April 1999: 114–123

Iron Transport Across Biologic Membranes

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Iron is essential for life, but is toxic in excess. Nearly all organisms have therefore developed regulated mechanisms for efficient transport of iron into cells. This paper reviews the current understanding of iron transport, focusing on valuable lessons from studies of yeast iron transport and the discovery of the first mammalian transmembrane iron transporter.

Introduction

Iron is one of the most abundant metals in the earth's crust and is crucial for the survival of nearly all organisms, from bacteria to humans. Its flexible redox potential has been exploited by a variety of proteins that bind oxygen or transfer electrons. In an aerobic environment, iron exists primarily in its ferric form, which is highly insoluble at neutral pH. Organisms have developed a variety of strategies for solubilizing iron and transporting it into cells. Because iron is toxic in excess, transport is meticulously regulated and chelating compounds are produced to detoxify both extracellular and intercellular iron. This review will discuss the various mechanisms that have developed to accomplish these tasks.

Transmembrane Iron Transport in Yeast

Studies of baker's yeast, *Saccharomyces cerevisiae*, have helped define several important aspects of mammalian iron uptake. Most notably, yeast studies have substantiated the considerable interdependence of iron and copper metabolism and have elucidated several genes and gene families important for these processes in mammals.

Iron uptake in yeast is mediated by two distinct transport systems: a low-affinity system with an apparent K_m of 30 μM that is responsible for iron accumulation in iron-replete media[1] and a high-affinity system with an apparent K_m of 0.15 μM that is induced by iron deprivation.[2] Each has a requirement for ferrous (Fe^{2+}) iron. Because iron has a tendency to be oxidized into insoluble ferric (Fe^{3+}) hydroxides, its bioavailability is limited by its reduction state. Indeed, extracellular reduction of iron appears to be the rate-limiting step in yeast iron uptake.

To surmount this problem, yeast express an externally directed cell-surface ferrireductase activity. Ferric reductase 1(FRE1) is a major membrane structural component of this system. It is a glycosylated transmembrane protein homologous to the large subunit of the neutrophil cytochrome b_{558} (gp91*phox*).[3,4] In conjunction with other membrane and cytoplasmic proteins, this neutrophil protein is responsible for the transmembrane transfer of electrons from cytoplasmic reduced nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen located within phagocytic vacuoles, generating superoxide anion. Importantly, the protein motifs in gp91*phox* thought to be responsible for binding the cofactors flavin adenine dinucleotide (FAD) and NADPH are conserved in FRE1.[5–7] The spectral characteristics of isolated yeast membranes also support the hypothesis that FRE1 is a flavocytochrome. Analogous to the mammalian system, it is hypothesized that FRE1 participates in the reduction of extracellular ferric iron to ferrous iron by transferring an electron from NADPH to Fe^{3+}, possibly involving superoxide anion as an intermediate. Consistent with a role in iron metabolism, surface ferrireductase activity and FRE1 mRNA levels are regulated by iron. In addition, FRE1 also participates in the reduction of extracellular copper,[8,9] which is required for the proper function or cell-surface expression of several components of the iron uptake system (see below). A single protein that modifies FRE1 function, UTR1, has been described;[10] however, the mechanism by which it can act with FRE1 to increase cell-surface ferrireductase activity is uncertain.

At least six other proteins homologous to FRE1 are present in the yeast genome, and each is subject to metal-dependent transcriptional regulation by iron (FRE2-FRE6), copper (FRE7), or both (FRE1).[8,11–13] Deletion strains lacking *fre1* have limited residual surface ferrireductase activity. In a *fre1 fre2* null strain, this activity is essentially undetectable. Consequently, other FRE homologues may be of lesser functional importance or may act at intracellular sites.[13]

High-affinity iron uptake in yeast is mediated by a permease-oxidase complex encoded by the *FTR1* and *FET3*

