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Mechanisms of Cellular Iron Acquisition: Another Iron in the Fire

Minireview

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Iron transport occurs by the well-known transferrin (Tf)-transferrin receptor (Tf receptor) system and by a second as yet uncharacterized system. Two reports in the current issue of *Molecular Cell* suggest an unexpected candidate for the Tf-independent system.

Iron is an essential element required for many redox processes in all eukaryotes and most prokaryotes. Iron physiology is dominated by the fact that there is no excretory route for iron in either uni- or multicellular organisms. Consequently, iron homeostasis is regulated at the level of uptake. In multicellular organisms, iron must be distributed from the site of absorption to cells that require iron. Genetic and molecular approaches have defined the mechanisms of iron uptake into absorptive cells and the subsequent transport of iron into plasma. This review will discuss the transporters involved in intestinal iron acquisition. It will also discuss how iron is distributed from the site of absorption to other cells. Transport occurs by the well-described transferrin (Tf)-transferrin receptor (Tf receptor) system and by a second Tf-independent system of which, until recently, little was known.

Iron Transport by the Intestine

Environmental and dietary iron is most often present as Fe(III), a form that is sparingly insoluble and generally bio-unavailable. In vertebrates, iron entry into the body occurs primarily in the duodenum, the most proximal portion of the intestine (Figure 1). In the intestinal lumen, Fe(III) is reduced to the more soluble Fe(II) by a heme containing ferrireductase (DcytB), which transports electrons from cytosolic NADPH to extracellular acceptors such as Fe(III) and possibly Cu^{2+} (McKie et al., 2001).

A transmembrane permease, Nramp2 (also known as DMT1 or DCT1), transports Fe(II) across the intestinal surface (Fleming et al., 1997; Gunshin et al., 1997). Nramp2 is a H^+/divalent metal symporter that transports other transition metals in addition to iron; most important physiologically is Mn^{2+} and most important pathologically is Pb^{2+}. Nramp2 is the most conserved of the known iron transporters, with homologs in bacteria, yeast, and plants. Defects in Nramp2 result in impaired intestinal iron transport and, as will be discussed below, defective endosomal iron transport.

Iron entering the absorptive enterocyte is either stored as ferritin or transported across the basolateral surface. Mature absorptive cells have a limited life span (2–5 days) so that iron retained as ferritin may become unavailable for absorption. Iron not sequestered as ferritin is transported across the basolateral surface by a transmembrane permease termed ferroportin (also referred to as IREG1, MTP, and Slc11a3) (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2001). The mechanism by which this permease affects iron transport is unknown. As opposed to Nramp2, ferroportin shows no homology to transporters in single-cell organisms. Ferroportin is not specific to the intestinal cells but is present on all cells in which iron export is a major function, such as placental cells (or yolk sac of nonmammalian vertebrates) and macrophages. While the intestine is the site of net iron absorption into the body, most iron entering plasma results from iron recycled from senescent or damaged red blood cells. Transcription of ferroportin is iron regulated, but again the mechanism of regulation is unknown.

A strong candidate for a regulator of intestinal iron absorption has recently been identified. Hepcidin is a protein that was discovered simultaneously as an antibacterial protein found in human urine (Park et al., 2001) and as a protein preferentially expressed in iron-loaded murine liver (Pigeon et al., 2001). Hepcidin is a heavily disulfide bonded peptide of 20–24 amino acids that is synthesized from a larger precursor secreted by the liver. A gene deletion of hepcidin resulted in massive iron-liver loading (Nicolas et al., 2001), whereas transgenic expression of hepcidin led to severe anemia (Nicolas et al., 2002). This latter result was confirmed by the discovery that humans who have liver adenomas that express high levels of hepcidin present with a chronic anemia (Weinstein et al., 2002). In sum these results indicate that hepcidin is produced by iron-loaded liver and can regulate intestinal absorption. The receptor for hepcidin is unknown and clearly its discovery will represent an important milestone in understanding the mechanism of regulation of iron absorption.

