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Separate pathways for cellular uptake of ferric and ferrous iron

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Conrad, Marcel E., Jay N. Umbreit, Elizabeth G. Moore, Lucille N. Hainsworth, Michael Porubcin, Marcia J. Simovich, Marian T. Nakada, Kevin Dolan, and Michael D. Garrick. Separate pathways for cellular uptake of ferric and ferrous iron. *Am J Physiol Gastrointest Liver Physiol* 279: G767–G774, 2000.—Separate pathways for transport of nontransferrin ferric and ferrous iron into tissue cultured cells were demonstrated. Neither the ferric nor ferrous pathway was shared with either zinc or copper. Manganese shared the ferrous pathway but had no effect on cellular uptake of ferric iron. We postulate that ferric iron was transported into cells via β_3 -integrin and mobilferrin (IMP), whereas ferrous iron uptake was facilitated by divalent metal transporter-1 (DMT-1; Nramp-2). These conclusions were documented by competitive inhibition studies, utilization of a β_3 -integrin antibody that blocked uptake of ferric but not ferrous iron, development of an anti-DMT-1 antibody that blocked ferrous iron and manganese uptake but not ferric iron, transfection of DMT-1 DNA into tissue culture cells that showed enhanced uptake of ferrous iron and manganese but neither ferric iron nor zinc, hepatic metal concentrations in mk mice showing decreased iron and manganese but not zinc or copper, and data showing that the addition of reducing agents to tissue culture media altered iron binding to proteins of the IMP and DMT-1 pathways. Although these experiments show ferric and ferrous iron can enter cells via different pathways, they do not indicate which pathway is dominant in humans.

mobilferrin; calreticulin; integrin; divalent metal transporter-1; Nramp-2

ALL CELLS REQUIRE IRON TO SURVIVE (6). Iron is acquired from the diet and transported across the intestinal mucosa as either inorganic iron or heme iron. Little is known about the mechanism for transport of heme iron into the absorptive cell as a metalloporphyrin, but the transport of nonheme iron has been the object of considerable recent study. Because the lumen of the intestine does not contain adequate amounts of transferrin and the luminal surface of the enterocyte lacks transferrin receptors (34, 35), the transport of iron by intes-

tinal cells differs from that of other cells. While nonintestinal cells are capable of transporting iron in the absence of transferrin, plasma has only low concentrations of nontransferrin bound iron, and plasma iron is mostly transported in association with transferrin. Iron is largely transported into nonintestinal nucleated cells by either of two transferrin transport mechanisms: the classical pathway utilizing the transferrin receptor and another poorly understood low-affinity system that does not utilize the classical transferrin receptor (25, 39). Recent discovery (26) of another transferrin receptor may provide insight into the low-affinity, high-capacity system. In iron-overloading disorders, ferric citrate may serve as an important contributor for delivery of iron to nonintestinal cells (24).

Despite the importance of transferrin, nonintestinal cells retain the capability to transport inorganic iron free of transferrin (3, 7, 19, 22, 25, 32, 37, 39, 42). Tissue culture cells possess a similar capability (15, 27, 36). In one of the best studied cell lines, erythroleukemia-like K562 cells transport ferric iron by the β_3 -integrin-mobilferrin pathway (IMP) (15). This is the same pathway demonstrated to transport ferric iron into the intestinal mucosa of rats and humans (10, 13, 14, 17). Ferric iron is not soluble at physiological pH and must be chelated to remain soluble above pH 3. In the intestine, the chelator mucin is available (11), but in tissue culture systems a chelator must be added, usually citrate or nitrilotriacetic acid, to solubilize the ferric iron. The concentrations of chelators must be controlled or else the chelators both compete with cells for iron and can cause cell damage. Ferric iron is bound to a cell surface β_3 -integrin (10, 16) and transferred to a calreticulin-like chaperone protein called mobilferrin (17). Before the iron can be used for heme synthesis it must be converted to the ferrous valence. A cytoplasmic protein complex, containing mobilferrin, β_3 -integrin, and flavin monooxygenase as well as other peptides, uses an electron-transport chain with energy

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derived from NADPH to accomplish and maintain ferrereduction intracellularly (40, 41).