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genes.[14] FTR1, the permease component, contains a probable leader sequence and six putative transmembrane domains. In addition, it has a motif of five amino acids, REGXE, that is present in mammalian ferritin light chains that are thought to interact with iron.[14] FET3 is a type 1 transmembrane protein with a predicted extracellular domain that is homologous to blue copper oxidases.[15] Similar to other members of this protein family, FET3 is a cuproprotein that catalyzes the oxidation of four substrate molecules, in this case Fe^{3+}, with concomitant reduction of one molecule of oxygen to two molecules of water.[16,17] In this way, the function of FET3 is similar to the mammalian blue copper oxidase ceruloplasmin, which is thought to facilitate the loading of iron onto transferrin.[18] Yeast mutants deficient in FET3 lack high-affinity iron transport not only because of a lack of the oxidase itself, but also because a mature copper-loaded FET3 polypeptide is required for the FTR1 permease component to be translocated to the plasma membrane.[14] This FET3-FTR1 interdependence is further illustrated by the observation that FTR1 is required for FET3 to be loaded with copper, to acquire other post-translational modifications, and to express oxidase activity.[14] It is therefore not surprising that mutations in other proteins important for yeast-copper metabolism also adversely affect iron transport. Among these are the cell-surface copper transporter, CTR1,[19] the post–Golgi compartment copper transporter, CCC2,[20] which is homologous to the Menkes disease and Wilson's disease proteins, and the small intracellular copper-binding protein ATX1.[21,22] Other post-Golgi proteins, including GEF1 and VPS41, do not directly mediate copper transport but participate in the maturation of the FET3 protein and give rise to a high-affinity iron transport defect when mutated.[23–26] Homologues of both FTR1 and FET3, named FTH1 and FET5, respectively, are present in the yeast genome. The activity of FTH1 has not been investigated, but FET5 has an oxidase function and its overexpression can suppress the mutant phenotype of *fet3 fet4* double-deletion yeast, suggesting a possible role in iron uptake.[27]

It is uncertain why both cell-surface reduction mediated by FRE1 and subsequent reoxidation by FET3 should be required for high-affinity iron transport. It is likely that the initial reduction enhances the bioavailability of iron; the reoxidation coupled to the permease may enhance the substrate specificity of the transporter.

By contrast, low-affinity iron transport appears to be much less complex and not so intertwined with copper metabolism. FET4, the low-affinity ferrous iron transporter, was cloned as a suppressor of iron-limited growth in a *fet3* deletion strain, which lacked high-affinity uptake.[1] FET4 utilizes ferrous iron like the FTR1/FET3 complex, but does not require FET3 for function. The FET4 open reading frame predicts a protein with a molecular mass of 63 kD with six putative transmembrane domains.[1] It bears no homology to any other known protein or expressed sequence in the database (Genbank 12/98), and it does not contain any known metal-binding motifs. FET4 localized to the plasma membrane.[28] Overexpression and disruption of the protein stimulate and eliminate low-affinity iron uptake, respectively.[1] The fact that mutations in FET4 alter the apparent K_m of the low-affinity system for Fe^{2+} uptake supports the conclusion that FET4 is the low-affinity transporter.[28]

Iron deprivation induces the expression of multiple components of the high-affinity iron uptake system in yeast. This effect is mediated at the level of transcription by a sequence-specific, iron-responsive transcription factor called AFT1. Although it is not homologous to other known proteins, the N-terminal region contains clusters of basic amino acids and the C-terminal region contains numerous glutamine residues, features consistent with DNA-binding and transactivation domains, respectively. A dominant mutant allele of *AFT1, AFT1*-1^{up}, results in high levels of surface ferrireductase activity and iron uptake that are not repressed by high extracellular iron concentrations. Conversely, an *aft1* disruption mutant exhibits low surface reductase activity, impaired high-affinity iron uptake, and sensitivity to iron deprivation. Transcription of genes encoding *FRE1-FRE6*,[13,29] *FET3*,[29] *FTR1*,[12,14] *FTH1*, and *CCC2*[12] has been shown to be AFT1 dependent. Transcription of the gene encoding *FET4*, the low-affinity transporter, is not modulated by AFT1.[28] AFT1 binds to an eight-base-pair consensus sequence (PyPuCACCCPu) present in the promoter regions of genes encoding *FET3, FRE1, FRE2, FRE3, FRE6, FTR1, CCC2,* and *FTH1*.[12,13] Variant sequences are also found in the promoters of genes encoding *FRE4* and *FRE5*.[13] In vivo footprinting has shown that this sequence in the *FET3* promoter is occupied in iron-deficient wild-type and *AFT1*-1^{up} cells and exposed in iron-replete wild-type and *aft1* null cells.[12]

