for iron has provided a possible explanation for the previously known absorptive interaction of various divalent metal ions with each other. It has been demonstrated that manganese absorption is impaired in Belgrade rats [presumably by the mutation in DMT1 (8)]. Similarly, the ability of DMT1 to transport other ions such as Pb or Cd (55) provides some explanation for the increased absorption of these toxic metals in iron deficiency (24). On the other hand, Zn and Cu have been reported to regulate DMT1 expression (79, 93), providing another possible mechanism for metal interactions with iron absorption.

APICAL FERRIC REDUCTASE DCYTB Because DMT1 only transports ferrous iron, whereas dietary iron is likely to be mostly ferric, a ferric reductase was predicted to be present in the duodenal mucosa. The presence of such a surface ferric reductase activity in the duodenal mucosa was first described some time before the discovery of DMT1 (66). In that study it was shown that a ferric reductase activity could be measured at the surface of the duodenal mucosa. The reductase activity was strongly stimulated by hypoxia and iron deficiency, both of which stimulate increased absorption of dietary iron. In addition, it was found that the activity was highest in the duodenum and lowest in the ileum, compatible with the profile of iron absorption along the gut, which is highest in duodenum and lowest in ileum. Attempts to purify the protein responsible for this activity provided evidence that

the activity was associated with a b-type heme protein that was immunologically distinct from the NADPH oxidase GP91-Phox (62). The protein was never successfully purified using biochemical methods because the heme was lost early in the purification. The gene responsible for this activity, Dcytb (for duodenal cytochrome b), was eventually identified with a subtractive cloning strategy (46). The protein sequence of Dcytb was homologous to cytochrome b561. A b-type heme transmembrane dehydroascorbate reductase, b561 is highly expressed in chromaffin granule membranes in the adrenal medulla (17, 75). The role of b561 is to reduce granular dehydroascorbate to ascorbate by transporting an electron donated by cytoplasmic ascorbate across the granular membrane (27). In addition to b561, Dcytb was identical to the N terminus of a protein called P30 (19, 20). When expressed in either Xenopus oocytes or cultured cells, Dcytb induces ferric reductase activity. It is noteworthy that Dcytb, although a b-type cytochrome, does not share any homology with the GP91-phox or any of the ferric reductase proteins described so far in yeast or plants (38). This indicates that the mammalian ferric reductases evolved independently to yeast and plant systems.

Proteins Involved in Basolateral Transfer of Iron

IREG1/FERROPORTIN/MTP1 Ireg1 was isolated following the same strategy as for Dcytb (48). The mRNA for Ireg1 contained a functional IRE sequence, was sharply localized to the duodenum and was regulated by several independent stimulators of iron absorption. The gene encoded a novel highly hydrophobic membrane protein with 10–12 transmembrane-spanning domains. Transfecting polarized epithelial cells with a tagged Ireg1 showed the protein was targeted to the basolateral membrane. This finding led to the hypothesis that Ireg1 was an iron-regulated protein involved in the transfer of iron to the circulation across this membrane. The Xenopus oocyte expression system demonstrated that Ireg1 did indeed stimulate efflux of iron (48). Ireg1 was independently identified in two other labs and has been named ferroportin (16) and MTP1 (1). In one of these reports, Donovan et al. (16) used positional cloning to identify the gene responsible for the *wiessherbst* (weh) mutant phenotype in zebra fish, so called because their lack of hemoglobin gives them a pale appearance. The gene they identified was the zebra fish homologue of Ireg1, named ferroportin. In zebra fish, ferroportin is expressed in the yolk sac and is likely to be responsible for transfer of iron from the maternally derived iron stores to the circulation. In the other report, an approach of enriching for cDNA containing IREs (1) was used. In addition to its role in transfer of iron from the intestine to the circulation, Ireg1 likely also plays an important role in iron transport in other organs, notably the placenta, where iron transfers between maternal and fetal circulations (48). Similarly, recycling of iron into the circulation from the breakdown of hemoglobin in the Kupffer cells of the liver and red pulp for the spleen also probably involves Ireg1 (see Figure 1).