Tissue culture cells also directly transport ferrous iron, which is soluble at physiological pH but rapidly oxidized to ferric iron in an aerobic environment. A reducing agent, often ascorbic acid, must be added to maintain transiently the ferrous valence. It is believed that the ferrous pathway in nonintestinal cells is the same as in the intestine and that the iron is transported via a transmembrane pump protein, divalent metal transporter-1 (DMT-1; Nramp-2) (20, 21, 23). Although an iron-transport function has never been directly demonstrated for this protein in the intestine, the levels of the protein in enterocytes are 100-fold increased in iron deficiency (8, 38). Defects in DMT-1, expressed in the mk/mk mouse and the Belgrade rat (20, 21), result in a viable but iron-deficient animal. Once inside the cell, the ferrous iron pathway for delivery to the mitochondrion for insertion into porphyrin is not known. Iron transport into the mitochondrion was inhibited by manganese, but not by zinc, and required the ferrous valence, similar to DMT-1 (9, 29).

The IMP is unusual in that it does not involve a classical ion pump, but rather a series of interacting proteins that bind iron *in vivo* and *in vitro*. This has led some to suggest it plays no role in iron transport and that ferric iron must be reduced by an extracellular, membrane-bound ferrereductase to ferrous iron before it can be transported into the absorptive cell, presumably by the DMT-1 protein (30).

The relationship between ferric and ferrous iron-transport systems is critical for understanding the transport of inorganic iron independent of transferrin. In this study, we demonstrate that ferric iron and ferrous iron are transported by two separate pathways in K562 cells, obviating the requirement for an extracellular ferrereductase. Ferric iron does not require reduction to ferrous iron before transport. Because the pathways are believed to be the same as in the intestinal cell (15, 20), these results can be extrapolated to the *in vivo* situation. By utilizing tissue culture cells, it is possible to control the valence state with precision not possible *in vivo*.

METHODS AND MATERIALS

Tissue culture. K562 human erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, MD). A human kidney cell line (HEK293T) was provided to M. D. Garrick by Dr. Mark Fleming of Children's Hospital (Boston, MA). The K562 cells were grown in suspension in RPMI 1640 and 10% heat-inactivated fetal bovine serum (Life Technologies). Cells were incubated in a 5% CO_2 incubator (Shel-Lab, Cornelius, OR). Cells were grown to a density of 5×10^6 cells. The cells were carefully washed in buffer free of fetal calf serum three times (10 mM HEPES, 0.15 M NaCl, 0.1% glucose, and 1 mM calcium and magnesium, pH 7.4). Cell viability was judged by exclusion of 0.4% trypan blue; it always exceeded 95% except in the presence of a high concentration of certain metals ($>10^{-4}$ M) when cell death and an enhanced nonspecific binding of metals occurred. Cells were centrifuged at 1,500 rpm for 5 min to wash and concentrate cells for study (Beckman TJ6R, Palo Alto,

CA). Antibiotics were not added to tissue culture flasks to avoid difficult-to-detect contamination (L forms) and because many antibiotics bind iron. Approximately 1.5×10^6 cells, monitored by using a hemocytometer, were used in each test mixture. HEK293T cells were grown in DMEM and 10% heat-inactivated fetal bovine serum. Media removal and washes were accomplished by pipette without centrifugation. Otherwise, preparation was similar to that described for K562 cells. Cells were freed from the plates by the addition of detergent solution (1% deoxycholic acid, 1.5% Triton X-100, 0.1% SDS, and 25 mM HEPES) for transfer to test tubes for quantification of radioactivity in an automated gamma detector. All buffers were prepared using doubly distilled deionized water.

Transfection of HEK293T cells. HEK293T cells were plated at 2×10^5 cells/well in six-well plastic Biocoat plates coated with poly-D-lysine (Fisher Scientific, Norcross, GA). Opti-Mem (0.5 ml) (GIBCO, Grand Island, NY) was mixed with 6 μl DMR1E (Gibco) in Falcon 2027 tubes (Becton Dickinson, San Jose, CA). One microliter of rat DMT-1 construct DNA (1 $\mu\text{g}/\mu\text{l}$) was mixed with 0.5 ml of Opti-Mem in Falcon 2027 tubes. The mixtures were combined and incubated for 45 min at 25°C. HEK293T cells were washed with PBS, and the mixture was added to each well and incubated for 5 h in a CO_2 incubator at 37°C. Then 1.2 ml of DMEM with 20% fetal calf serum and 2 mM L-glutamine were added to each well. The cells were used for assay 48–72 h later.