These data indicate that ATF1 has a singular role in the coordinate expression of components of the yeast high-affinity uptake system. It is worth emphasizing that AFT1 influences not only the expression of the reductases and permease/oxidase complex, but also the expression of the intracellular copper transporter, CCC2, required for FET3 cytoplasmic maturation. The transcriptional cross-talk between the iron and copper metabolic pathways also extends to the major transcriptional regulator of yeast copper metabolism, MAC1.[30] The transcriptional activation activity of MAC1 is copper dependent. Under copper-limiting conditions, MAC1 promotes the expression of the copper transporters CTR1 and CTR3 and the cell-surface reductases FRE1 and FRE7.[13,31–34] In coupling the expression of copper transport proteins to the major surface ferrireductase, yeast can, in effect, anticipate the iron deficiency that necessarily accompanies copper deficiency.

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A major site of iron utilization in the yeast cell is the mitochondrion, where iron is incorporated into heme as well as into several iron-containing components of the respiratory system. How iron is shuttled from the plasma membrane to mitochondria and other sites is unknown. It is possible that an intracellular chelator similar to the copper homeostasis protein ATX1 also exists for iron. Mitochondrial iron transport is not as well delineated as plasma membrane iron assimilation. Perhaps the best candidate yeast mitochondrial iron transporter is the protein ATM1. ATM1 is an ATP-binding cassette (ABC) "half transporter" that localizes to the inner mitochondrial membrane.[35] Disruption of *ATM1* results in mitochondrial iron overload, cytochrome deficiency, and oxidative damage.[35–37] In addition, *atm1* null yeast are more sensitive to iron deprivation than are wild-type strains.[36] Because of the functional mitochondrial iron deficiency seen in the setting of excess intramitochondrial iron, it is possible that ATM1 mediates transport of iron across the inner mitochondrial membrane into the matrix, where it can be utilized by the terminal enzyme in heme biosynthesis—ferrochelatase—and incorporated into the iron-sulfur enzymes of the respiratory complexes. The iron accumulation seen in *atm1* mitochondria is similar to the mitochondrial iron deposits detected in sideroblastic anemias. Interestingly, the human homologue of *ATM1, ABC7,* maps to chromosome Xq13.1-q13.3, a region associated with X-linked sideroblastic anemia with spinocerebellar ataxia, suggesting *ABC7* as a candidate gene for this disorder.[38]

Two other possible yeast mitochondrial iron transporters have been identified by complementing the low iron growth defect of a mutant in an iron-dependent protein. These proteins, MFT1 and MFT2, belong to a family of transmembrane transition metal transporters that are found in diverse species, ranging from bacteria to mammals. Each localizes to mitochondria, and overexpression of either protein results in mitochondrial iron accumulation similar to that found in ATM1-deficient strains.[39]

Abnormal mitochondrial iron accumulation is also seen in strains deficient for the yeast homologue (YFH1) of the human protein frataxin, which is responsible for the neurodegenerative disease Friedreich's ataxia. YFH1 is a small, 174-amino-acid protein that localizes to mitochondria. It does not co-localize with the outer mitochondrial membrane protein porin, suggesting that it is an inner membrane or matrix protein.[40–42] Disruption of the gene results in respiratory deficiency associated with loss of mitochondrial DNA.[40–43] There is an increase in cellular iron concentration and marked mitochondrial iron accumulation.[40,43] Iron overload in these cells is mediated by constitutive induction of the high-affinity cell-surface iron transport system, including FRE1 and FET3. Of note, *AFT1*-1^{up} regulatory mutants similarly overexpress the high-affinity uptake system, but do not accumulate excess mitochondrial iron or exhibit respiratory deficiencies,[40] indicating that the *yfh1* null phenotype is not solely attributable to elevated intracellular iron concentrations. The excess mitochondrial iron renders *yfh1* strains more susceptible to oxidative damage.[40,43] A mitochondrial phenotype similar to *yfh1* mutations is also seen in yeast that lack the mitochondrial heat shock protein (mt-Hsp70) homologue SSC2 (alternatively SSQ1). It has been shown recently that this protein plays an essential role in the maturation of YFH1 within mitochondria.[44] Overall, these findings demonstrate that YFH1 influences mitochondrial iron homeostasis and suggest a possible role for mitochondrial iron toxicity in the pathophysiology of Friedreich's ataxia.