Delivery of Iron to Cells by the Tf Receptor System

For effective utilization of iron, Fe(II) exported from the intestine must be converted to Fe(III) and subsequently bound to Tf. This protein, found in plasma, vitreous fluid, and brain, is the major extracellular iron binding protein. Conversion of Fe(II) to Fe(III) is catalyzed by a specific class of copper-containing enzymes, multicopper oxidases, which accomplishes the oxidation of Fe(II) without generating reactive oxygen intermediates. The basolateral surface of the intestine utilizes a membrane bound putative multicopper oxidase termed hephaestin (Vulpe et al., 1999). Mutations in hephaestin in mice lead to defective intestinal iron transport resulting in microcytic anemia. Most mammalian cells rely on the plasma multicopper oxidase ceruloplasmin to effect oxidation of newly exported Fe(II) to Fe(III) (for review see Hellman and Gitlin, 2002). Ceruloplasmin is the major copper-containing protein in plasma. Decreased plasma ceruloplasmin results in decreased cellular iron export and increased tissue iron content. One of the phenotypic manifestations of ceruloplasmin mutations in humans is ataxia due to iron accumulation in basal ganglia.

Transferrin (Tf) delivers iron to cells by binding to cell

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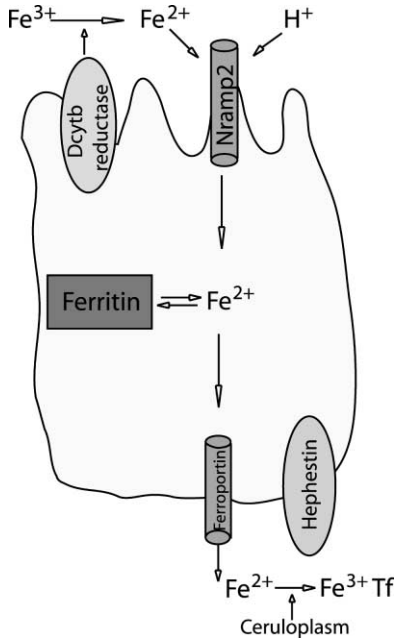

Figure 1. Intestinal Iron Uptake

Ferric (Fe(III)) iron is reduced in the lumen of the intestine by the membrane reductase DcytB. Iron (Fe(II)) is transported across the plasma membrane by the H^+/divalent cation symporter Nramp2. Within cells, iron is stored as ferritin or exported across the basolateral surface by ferroportin. Exported Fe(II) is converted to Fe(III) by either membrane bound hephaestin or soluble ceruloplasmin, which is then bound to transferrin.