Recently, several groups have reported on families with mutations in Ireg1 that cause hemochromatosis (15, 49, 67) with an autosomal-dominant

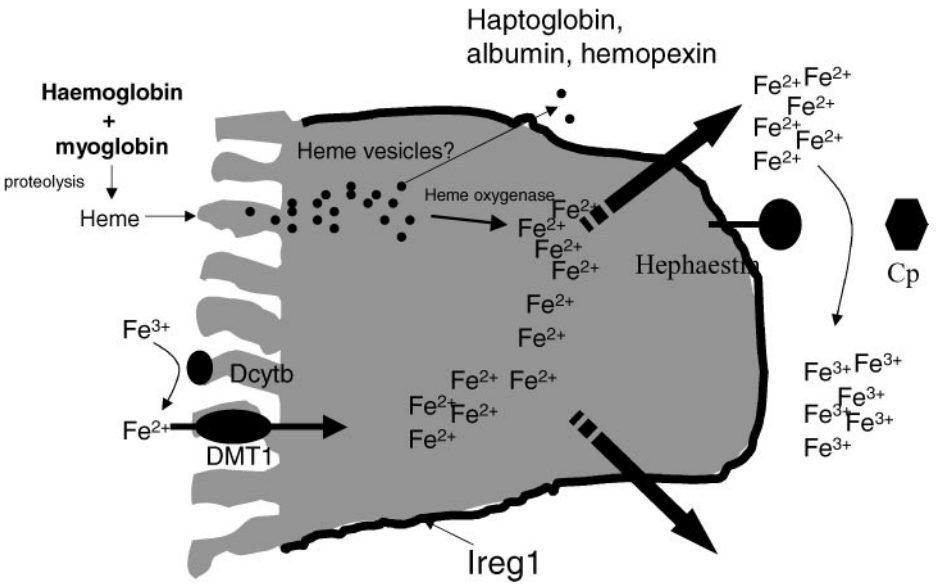

Figure 1 Pathway for intestinal inorganic iron and heme absorption.

inheritance distinct from HFE mutations. The clinical data from these patients show distinct differences from patients with HFE mutations (49). In patients with Ireg1 mutations, serum ferritins are very high and reticuloendothelial (RE) cells, are severely iron loaded in contrast to HFE patients, where these calls are free of iron deposits (49). These data would indicate a fault with recycling iron via RE cells, and since Ireg1 is highly expressed in these cells the mutation may adversely affect Ireg1 function. Important questions remain unanswered regarding the mechanism of iron transport by Ireg1, such as the driving force and possible interaction of cooperating proteins such as hephaestin and/or ceruloplasmin. The protein sequence of Ireg1 does not contain Walker motifs, which suggests that ATP-driven transport can be ruled out. Ireg1 is a member of the solute linked carriers (SLC) protein family that may suggest a facilitated diffusion mechanism. However, the movement of iron requires the movement of two positive charges, which would require either the movement of two electrons in the same direction, or the movement of two other positive charges in the opposite direction to maintain the membrane potential. The identity of such counter ions is not yet known, but Na^+ or H^+ are candidates. Another possibility is that transfer is driven by the oxidation of ferrous iron to ferric iron by either hephaestin or ceruloplasmin. This would maintain a steep concentration gradient for release of ferrous iron. Further work is required to address these questions.

INTESTINAL FERROXIDASE—HEPHAESTIN Mice with sex-linked anemia develop anemia due to a defect in intestinal transfer of iron to the circulation. As a

consequence, iron builds up in the intestinal cells and the animals become anemic (3, 58). A positional cloning approach was used to identify the defective gene (83). Interestingly, the protein sequence encoded by the gene (hephaestin) was very similar to ceruloplasmin, a copper-containing protein with ferroxidase activity. However, unlike ceruloplasmin, which is a serum ferroxidase, hephaestin contains a single putative transmembrane domain at the C terminus of the protein, which may serve to anchor the protein into a membrane. Hephaestin is highly expressed in small intestine, though not predominantly in the duodenum like Ireg1, Dcytb, or DMT1. In fact, hephaestin is expressed along the length of the gut with no obvious gradient. This suggests hephaestin may have additional roles in the intestine unrelated to iron absorption. The subcellular location of hephaestin is also puzzling for a protein implicated in transfer of iron to the circulation. Studies indicate the protein is not found on the basolateral membrane but rather in some intracellular perinuclear vesicles (29). The transport of iron through the enterocyte itself is an important aspect of the absorptive process about which little is known. It is possible that hephaestin has a role in this process.