Transmembrane Iron Transport in Mammals

Unicellular organisms have evolved efficient mechanisms to regulate iron uptake and natural defenses against iron toxicity. Iron toxicity is a greater problem for multicellular organisms, because iron must move safely from the site of initial absorption to sites of utilization. To accomplish this, multicellular organisms produce transferrins or transferrin-like proteins that chelate iron very effectively. Transferrin (Tf) is an 80 kD glycoprotein that can bind two atoms of iron.[45] It is abundant in plasma. Consequently, normal individuals never have full saturation of all Tf iron-binding sites. This provides a safety margin to accommodate fluctuation of iron levels that accompany dietary and environmental changes. Complete saturation of Tf is seen only in pathologic states, such as hereditary hemochromatosis and transfusional iron overload.

In addition to chelating iron to prevent its toxic effects, Tf also facilitates cellular iron uptake. Its role is superficially similar to that of bacterial siderophores in that it binds to specific cell-surface receptors, but the iron-uptake mechanism differs considerably. Tf receptors (TfRs) are found in abundance on rapidly dividing cells, placental cells, and erythroid precursors. Their function has been studied in most detail in erythroid precursors, although it has been assumed that they have similar activity in other cell types. Diferric Tf binds to TfR, and the complex is internalized by receptor-mediated endocytosis. At that point, the analogy to bacterial siderophore transport breaks down. Specialized endosomes form and undergo acidification through the action of a proton pump. This promotes release of iron from Tf and stabilization of the TfR/apo-Tf interaction. The iron is reduced by an endosomal ferric reductase. It is then exported from the endosome (see below) and the TfR/Tf complex is returned to the cell surface for reuse, completing an elegant and efficient cycle (reviewed in reference 46).

The purpose of the Tf cycle is not completely understood. There are mutant animals and human patients that lack Tf (hypotransferrinemic mice and atransferrinemic people).[47–49] They develop severe anemia, but other tissues are spared and actually become iron overloaded. This

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makes it clear that the Tf cycle is not obligatory for iron uptake by all cells. It seems likely that the Tf cycle evolved for the purpose of concentrating iron in a low-pH environment. This probably makes the iron more accessible to ferrous transporters. The nature of the transport system that exports iron from Tf-cycle endosomes will be addressed later.

Until recently, little was known about how iron enters the body through the intestine, where it must traverse both membranes of the mucosal cell layer. Little Tf is found in the intestinal lumen, and the Tf that is present has been excreted in bile.[50] Because this is insufficient to account for dietary iron absorption, at least one non-TfR-mediated uptake system must exist in the intestine. In general, two distinct forms of iron are present in food—heme iron and nonheme iron. The bulk of intestinal nonheme iron is absorbed in the first section of the duodenum.[51] The acidic pH in the proximal intestine helps solubilize Fe^{2+}, which is produced from dietary Fe^{3+} through the action of reductants such as ascorbate[52–55] and a brush border ferrireductase.[56] However, the mechanism by which Fe^{2+} is subsequently absorbed in the intestine is poorly defined, and a candidate iron transporter was only recently reported in 1997. Using two different approaches, two research groups identified Nramp2 as a major transmembrane iron transporter.

Nramp2, now referred to as divalent metal ion transporter 1 (DMT1),[57] was first identified based on its homology to Nramp1 (natural resistance-associated macrophage protein 1).[58] Nramp1 is involved in host resistance to infection by intracellular pathogens, but its mechanism of action is unknown.[59] The predicted secondary structure of DMT1 is very similar to that of Nramp1, with nearly identical hydropathy profiles and predicted membrane organization. Each of these genes encodes 12 putative membrane-spanning domains, predicted glycosylation sites in the fourth extracellular loop, and a sequence bearing weak homology to bacterial consensus transport motifs in the fourth intracellular loop.[60]

Expression Cloning of DMT1

Gunshin et al.[61] reported the isolation of DMT1 in an expression cloning experiment, using a radiotracer assay for iron uptake by cRNA-injected *Xenopus* oocytes in the presence of ascorbic acid, at pH 6.2. Oocytes injected with mRNA from iron-deficient rat duodenum had a sevenfold increase in $^{55}Fe^{2+}$ uptake compared with water-injected oocytes. Size fractionation established that the peak in $^{55}Fe^{2+}$ uptake was associated with mRNA of 4.0–4.5 kilobases (kb) in length. Screening of the 4.0–4.5-kb mRNA pool resulted in the isolation of a single cDNA encoding DMT1. When expressed in oocytes, this cDNA increased the uptake of $^{55}Fe^{2+}$ more than 200-fold compared with control oocytes.