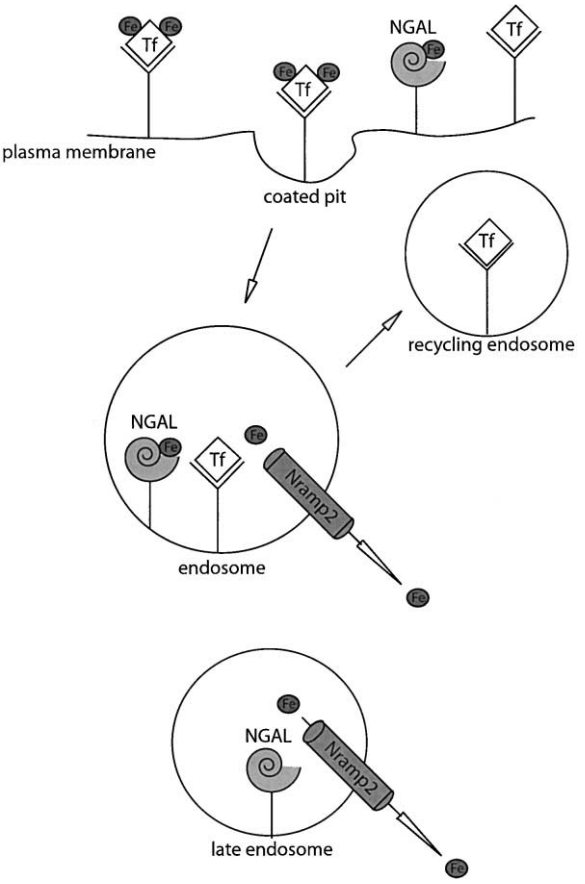

Figure 2. Iron Delivery to Tissues

Transferrin (Tf) iron complexes bind to cell surface Tf receptors and the receptor ligand complex is internalized through clathrin-coated pits. Iron dissociates from receptor bound transferrin within an early endosome, where Nramp2 then transports it out of the endosome into the cytosol. NGAL/M24p3 with a bound siderophore-Fe(III) is also internalized by clathrin-mediated endocytosis. Once internalized, however, NGAL/M24p3 appears to separate from Tf and is trafficked to late endosomes. Iron dissociates from NGAL/M24p3 in this compartment, which is more acidic than early endosomes. The siderophore may remain associated with NGAL/M24p3, and the NGAL/24p3-apo siderophore complex is recycled to the cell surface.

surface Tf receptors (Figure 2). The delivery of transferrin bound iron to cells was recognized almost fifty years ago and the mechanism of iron delivery is known in some detail (for review see Richardson and Ponka, 1997). The Tf-Fe(III)-Tf receptor complex is internalized into endosomes, where iron then is released from Tf. While iron binds to Tf with a K_d of 10^{-24} at neutral pH, the binding of Tf to Tf receptors at the acidic pH of endosomes dramatically lowers the affinity of Tf for iron, permitting its release from receptor bound Tf. Additionally, there is good evidence that a ferrireductase is present in the endosome, as iron is transported across the endosomal membrane by the divalent metal transporter Nramp2. Thus, defects in Nramp2 result in both impaired intestinal iron transport and endosomal iron delivery.

Tf is not the only mammalian protein that binds iron with high affinity. Lactoferrin is a second vertebrate iron binding molecule that, like Tf, binds two atoms of Fe(III). Rather than provide iron to cells, the function of lactoferrin is to withhold iron from infectious agents. Lactoferrin is stored in neutrophil azurophilic granules and is released when these granules fuse with the cell surface. There are a wide variety of inflammatory agents that induce release of granular contents. By binding iron, lactoferrin is thought to be bacteriostatic, although other roles have been suggested. Lactoferrin is one of a number of antibacterial proteins released by neutrophils; other examples include vitamin B12 binding protein and myeloperoxidase.

Iron Delivery by a Tf-Independent Transport System

There is strong evidence that cells must also express a Tf-independent iron transport system. For example, mice and humans lacking Tf, while anemic, show iron overload in parenchymal tissues such as liver and spleen. Similarly, when iron enters plasma in excess of the Tf binding capacity (as seen in hereditary hemochromatosis), iron is cleared from plasma by uptake into parenchymal tissues. Further, Tf receptor knockout mice die as an early embryonic lethal, but there is some tissue development, suggesting a second iron uptake mechanism (Levy et al., 1999).

It is in this context that the discovery that neutral gelatinase-associated lipocalin (NGAL) binds iron is both interesting and important. Lipocalins are a large group of proteins that are related more in structure than sequence and which bind small molecules (for review see Akerstrom et al., 2000; Flower et al., 2000). Lipocalins have been implicated in the transport of small organic molecules such as retinol, prostaglandins, fatty acids, and oderants. Structural studies reveal that lipocalins have a fold capable of binding hydrophobic li-

gands. Lipocalins have also been implicated in a wide variety of cellular processes including apoptosis, immune modulation, and growth regulation. NGAL is secreted by neutrophils and epithelial cells. NGAL was thought to have an immune regulatory effect resulting from the binding of the hydrophobic bacterial chemoattractant F-Met Leu Phe. Goetz et al. (2002), however, considered that the ligand binding fold in NGAL is shallow and lined with polar and positively charged residues compared that of other lipocalins, which are deeper and more hydrophobic. In order to define the ligand binding characteristics of NGAL, Goetz et al. expressed the protein in *E. coli* and discovered that a red chromophore copurified with the protein. The chromophore was tightly but not covalently associated with the purified NGAL. Examination of the protein chromophore complex by X-ray crystallography showed the presence of a strong novel signal emanating from the center of the ligand complex. The signature of the signal indicated a heavy atom, and both atomic absorption and X-ray fluorescence identified it as iron. Further analysis, including both analytical and binding studies, revealed that the red chromophore was iron-enterobactin. Enterobactin is one of a number of high-affinity iron binding molecules called siderophores, which are produced by bacteria or fungi in response to iron need (for review see Braun, 2001). Siderophores bind iron with extraordinarily high affinity and iron-siderophore complexes are then taken up by specific receptors. Goetz et al. also demonstrated that NGAL binds enterobactin with an affinity that rivals the *E. coli* enterobactin transporter. Consequently, the binding of enterobactin by NGAL can prevent bacterial iron uptake. This observation suggests that NGAL might act as a bacteriostatic agent like lactoferrin. The finding that NGAL binds enterobactin might explain why *E. coli* does not require enterobactin for mammalian colonization, as deletions in genes that synthesize or accumulate enterobactin do not affect growth of *E. coli* in mammals. Aerobactin, another siderophore secreted by *E. coli*, is required for mammalian colonization. Goetz et al. point out that NGAL binds enterobactin but does not bind aerobactin. A clear prediction is that because of NGAL, enterobactin is not a pathogenicity factor but would be in a NGAL-deficient animal.