Expression constructs. Full-length rat DMT-1 wild-type and mutant (G185R) was provided by M. D. Garrick. They were subcloned into the *EcoR* I site p tracer CMV 2 (Invitrogen, Carlsbad, CA). The vector contained the p Tracer-CMV 2 promoter for high-level expression of DMT-1. The vector also contained a human elongation factor-1 α promoter for expression of the green fluorescent protein-Zeocin fusion protein that was used to monitor transfection efficiency. Transfection efficiencies were obtained using fluorescence microscopy (Olympus model BX 60, Melville, NY). The percentage of fluorescent cells was quantified visually and showed that 25–30% of cells in our studies were transfected with DMT-1 wild type and mutant.

Radionuclide uptake studies. $^{59}\text{FeCl}_3$ (12.8 mCi/mg) or $^{59}\text{FeSO}_4$ (13.4 mCi/mg), $^{65}\text{ZnCl}_2$ (67.5 mCi/mg), and $^{54}\text{MnCl}_2$ (40 mCi/mg) were obtained from NEN (Boston, MA). $^{64}\text{CuCl}_2$ (9.3 mCi/mg) was obtained from the University of Missouri (Columbia, MO). One milliliter of citrate (10^{-5} M) was added to the $^{59}\text{FeCl}_3$ at pH 2.2 to maintain the iron soluble as a chelate at neutral pH when it was buffered with HEPES to pH 7.4. One milliliter of ascorbic acid (10^{-5} M) was added to the $^{59}\text{FeSO}_4$ before dispensing it from the manufacturer's anaerobic container. In certain experiments, the radiolabeled ferrous ascorbate was dispensed into tissue culture cell buffer. It contained 2-mercaptoethanol (10^{-4} M) to maintain the iron in a reduced state (this concentration is commonly employed in cultures of embryonic stem cells). Approximately 0.1 μCi of radionuclide in 10 μl was added to 1 ml of serum-free HEPES buffer (as described above) containing 1.5×10^6 cells. It was incubated for 30 min at 37°C. Then the cells were washed with three changes of a cold serum-free buffer (4°C) before the radionuclide uptake by cells was measured in a gamma detector. In certain experiments, nonradioactive metal salts were added in various molarities varying from 10^{-9} to 10^{-4} M. Higher concentrations of some metals produce cell injury as determined by trypan blue exclusion and impaired uptake of [^{14}C]alanine. These cells show increased nonspecific binding of metals to the cellular structures. In uptake experiments of radioactive metals, carrier iron of the

same redox state was added in sufficient quantities to make the final solution 10^{-7} M.

Antibodies. A monoclonal anti- β_3 -integrin antibody (lot JG 10396) was provided by Centocor. The antibody contained 2 mg protein/ml. The antibody was incubated with triply washed cells for 10 min at $37^\circ C$ before addition of radionuclide to the tissue culture cells. Unless specified, 10 μ l of the antibody were added to incubation mixtures designated for antibody exposure before addition of radionuclide. A polyclonal antibody was raised against a polypeptide sequence of human DMT-1 (amino acids 325-339, KTNEQVVEVCT-NTSS) (28). Each rabbit was immunized with the polypeptide construct in Ribi adjuvant at 2-wk intervals for three multiple subcutaneous inoculations. Development of antibody against the polypeptide construct was monitored in rabbit serum by an ELISA method. Preimmunization and postimmunization serum was used for purification of IgG (Immunopure A/G purification kit, Pierce, Rockford, IL) for use in blocking radiolabeled uptake of metals by K562 cells as described above. To test for specificity, whole lysates of K562 cells were electrophoresed on 7.5% polyacrylamide gels and transferred to Western blots. The purified anti-DMT-1 antibody reacted with a single band of ~ 58 kDa using chemiluminescence (Amersham, Arlington Heights, IL) (Fig. 1). Chemiluminescence was quantitatively blocked by addition of increasing amounts of the immunizing peptide to antibody before developing the Western blot. Molecular mass was estimated by comparison to high-range rainbow molecular weight standards (Amersham Pharmacia Biotech, Piscataway, NJ).