In oocyte iron uptake experiments performed in the nominal absence of Ca^{2+} and Mg^{2+}, transport of $^{55}Fe^{2+}$ followed Michaelis-Menten-type saturation kinetics. The apparent affinity constant for iron ($K_{0.5}$Fe) was approximately 6 µM. A second, low-affinity iron uptake activity appeared to be associated with an mRNA fraction of 2.0–3.0 kb, suggesting that there may be another iron transport activity represented in the duodenal mRNA sample. $^{55}Fe^{2+}$ uptake was independent of sodium and chloride ion concentrations, and was potently inhibited in the presence of several divalent metal cations (Cd, Mn, Co).[61] DMT1 displays an unusually broad substrate selectivity, with transport capacity decreasing in the order Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}.[61]

Two microelectrode voltage-clamp studies in oocytes revealed that divalent metal ion transport mediated by DMT1 is electrogenic, with Fe^{2+} evoking currents of up to -1000 nano-amps.[61] The Fe^{2+}-evoked currents were both voltage dependent and pH dependent; transport was driven at higher rates at hyperpolarized potentials and/or low extracellular pH. Superfusion of Fe^{2+} resulted in a profound intracellular acidification in oocytes expressing DMT1, but not in control oocytes, indicating that divalent metal iron transport mediated by DMT1 is proton coupled.[61] H^+ activation of the Fe^{2+}-evoked currents revealed Hill coefficients (n_H) for H^+ of approximately 1 (as for Fe^{2+}), suggesting that the transport stoichiometry for DMT1 is 1 H^+:1 Fe^{2+}. At physiologic membrane potentials, the apparent affinity constant for H^+($K_{0.5}$H) was 1–2 µM. According to microclimate pH measurements made in situ, brush border extracellular pH is no more than 6, even when the bulk luminal pH is significantly greater.[62] There are several possible reasons for obligate cotransport of divalent metal ions and protons. First, movement of H^+ down its electrochemical gradient should facilitate uptake of divalent metal ions, even when present in trace amounts. Second, lowering of pH at the intracellular surface of the membrane may help maintain iron in its soluble, Fe^{2+} state, and this may aid in intracellular distribution.

Mutations in DMT1 in Animals with Iron Transport Defects

In a parallel effort to identify transmembrane iron transporters, Fleming et al.[63,64] used a positional cloning approach to find the genes defective in microcytic (*mk*) mice and Belgrade (*b*) rats. Both animal models had been shown to have severe iron deficiency anemia, owing to autosomal recessive iron transport defects at several levels.

The *mk* defect had been particularly well characterized in the intestine. Measurements of iron transport in isolated gut loops indicated that the *mk* defect affected apical iron uptake.[65] Little iron was transferred from the intestinal lumen into mucosal cells, but the iron that was taken up was efficiently exported across the basolateral

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cell border. However, the defect also affected erythroid iron utilization, because the *mk* anemia could not be corrected by parenteral administration of iron.[66,67] Reciprocal bone marrow transplant experiments confirmed that the defect was present both in the intestine and in bone marrow–derived hematopoietic cells.[67] The simplest explanation was that the *mk* mutation altered an iron transporter protein that was needed at both sites.

The *b* defect was also known to affect intestinal iron uptake,[68] but it had been studied primarily in erythroid cells.[69–73] Animals had a late defect in TfR-mediated iron uptake. Dual labeling experiments using radiolabeled iron bound to ^{125}I-Tf indicated that Tf binding and internalization were normal but that iron was not retained within cells.[72,73] It appeared that the Tf cycle was futile; iron could not be exported from endosomes, and it was inappropriately recycled to the cell surface along with Tf and TfR. Although the mechanism of the *b* defect was unclear, it seemed likely that the *b* mutation affected a protein important for iron transport.