Regulation of Dietary Iron Absorption

Iron stores and erythropoiesis have major effects on iron transport; other stimulators include hypoxia, pregnancy, and inflammation. Systemic signals received by the intestine from these stimuli act to alter iron absorption through regulation of the levels, activity, and localization of the transporters. It has been postulated that at least two humoral factors regulate iron balance (23). Although the molecules responsible have not been defined, they have been referred to as the "stores regulator" and the "erythropoietic regulator" (23). The "erythropoietic regulator" would balance the rate of erythropoiesis in the bone marrow with duodenal iron absorption, independently of iron stores. The signal could be a soluble plasma component that would probably originate in the bone marrow. This signal would target the storage sites to mobilize iron stores as well as stimulate the duodenum to increase iron absorption. The pathway targeted in the duodenum might be distinct from that tuned by the "stores regulator." It has been known for some time that inflammation leads to anemia and that injection of endotoxin into experimental animals reduces plasma iron by 50% in one hour (37). This effect seems to be mediated by blocking release of iron from the reticuloendothelial cells but also seems to have an effect on transfer of duodenal iron to the body. The activities of the iron regulatory proteins (71) are likely to be important regulators in the enterocyte since both the main transporters on the apical and basolateral membrane contain IRE sequences.

One of the central unresolved questions in iron metabolism is how the duodenal mucosa is able to sense the level of body iron stores or changes in demand for iron. Iron content of the mucosa cells is likely to be an important factor in regulating intestinal iron transport as has been suggested for many years (11). Another key aspect of iron absorption regulation is the spatial and developmental separation of the iron absorption mechanism from the presumed sensing mechanism for its

regulation (11). This early proposal has been strikingly supported by recent findings on the crypt-villus localization of proteins and activities associated with iron absorption. Thus Ireg1, Dcytb, and DMT1 are found, together with reductase activity and iron uptake, in mature enterocytes in the mid-villus to villus-tip region (46, 48, 53, 80). In contrast, transferrin receptor and HFE are found predominantly in the crypt-lower villus. Schumann et al. (72) have shown how these different villus regions may be linked by changes in iron regulatory protein activity following a pulse of intravenous iron.

Regulation of Iron Absorption Genes

MUCOSAL UPTAKE Duodenal DMT1 mRNA levels are increased some 10-fold in iron deficiency (36, 94). A similar increase is seen in Dcytb mRNA and protein levels in the duodenum. DMT1 and Dcytb seem to be regulated in synchrony, as the levels of expression of these genes are closely correlated (18, 31). This supports the idea that Dcytb provides the reduced iron [Fe(II)] for DMT1 to take into the cell. After high oral doses of iron, Dcytb and DMT1 were rapidly downregulated by mucosal iron, which suggests that the "mucosal block" operates by decreasing the levels of proteins required for uptake of iron (30). Interestingly, unlike DMT1, which contains a 3′ IRE, the Dcytb mRNA seems not to contain a recognizable IRE, yet the regulation by iron follows an almost identical pattern. This points to other regulatory mechanisms such as transcription or IRE-independent post-transcriptional regulation, as has recently been suggested for DMT1 (39, 78). Other studies also suggest that DMT1 protein localization changes in the iron-deficient and iron-loaded state. DMT1 was found in the apical membrane of duodenal enterocytes in iron deficiency and in the cytoplasm in iron-loaded animals (80). It is not known if the same trafficking occurs for the Dcytb protein localization in enterocytes.

TRANSFER In contrast to regulation of genes involved in uptake, DMT1 and Dcytb, the regulation of the genes involved in basolateral transfer, Ireg1 and hephaestin, is modest (30, 46, 48). While there was a 10-fold increase in DMT1 and Dcytb mRNA levels in chronically iron-deficient mice, there was only a 2–3-fold increase in duodenal messenger RNA levels of Ireg1 (6, 48). In homozygote hpx mice there was a 10–20-fold increase in Dcytb and DMT1 and a 3-fold increase in Ireg1 levels compared to the wild-type mice (48). Therefore, the increase in expression of Ireg1 is similar in magnitude to increases in duodenal iron absorption in iron deficiency and hypotransferrinemia. Hephaestin does not appear to be very strongly regulated by iron stores (31), although the influence of regulators such as hypoxia or erythropoiesis on hephaestin is unknown. Oral dosing with iron also seems to have less effect on Ireg1 and hephaestin levels compared with Dcytb and DMT1 (30). The differing magnitude of regulation of transfer versus uptake has been observed in some studies, leading to the suggestion that transfer is not regulated at all (71). Other studies, however, do seem to show regulation of iron transfer (reviewed in Reference 47).