Protein purification. Mobilferrin, β_3 -integrin, and paraferitin were isolated to near homogeneity as described previously (10, 13, 14, 16, 17, 40). DMT-1 was purified from water-insoluble, Triton X-100-insoluble fractions that were prepared as described previously to separate DMT-1 from other iron-binding proteins in cell homogenates (10). The Triton X-100 precipitate was solubilized in 6 M guanidine (10 mM

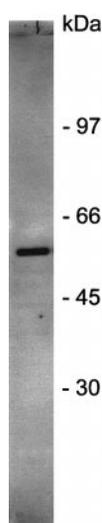


Fig. 1. Western blot of whole cell lysates of K562 cells. Approximately 1×10^6 K562 cells were collected, washed twice with PBS, and then resuspended in 0.5 ml of Laemmli sample buffer and heated at $100^\circ C$ for 5 min, and 25 μ l were applied to a 7.5% SDS gel. A single band at ~ 58 kDa was detected using the antibody to the divalent metal transporter-1 (DMT-1) peptide and enhanced chemiluminescence (Amersham). Incubation of the blot with peptide (2 μ g/ml) with the anti-DMT-1 antibody before development of the chemiluminescence ablated the band, but other nonspecific peptides had no effect.

HEPES, pH 7.4) overnight on a rotator at $4^\circ C$. The supernatant was dialyzed against 4 M urea, which maintained the radioiron in solution. The supernatant was used in the experiment described in this study and contained no evidence of other known iron-binding proteins (ELISA). DMT-1 protein was identified in this labeled isolate by Western blot. Additional purification of DMT-1 may be accomplished by sequentially using sizing and anion-exchange columns (ACA 22, DE-52).

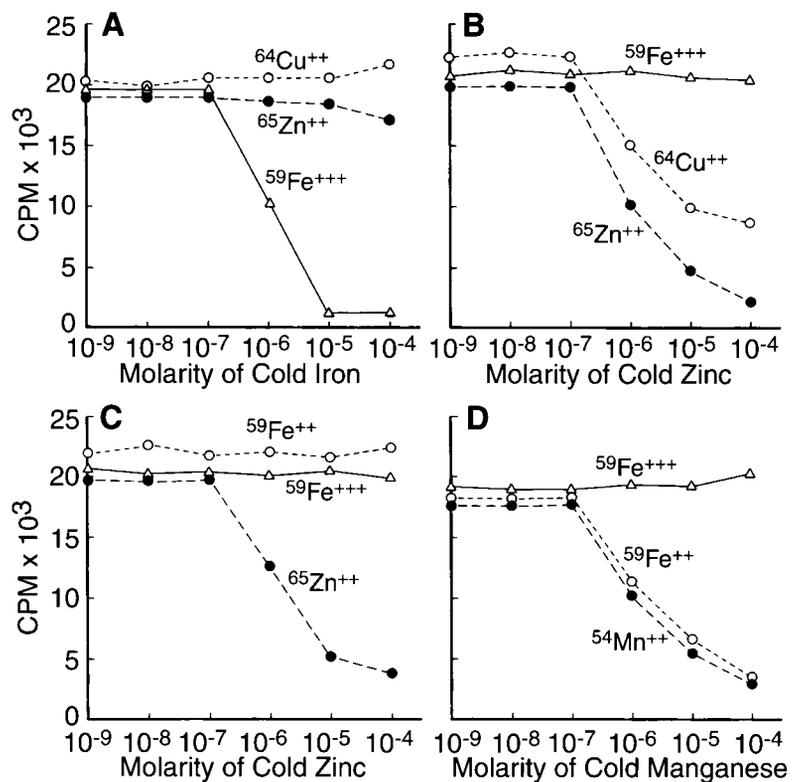
Statistical analyses. Statistical analyses were performed using an unpaired *t*-test.