A positional cloning/candidate gene strategy initially pinpointed the DMT1 gene as the site of the *mk* mutation.[63] When this was first discovered, the function of DMT1 (then known as Nramp2) was unknown. However, it had recently been reported that a yeast homologue, SMF1, served as a manganese transporter.[74] A single point mutation in DMT1 in *mk* animals substituted an arginine residue for a glycine residue (G185R) within predicted transmembrane domain 4.[63] On the strength of the accumulated data—that the DMT1 gene was nonrecombinant with *mk* in a large genetic backcross, that a homologue could transport Mn, and that *mk* animals carried a nonconservative mutation—it was concluded that G185R is the disease-causing mutation.[63] When expression cloning of DMT1 by Gunshin et al.[61] was reported, this conclusion became indisputable. Taken together, these data indicated that DMT1 is the apical transmembrane iron transporter functioning in absorptive enterocytes.

Soon afterward, directed studies of DMT1 in *b* rats showed that these animals also carried a mutation; in fact, they had exactly the same G185R mutation as *mk* mice.[64] This result, taken in the context of the results on defective TfR-mediated iron uptake in *b* erythroid cells, strongly suggests that DMT1 is the endosomal iron transporter responsible for export of iron from Tf-cycle endosomes. Thus, transmembrane iron transport in the intestine and endosomal iron transport during the Tf cycle have the common feature of transmembrane iron transport carried out by DMT1.

It is not known why the mutation occurred at the same site in two different species. When expressed in transfected mammalian cells, wild-type DMT1 has been shown to confer a large, approximately 75-fold increase in cellular iron uptake.[75] The *mk* or *b* mutation severely impairs iron uptake in similar experiments, indicating that it results in near total loss of DMT1 activity.[75] This result makes it unlikely that the mutation is functionally unique. The most likely explanation for the identical mutations is that there is some structural feature of the DNA sequence encoding transmembrane domain 4 that predisposes both mice and rats to the G185R mutation. This conclusion is supported by the fact that the G185R mutation has been observed on at least two independent occasions in mice.[63] Furthermore, a naturally occurring mutation in the homologous protein, Nramp1, has occurred at an adjacent residue. When DMT1 and Nramp1 proteins are aligned, glycine 169 of Nramp1 corresponds to glycine 184 of DMT1. A mutation at this residue is responsible for increased susceptibility to certain kinds of infection in *bcg* strains of mice.[59] To date, no mutations in DMT1 (at this site or other sites) have been reported in humans.

Expression Pattern of DMT1 In Vivo

Northern blot analysis and in situ hybridization experiments have shown that DMT1 is widely expressed.[58,61] Although detectable in all tissues tested, DMT1 mRNA levels are generally quite low. Expression is high in the duodenum and decreases along the proximal-to-distal axis of the intestine.[61] This pattern corresponds to the anatomical pattern of intestinal iron absorption.[51,76] Iron homeostasis is principally maintained by regulated absorption in the proximal intestine. DMT1 mRNA is most abundant in the villus crypts and decreases along the crypt-to-tip axis, with no mRNA detected at the villus tip.[61] Following diet-induced iron deficiency, DMT1 mRNA expression was dramatically increased in duodenum and in other tissues examined, suggesting that DMT1 expression is regulated in response to iron status. One possible mechanism for this regulation will be described below.

In kidney, DMT1 mRNA was expressed at highest levels in S3 proximal tubule segments,[61] suggesting that DMT1 might be involved in the reabsorption of divalent metals. Less intense expression was detected over the entire length of the collecting ducts, where it might be involved in the final reabsorption of metal ions. DMT1 was also evident in the brain. DMT1 mRNA was consistently found in neurons, but not in glial or ependymal cells.[61] Most neurons expressed DMT1 mRNA at low levels, with more prominent expression in densely packed cell groups, including the hippocampal pyramidal and granule cells, cerebellar granule cells, and the preoptic nucleus and pyramidal cells of the piriform cortex. DMT1 mRNA was present at moderate levels in the substantia nigra.