Comparing the expression profiles of the genes along the length of the intestine, there is a sharp fall in Ireg1 expression levels distal to the duodenum, underlining the importance of this organ in iron absorption (29). DMT1 expression also falls away along the small intestine but to a lesser extent than Ireg1. In contrast, hephaestin expression is fairly constant along the whole length of the small intestine (29). This compares with the report of a very sharp fall in transfer of iron to the body when going distally from duodenum to jejunum and ileum (86). Uptake of iron into the mucosa is less affected at these locations although it still falls. Dcytb expression seems to be virtually undetectable in duodenum by Northern blotting in normal iron-replete mice, but is strongly induced by iron deficiency in duodenum with no expression detected in ileum even in iron deficiency. The finding of relatively high expression of Ireg1 in colon is noteworthy (29). The function of the expression of Ireg1 and other genes in the colon is not known as there is not thought to be much iron absorption at this site.

In summary, the regulation of the above transporters accounts for the ability of the intestine to adapt iron absorption to meet the different states of iron repletion. The regulatory process is very effective over a wide range of dietary iron apart from at very low or very high dietary iron where absorption is unable to cope, resulting either in iron deficiency or iron overload.

HFE: SENSING IRON HFE was discovered as the causative gene for genetic hemochromatosis in 1996 although its physiological function in iron metabolism has until recently remained unclear. Two mutations (C282Y and H63D) account for greater than 90% of hemochromatosis. Wild-type HFE protein and mRNA are ubiquitously expressed but both have been detected in crypt cells of the duodenal mucosa (56). HFE and TFR1 form a complex and have been co-crystallized (42). Experiments in cultured cells indicated that as a consequence of expression of wild-type HFE, there was a reduction in the binding of TfR to its natural ligand, diferric transferrin. In contrast, expression of the mutated form of HFE had no effect (22). It has been thought for some time that the level of iron in the crypt cells, which eventually migrate along the villus to become mature enterocytes, is critical to program the cell to absorb more or less iron from the diet. Because the crypt cells of the duodenum receive iron from the circulation and have been shown to express both TfR1 and HFE, it was suggested that the two molecules could be involved in the mechanism of sensing body iron levels. However, from the above data one would predict that hemochromatosis patients with the C282Y mutation would have iron-loaded crypt cells. This was in conflict with previous studies on patients' hemochromatosis where it appeared that rather than being iron-loaded the duodenal mucosa was iron-deficient (60). Two studies have recently shed light on these inconsistencies. First, Waheed et al. (84) demonstrated in CHO cells that overexpression of HFE can either enhance or inhibit Fe-transferrin uptake depending on whether β_2-microglobulin is coexpressed in cells. In this system, they found that wild-type HFE in fact enhanced uptake of transferrin-bound iron, but only if β_2-microglobulin was also expressed. This led to the proposal that in the

presence of a stable wild-type HFE-β_2-microglobulin complex, TfR1-mediated cellular uptake of transferrin-bound iron was increased. Previous studies were carried out without simultaneous expression of β_2-microglobulin and this may explain the contradictory results.

Another in vivo study demonstrated a significant impairment in the uptake of transferrin-bound iron by the duodenum of Hfe knockout mice compared to the wild-type controls (81). This finding indicates that in the absence of HFE, iron uptake by the duodenum still reflects iron status but this process was less sensitive to iron stores. This effect was specific for the duodenum, and no differences were found in the uptake of transferrin-bound iron by the liver or kidney in the Hfe knockout mice compared to the wild-type controls (81). Therefore, it appears that the HFE-β_2-microglobulin complex is the key to sensing the levels of transferrin-bound iron in the plasma and that it facilitates the uptake of transferrin-bound iron by duodenal crypt cells where iron absorption is initially set. HFE mutations that impair this function in hemochromatosis patients would decrease iron uptake by the crypt cells, so intracellular iron levels would not reflect the high plasma iron levels. As a result, differentiating enterocytes would be programmed to absorb iron inappropriately, resulting in the increased iron absorption as shown in such patients. Interestingly, ß$_2$-microglobulin-deficient mice develop iron overload similar to Hfe knockout mice (14).

HEPCIDIN: THE KEY REGULATOR? Over the years, several plasma components have been investigated as the possible "stores regulator": serum ferritin (7, 34) or serum transferrin receptor (4). Recently, hepcidin has been put forward as a candidate "stores regulator." Hepcidin was initially found in humans and named liver-expressed antimicrobial peptide (LEAP-1) (41). Hepcidin was discovered by two groups independently as an antimicrobial peptide synthesized in the liver and found in plasma and urine (54, 61). Homologues have been found in pig, mouse, and rat. Expression of hepcidin mRNA was mainly found in the liver with a lower level in the heart. The transcript encodes an 84-amino acid precursor, with a putative 24 amino acid leader peptide characteristic of secreted proteins. Hepcidin is proteolytically processed and released to the circulation where it has been found as a small peptide with the C-terminal 25 amino acids containing eight cysteine residues that form four disulfide bonds. These cysteine residues are well conserved among several species. The two predominant forms in urine contain 20 and 25 amino acids and both have the cysteines and corresponding disulfide bonds. Hepcidin exhibits antifungal and antimicrobial activity and shares structural features with other known peptide antibiotics such as defensins (43).