RESULTS

Inhibition of cellular metal uptake. DMT-1 was reported to transport several metal cations in model systems using frog oocytes and metallic solutions at pH 5.5 (23). On the other hand, the IMP has been reported to be specific for ferric iron (10, 15). In K562 cells, metals inhibited the uptake of that metal but not most other metals tested, suggesting that metals entered cells by different pathways (Fig. 2). Cell suspensions of K562 cells were incubated with radiolabeled isotopes of either ferric iron, ferrous iron, copper, zinc, or manganese. Increasing amounts of unlabeled metals were added to the incubation mixtures to compete for the binding site of the metals on the cell surface. After incubation at $37^\circ C$ for 30 min, the cells were collected and the amount of radioactivity incorporated into cells was determined. At $\sim 10^{-6}$ M of an unlabeled metal, the uptake of that metal was 50% decreased, reflecting the binding coefficient of the cell transporter for that metal. The unlabeled metal attached to a specific metal binding site decreased the uptake of label. Ferrous iron, ferric iron, zinc, and copper did not inhibit the uptake of each other, indicating they were taken up by different cellular pathways (Fig. 2, A, B, and C). The ability of manganese to decrease ferrous iron uptake indicated that both metals compete for the same cellular transporter (9). On the other hand, manganese exerted no effect on the uptake of ferric iron (Fig. 2D), showing that ferric iron was capable of being transported into cells by a different pathway than either ferrous iron or manganese. It also suggested that manganese may be a useful surrogate for ferrous iron in transport experiments; manganese is soluble at physiological pH, does not require reducing agents to maintain its redox state, and appears to be less toxic than most other metals tested at high concentrations ($>10^{-4}$ M).

In these experiments, ferrous iron was maintained in the ferrous state by a combination of ascorbic acid and 2-mercaptoethanol. The ascorbate was continuously oxidized by ferric iron formed by the spontaneous oxidation of ferrous iron. The 2-mercaptoethanol was required to re-reduce the ascorbate. In addition, ascorbate acted as a chelate to maintain the iron in solution, so that it could be reduced. In the absence of 2-mercaptoethanol a variable amount of the ferrous iron (between 20% and 50%) was spontaneously oxidized to ferric iron during the incubation, even in the presence of ascorbic acid, as demonstrated by the ability of the

Fig. 2. Competitive inhibition studies in K562 cells showed that increasing concentrations of nonradioactive iron inhibited uptake of $^{59}\text{Fe}^{3+}$ citrate but not ^{64}Cu or ^{65}Zn (A), nonradioactive zinc inhibited uptake of ^{65}Zn and ^{64}Cu but not $^{59}\text{Fe}^{3+}$ citrate (B), nonradioactive zinc failed to inhibit uptake of either $^{59}\text{Fe}^{3+}$ citrate or $^{59}\text{Fe}^{2+}$ sulfate (C), and nonradioactive manganese inhibited uptake of ferrous but not ferric iron (D). Similar experiments using ^{54}Mn and increasing concentrations of cold ferric citrate (10^{-9} to 10^{-4} M) showed no inhibition of ^{54}Mn uptake by K562 cells. The ferrous sulfate solutions contained ascorbate (10^{-6} M) and 2-mercaptoethanol (10^{-4} M). These data indicate that ferric and ferrous iron utilized separate pathways to enter cells, ferrous iron and manganese share the same pathway, and zinc and copper did not share a pathway with either ferric or ferrous iron.



impermeable specific ferric iron chelator 4,5-dihydroxy-1,3 benzene disulfonic acid (Tiron) to inhibit iron uptake into K562 cells (1, 2, 5).

Blocking β_3 -integrin antibody. A monoclonal anti- β_3 -integrin antibody was raised (lot JG 10396, Centocor) that blocked $^{59}\text{Fe}^{3+}$ citrate uptake in K562 erythroleukemia cells (Fig. 3). The antibody produced no inhibition of iron uptake from solutions containing ferrous iron maintained in the appropriate valence by ascorbate and 2-mercaptoethanol. The lack of inhibition by