To date, anti-DMT1 antibodies have been inadequate for protein localization in cells or tissues. To circumvent this problem, the initial localization studies have been carried out using epitope tag-labeled DMT1 in transfected cells. Although the information derived from these stud-

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ies must be interpreted carefully because of the nature of the experiments, the results are still informative. Su et al.[75] have shown that DMT1 can be found both on the cell surface, as exhibited by biotinylation of intact cells, and within the cell in discrete endosomes. The intracellular pattern is consistent with localization in Tf cycle endosomes, and the pattern is nearly identical to that seen by uptake of Texas red Tf. This indicates that DMT1 can be expressed on the plasma membrane, as well as at the site of TfR-mediated iron uptake within the cells. These results are consistent with the DMT1 functions deduced from the study of *mk* and *b* animals.[63,64] They corroborate the conclusions that DMT1 is both the apical transmembrane iron transporter functioning in the duodenal mucosa and the endosomal iron transporter important for TfR-mediated iron uptake in erythroid cells. The *mk* or *b* mutation does not alter DMT1 localization in transfected cells, indicating that the consequent loss of function is not simply due to loss or mislocalization of DMT1 protein.[75]

Mutagenesis experiments were carried out to determine whether other transmembrane domain 4 residues, conserved among Nramp-like proteins, were also important for normal iron transport. Mutagenesis of glycine 184, the amino acid altered in the *bcg* mutant form of Nramp1, resulted in destabilization of the mature protein. Similarly, mutation of an unusual charged residue in transmembrane domain 4, aspartate 192, was deleterious only because it resulted in a lower level of DMT1 protein. Although not yet tested, it is intriguing to speculate that alteration of a phenylalanine residue, phenylalanine 196, might be informative. When transmembrane domain 4 of DMT1 is projected as an alpha helix, phenylalanine 196 lies adjacent to the G185R mutation found in *mk* mice and *b* rats. This is interesting because phenylalanine 196 is highly conserved across different species and it could serve as an attractive cation binding site. Phenylalanine is polar yet hydrophobic. It has been shown that cations can bind to the p face of an aromatic structure through a remarkably strong, noncovalent force, termed the cation-p interaction.[77]

Regulation of DMT1 Expression

Northern blot analysis demonstrated that dietary iron deficiency resulted in increased DMT1 mRNA levels in all tissues examined. It is not yet known whether this was caused by an increase in transcriptional activity, mRNA stabilization, or both. Interestingly, however, one of two alternative splice forms of DMT1 mRNA has a stem-loop structure in its 3´ untranslated region (UTR) that closely resembles canonical iron response elements (IREs, reviewed in reference 78). This stem-loop structure, located approximately 30 base pairs downstream from the translational stop codon, is reminiscent of IREs in the 3´ UTR of the transferrin receptor mRNA and the 5´ UTR of ferritin mRNAs.[79] It contains a consensus loop CAGUGN sequence after a single C-nucleotide bulge and a five-base-pair stem. There is an additional pyrimidine residue bulge on the opposite side of the stem, which is unique to DMT1.[61] One might expect DMT1 expression to be regulated in a fashion analogous to TfR. Under low iron conditions, iron regulatory proteins (IRPs) bind to the IREs in the 3´ UTR of TfR mRNA and protect the mRNA from degradation. Like the TfR mRNA, the DMT1 IRE may regulate its mRNA stability, increasing mRNA availability in iron-depleted states and decreasing mRNA stability when iron is abundant. This model has not yet been validated experimentally.

The IRE/IRP regulatory system is believed to be specific to iron regulation. Because DMT1 transports other metals in addition to iron, other regulatory mechanisms must exist to govern its expression. Interestingly, DMT1 is expressed as at least two different splice forms. One, containing the IRE sequence, encodes a 561-amino-acid protein. Another form does not contain a recognizable IRE and encodes a 568-amino-acid protein. It has been reported that both splice variants are widely expressed, but brain appears to express the highest ratio of IRE to non-IRE forms.[80] Spleen, thymus, and pancreas appear to have the highest ratios of non-IRE to IRE forms. The putative DMT1 5´ regulatory region contains two CCAAT boxes but no recognizable TATA sequence.[80,81] There are also five potential metal response elements, three potential SP1 binding sites, and a possible γ-interferon regulatory element. A segment containing as little as 246 base pairs upstream of the transcriptional start site has been shown to activate transcription of a luciferase report gene in HeLa cells,[81] but the details of DMT1 regulation remain to be elucidated.

SFT: Another Transmembrane Iron Transporter?