The finding that NGAL can function as an iron binding molecule is extended further in the companion paper by Yang et al. (2002). These authors studied the role of the murine homolog of NGAL, M24p3, in kidney development. Epithelial tubules in the kidney develop from mesenchymal cells through the influence of factors secreted by the ureteric bud. These authors previously identified the cytokine LIF as an inducing factor produced by the ureteric bud (Barasch et al., 1999). They noted the presence of a second factor, and in this communication they report the identity of this second factor as M24p3, the mouse ortholog of NGAL. Based on the structural studies of Goetz et al., which showed NGAL to be an iron binding molecule, Yang et al. metabolically labeled cultured ureteric bud cells with ^{59}Fe, purified M24p3, and demonstrated that the purified molecule had associated ^{59}Fe. Further, NGAL loaded with iron could donate iron to a number of cells in a biologically relevant manner, as shown by the fact that iron delivered by NGAL regulated iron-dependent genes. The authors probed the mechanism of NGAL-mediated iron delivery and showed that NGAL is internalized into endosomes and, like Tf, is recycled. However, the intracellular trafficking of NGAL/M24p3 is distinct from Tf, as it is internalized into what appears to be a late endosome. Further differences from Tf include the topological distribution of NGAL/M24p3 on polarized epithelial cells. Tf receptors are localized only on the basolateral surface, but NGAL/M24p3 can be internalized from either the basolateral or apical surface. Further, there are differences in tissue distribution. Yang et al. show that fluorescent Tf is accumulated by late epithelial progenitors and more differentiated renal tubule epithelia, whereas fluorescent NGAL was accumulated by peripheral cells, an earlier mesenchymal progenitor.

A conclusion from these studies is that differentiating epithelial cells utilize NGAL/M24p3-mediated iron delivery. Iron delivery by NGAL/M24p3 alone is probably not sufficient to promote tissue differentiation. The authors suggest that NGAL/M24p3-mediated iron delivery might be most important for tissue and organ development in the earliest stages of development, at a point before circulation of Tf and expression of Tf receptors is established. This hypothesis fits well with the finding that showed tissue development in the early embryo occurs in Tf receptor-deficient mice (Levy et al., 1999).

Unresolved and critical questions are what is the siderophore that binds to NGAL/M24p3, and what cell types synthesize it? That endogenous siderophores exist is a clear prediction from this study. The authors quote older studies that make such a prediction but this field has languished for years. It is exciting to consider that a NGAL-mediated iron transport may be the long-sought Tf-independent iron transport system. Equally exciting is that NGAL-mediated iron delivery may contribute in the anemia of chronic disease. As a consequence of an inflammatory state, plasma Tf iron levels drop dramatically, leading to an iron-limited erythropoeisis (Jurado, 1997). The mechanism(s) underlying this dramatic decrease in plasma iron has never been fully explained. Impaired iron recycling from macrophages is the favored hypothesis, but the ability of NGAL/M24p3 to bind Fe(III) may also interfere with delivery of Fe(III) to Tf. The discovery of NGAL as a potential iron delivery vehicle is exciting. A discovery that mammals produce siderophores would lead to an epochal change in the paradigm of mammalian iron homeostasis.

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