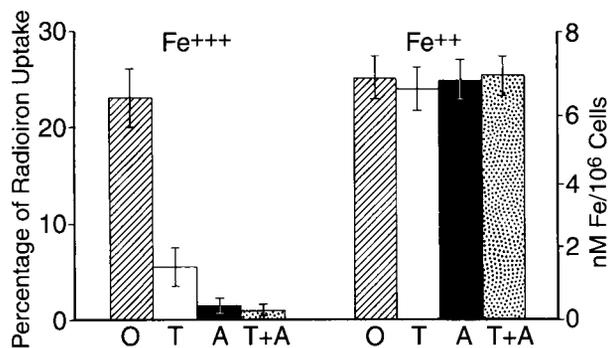


Fig. 3. Effect of anti- β_3 -integrin and 4,5-dihydroxy-1,3 benzene disulfonic acid (Tiron) on uptake of Fe^{3+} and Fe^{2+} in K562 cells. Uptake of Fe^{3+} from ferric citrate by K562 cells was markedly inhibited by both a blocking antibody against a β_3 -integrin (A) and Tiron (T) (GFS Chemicals, Columbus, OH), an impermeable Fe^{3+} chelator ($P < 0.01$). In contrast, neither Tiron (10^{-6} M) nor a β_3 -integrin blocking antibody had any significant effect on uptake of $^{59}\text{Fe}^{2+}$ from ferrous sulfate (10^{-8} M) in the presence of ascorbate (10^{-6} M) and 2-mercaptoethanol (10^{-4} M). O, cellular uptake of radionuclide in the absence of either antibody or Tiron. Results are means \pm SD of 4 determinations.

Tiron, an impermeable ferric iron chelator (1, 2, 5), indicated that the iron remained in the ferrous valence and that Tiron did not have a nonspecific toxic effect on the cells. This was in marked contrast to the effect of anti- β_3 -integrin antibody on uptake of ferric iron (as the citrate chelate). Ferric iron uptake by cells was virtually abolished by the antibody. Ferric uptake was also inhibited by Tiron as expected. The decrease in ferric iron uptake produced by anti- β_3 -integrin antibody in both K562 erythroleukemia cells was dependent on the quantity of antibody incubated with the cells before the addition of a radiolabeled ferric citrate (Fig. 4). The anti- β_3 -integrin antibody did not inhibit the cellular uptake of ^{65}Zn probably because zinc uti-

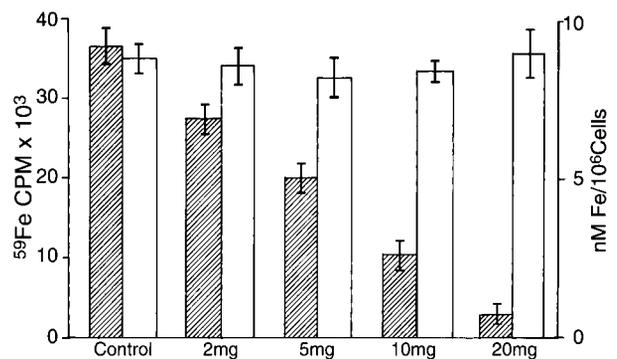


Fig. 4. Antibody to β_3 -integrin diminished uptake of $^{59}\text{Fe}^{3+}$ citrate in K562 erythroleukemia cells (hatched bars) in a dose-related manner. The antibody had no effect on uptake of ^{65}Zn citrate (open bars), suggesting that the effects observed were not due to toxicity and that Zn and Fe are transported into cells by different pathways. Results are the means of duplicate measurements.

lizes a β_1 -integrin to enter cells (15). Other antibodies (see below) had no blocking effect. Trypan blue exclusion in 96% of cells after incubation with antibody and radioiron indicated that no nonspecific toxicity to the cells was present. The differential effect of the antibody implied that at least one element in the ferric pathway was not shared by the ferrous pathway.

Blocking DMT-1 antibody. A purified polyclonal antibody was developed against a peptide sequence from human DMT-1 (KTNEQVVEVCTNTSS) (28). The antibody was shown to inhibit quantitatively the uptake of ferrous iron from a dose of ferrous sulfate ($^{59}\text{FeSO}_4$; ascorbic acid, 10^{-6} M; 2-mercaptoethanol, 10^{-4} M) and $^{54}\text{MnCl}_2$ (Fig. 5). Increasing amounts of antibody showed increasing ability to block the intracellular uptake of ferrous iron and manganese. Preimmune sera had no effect. These data were consistent with the inhibition of ferrous iron uptake by manganese in a shared divalent cation pathway (Fig. 2D). The antibody did not inhibit the cellular uptake of $^{59}\text{Fe}^{3+}$ citrate even at concentrations resulting in >80% inhibition of ferrous uptake. In similar experiments, the anti-DMT-1 antibody did not inhibit uptake of divalent ^{65}Zn . The polyclonal antibody was highly selective for DMT-1 because the antibody only showed activity against DMT-1 isoforms on Western blots. Even if the specificity was broader than demonstrated by Western analysis and included some activity against an otherwise unknown transport system, the antibody still clearly differentiated ferrous from ferric iron transport, providing convincing evidence for separate pathways.