Expression cloning in *Xenopus* oocytes has been used to identify a second protein that appears to play a role in iron metabolism. Microinjection of mRNA from K562 chronic myelogenous leukemia cells led to a modest increase in oocyte iron uptake, which was attributable to sequences encoding stimulator of Fe transport (SFT).[82] Forced expression of SFT in transfected cells also stimulates iron uptake, but this stimulatory activity has quite different properties from those of DMT1 and is not pH dependent.[83,84] SFT has been shown to co-localize with late Tf-cycle endosomes. Its precise function remains obscure; although it has multiple membrane-spanning regions, there is no direct evidence that it is an iron transporter. It may facilitate iron transport through some other mechanism.

Open Questions

The existence of a common intestinal absorptive mechanism for a variety of metals (Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+}) has important nutritional implications and em-

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phasizes the potential interplay among these essential trace minerals at the level of their absorption. Absorption of these divalent cations will proceed even at physiologic Ca^{2+} concentrations, although available data suggest that excessive luminal Ca^{2+} could potentially interfere with their normal absorption.[85] Of toxologic relevance, DMT1 also transports the toxic heavy metals Cd^{2+} and Pb^{2+}. The phenotype of the anemic Belgrade rat makes it clear that DMT1 plays an important role in other tissues as well. There is compelling evidence that DMT1 serves as the endosomal iron transporter in Tf-cycle endosomes, acting to export iron from the endosome into the cytoplasm of the cell. The role of DMT1 in other cell types remains to be established.[86–90] The low-pH, iron-rich milieu of the endosome is an ideal location for maximal activity of DMT1. It is not clear whether it functions in iron transport at the plasma membrane, where protons are harder to come by. The existence of plasma membrane, non-transferrin-bound iron uptake mechanisms is well established. Some of these are susceptible to interference from other divalent cations. It remains to be seen whether any of these activities are attributable to DMT1.

Based on existing animal models, it seems likely that human mutations in DMT1 might account for inherited hypochromic, microcytic anemias, but this has not yet been documented in the literature. It is possible that DMT1 may play a role in the etiology of certain neurodegenerative diseases by promoting the generation of reactive oxygen species by divalent cations, resulting in lipid peroxidation and damage to essential proteins. In Parkinson's disease there is substantial accumulation of iron in affected neurons of the substantia nigra (reviewed in reference 91), which has moderately high expression of DMT1.[61] Increased iron content there may contribute to neuron death by inducing the production of harmful hydroxyl radicals.

Collectively, the data presented constitute a major advance in our understanding of a critical physiologic question of how iron crosses cell membranes. Moreover, the existence of a single transport mechanism that serves a variety of divalent metal cations and is driven by the proton electrochemical gradient may have profound nutritional, clinical, and toxologic implications. Yet, several questions need to be addressed. If DMT1 is the apical iron transporter, what molecule serves as the basolateral intestinal iron transporter? How does DMT1 fit into the regulatory pathway that is perturbed in patients with hereditary hemochromatosis? Are there other, tissue-specific iron transporters that are operative in other tissues?

Finally, although it is indisputable that there is a connection between iron and copper metabolism in mammals as there is in yeast,[92] the molecular details have not been fully worked out. It has been known for many years that copper-deficient swine develop iron deficiency anemia.[93,94] The plasma copper protein ceruloplasmin functions as a ferroxidase and plays a role in the release of storage iron from cells.[18,95] As mentioned previously, it is homologous to the membrane-bound ferroxidase FET3 in yeast, but its function is probably not strictly analogous. Patients who lack ceruloplasmin have derangements of iron metabolism in some tissues (notably the central nervous system), but not all tissues.[96] Recently, a second mammalian ferroxidase has been discovered that is homologous to FET3 and ceruloplasmin.[97] This molecule plays a role in intestinal iron absorption, but its function is not fully understood. The precise interplay between mammalian iron metabolism and copper metabolism constitutes one of the major outstanding issues in this field.

Conclusions

Iron is an essential element for nearly all living organisms. Prevalent human metabolic and infectious diseases result from defects in iron procurement, transport, or sequestration. Recent discoveries have advanced our understanding of these processes and have introduced new areas for future investigation. The parallel study of mammalian and yeast iron transport processes has proved invaluable. It is clear that much remains to be learned and that important information is likely to come from further examination of the similarities between yeast and mammalian iron metabolism.

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