The relation between iron regulation and hepcidin was found later when Pigeon et al. (61) discovered that hepcidin was overexpressed in iron-overloaded mice. By performing a suppressive substractive hybridization between cDNA obtained from the liver of carbonyl iron-overloaded and control mice, they showed that hepcidin mRNA was overexpressed in iron-overloaded mice by 9.8-fold compared with the control mice. Hepcidin overexpression occurred also with other models of iron

overload such as iron-dextran injected mice and β_2-microglobulin knockout mice. Conversely, hepcidin expression was reduced in β_2-microglobulin knockout mice fed a low-iron diet. As with previous experiments, hepcidin transcripts were found exclusively in the liver. Interestingly, the injection of LPS, a classical inducer of acute-phase proteins involved in the inflammatory response, was also able to induce hepcidin expression.

Further evidence of the importance of hepcidin in iron metabolism was provided by Nicolas et al. (51). Their work showed lack of hepcidin expression in upstream regulatory factor 2 (USF2) knockout mice that leads to severe tissue iron overload. The disruption of the USF2 gene silenced the expression of the hepcidin immediately downstream. The knockout mice developed multivisceral iron overload that spared only the spleen. However, the role of USF2 in the process of iron overload could not be completely ruled out. A year later, Nicolas et al. (52) demonstrated that USF2 was not involved in the iron overload phenotype already described in the USF2 knockouts, which was more likely related to the lack of hepcidin. They also created transgenic mice overexpressing hepcidin in the liver. These transgenic animals died perinatally with severe iron-deficiency anemia. A further advance has been the finding that hepcidin levels are markedly downregulated in conditions of hypoxia and by severe anemia caused by phenylhydrazine treatment in experimental animals (52a). In addition, mutations in hepcidin cause early-onset iron overload in man known as juvenile hemochromatosis (67a).

Taken together, the results suggest that hepcidin, a molecule secreted into the plasma from the liver, is a key regulator of intestinal iron absorption and also of recycling of iron via reticuloendothelial cells. Increased hepcidin expression results in reduced iron absorption and iron recycling whereas reduced plasma hepcidin acts to increase iron absorption and release of iron through macrophages. These results suggest that hepcidin is a powerful negative regulator of iron absorption. However, how hepcidin acts on the enterocyte or, more precisely, where the interaction occurs and which molecules are involved in this process is not yet known. Nicolas et al. (51) have postulated that hepcidin might interact with the HFE-β_2-microglobulin complex, but further studies need to be done. Recently, levels of hepcidin have been shown to inversely correlate with DMT1 and Dcytb expression (31). Hepcidin does not explain all previous data, however, especially work that shows early effects of plasma iron on duodenal iron absorption (70), and it is likely that duodenal crypt cells sensing plasma iron also act as a "stores regulator." Hepcidin therefore may be more important as the erythroid regulator.

SUMMARY

Dietary iron may be taken up either as heme or nonheme forms. Absorption of dietary iron is affected by the contents of the lumen. Recent progress in the understanding of the uptake of nonheme iron indicates that uptake into the mucosa involves a ferric reductase (Dcytb) and a divalent cation transporter Nramp2/DMT1.

Transfer involves a probable ferrous transporter Ireg1 (or ferroportin or MTP1) in combination with a ferroxidase hephaestin as well as ceruloplasmin. The uptake step for iron via DMT1 appears to be driven by an inwardly directed proton gradient and membrane potential. The driving force for transfer of iron across the basolateral membrane is less clear and may be some kind of facilitated diffusion or involve oxidation of ferrous iron to ferric. With the exception of hephaestin, all the proteins are regulated by iron stores and other physiological regulators of iron absorption such as hypoxia and erythropoiesis. Overall, the identification of the proteins supports earlier physiological studies on the site of iron absorption and also the fact that uptake and transfer processes were distinct, involving different proteins. Several of the proteins have been implicated in genetic disease, such as the rodent anemias in the case of DMT1 and hephaestin and a rare form of autosomal dominant hemochromatosis in the case of Ireg1. The challenge now is to figure out how these genes and proteins are regulated.

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