Transfection of DMT-1 DNA into HEK-293T cells. Further evidence that the ferrous transport system in tissue culture cells involved the DMT-1 system and that this was a separate pathway from ferric iron transport was obtained by use of transient overexpression of the DMT-1 protein in HEK293T cells (Fig. 6). The transfection of DMT-1 DNA wild type into HEK293T kidney cells showed an ~30% increase in

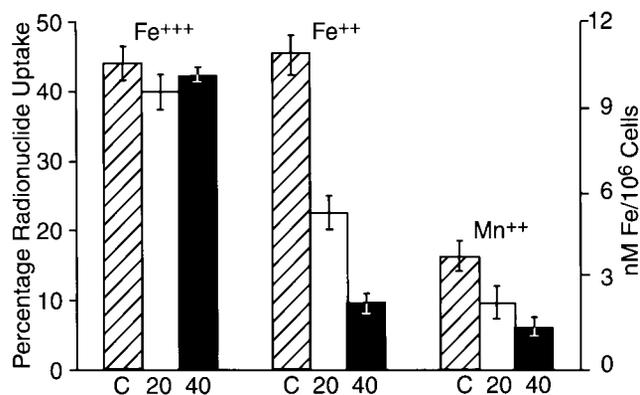


Fig. 5. Antibody to DMT-1 diminished uptake of $^{59}\text{FeSO}_4$ and $^{54}\text{MnCl}_2$ ($P < 0.01$) but had little effect on uptake of $^{59}\text{Fe}^{3+}$ citrate in K562 cells. Increasing the quantity of antibody (μg IgG) produced additional reduction in uptake of ferrous iron and manganese but had no effect upon the uptake of ferric iron. Control (C) specimens contained 20 μg of IgG derived from preinoculation rabbit sera. Values are means \pm SD of 4 determinations.

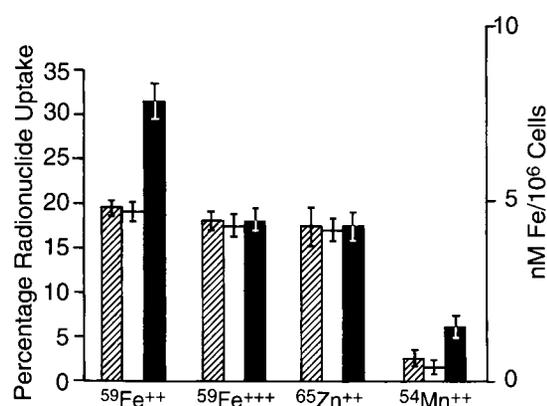


Fig. 6. Effect of transfection of DMT-1 DNA on uptake of radiolabeled metal cations by HEK293T kidney cells. Ferrous iron and manganese uptake by HEK293T kidney cells were upregulated by transfection of normal DMT-1 DNA (solid bars) ($P < 0.01$) whereas there was no increase in uptake of either ferric iron or zinc in comparison to either untreated cells (without DNA and DMRIE; hatched bars) or cells incubated with DNA containing the Belgrade rat mutation (G185R; open bars). Values are means \pm SD of quadruplicate measurements in representative experiments; 25% to 30% of cells were transfected in these studies. Observations reported are actual measurements without correction for %transfection.

uptake of ferrous iron and manganese compared with wild-type cells. Untransfected cells or cells receiving mutant DMT-1 DNA (Belgrade rat, G185R) failed to demonstrate an increase. In both cases ~20–30% of the cell population contained the plasmid as demonstrated by green fluorescent protein. The transfection of DMT-1 DNA had no effect on the uptake of ferric iron or zinc.

Metal concentrations in liver specimens from *mk/mk* and *+ /mk* mice. Specimens of liver were obtained from homozygous (*mk/mk*) and heterozygous (*+ /mk*) mice at autopsy. Aliquots were oven dried and weighed. Metals were extracted with TCA to precipitate heme and protein and avoid heme iron in the assay. The metal concentration in the extracts was measured in an atomic absorption spectrometer. The iron and manganese concentrations were significantly decreased in specimens from homozygous mice. The concentrations of both zinc and copper were similar in the livers of homozygous and heterozygous animals. These data indicate that the mutation in the *mk* mouse significantly affected acquisition of both iron and manganese but had little effect on the uptake and retention of zinc and copper (Table 1).

Iron-uptake pathways are altered in a reducing environment. Biochemical methods for isolating the iron-binding proteins of the IMP were described previously (10, 12–17, 39). DMT-1 was isolated to near homogeneity (Fig. 7). It was water and Triton X-100 insoluble but solubilized in 6 M guanidine from the Triton X-100-insoluble residue. The product of this isolation contained a 58-kDa band that was identified as DMT-1 by the specific antibody and chemiluminescence on a Western blot similar to Fig. 1. After a 10-min incubation of $^{59}\text{Fe}^{3+}$ citrate with K562 cells in the absence of reducing agents, the radioiron was recovered predom-

Table 1. Concentration of metals in livers of *mk* mice

Genotype	Fe	Zn	Cu	Mn
mk/mk	61 ± 21	703 ± 186	8.8 ± 0.9	0.88 ± 0.08
+mk	1229 ± 225	795 ± 240	7.4 ± 0.3	1.24 ± 0.09
<i>P</i>	0.007	0.82	0.30	0.05

Values are means ± SE in mcg/g dry wt; *n* = 6 mice/group. Metal cation concentrations are given for livers of mice either homozygous (mk/mk) or heterozygous (+mk) for the *mk* mutation of divalent metal transporter-1. All animals consumed the same commercial mouse diet. Metals were acid extracted, and measurements were made in a Varian atomic absorption spectrometer. Data indicate that mk/mk mutants have less Fe and Mn in their livers than heterozygotes. However, there was no significant deficiency of either Zn or Cu.

inantly in proteins of the IMP. However, when the cells were pretreated with 2-mercaptoethanol most of the recovered radioiron was found in association with DMT-1. This indicates that both pathways were operative simultaneously under the two conditions of the experiment but that the quantity of ferric iron that becomes available to the DMT-1 pathway is increased with ferrireduction. The binding of small quantities of radioiron to DMT-1 in K562 cells in the absence of 2-mercaptoethanol could be the result of either extracellular ferrireductase activity or intercourse between the IMP and DMT-1 pathways or both. However, most of the recovered radioiron was bound to proteins of the IMP in the absence of 2-mercaptoethanol.

DISCUSSION

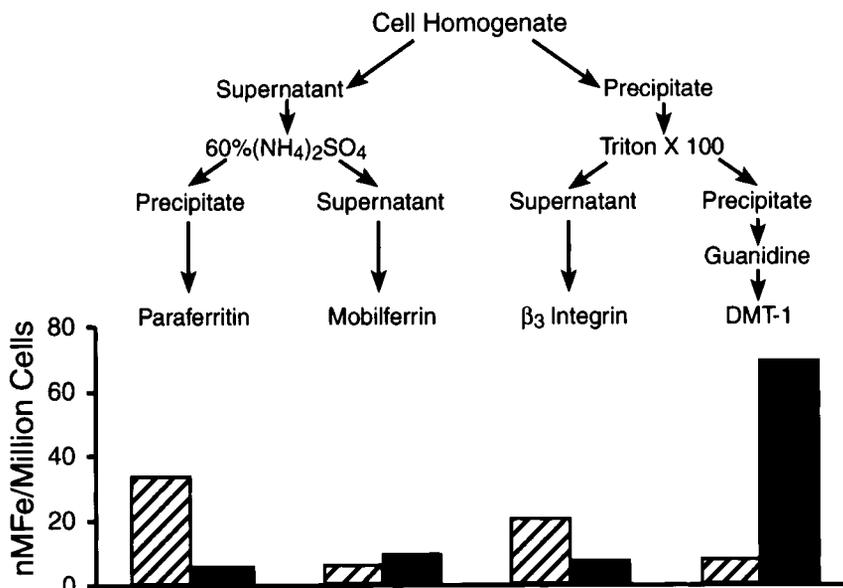
Iron is vital for all living organisms because it is essential for multiple metabolic processes including oxygen transport, DNA synthesis, and electron transport (6). Iron homeostasis is accomplished in mammals

largely by regulating absorption in the proximal small intestine. Iron excretion is limited and plays a more passive role. Absorption of iron as ferrous, ferric, and heme iron balances body losses of iron. Consistent failure to maintain this equilibrium results in either iron deficiency or siderosis. Nonintestinal cells acquire most of their iron from plasma transferrin, utilizing either the classical transferrin-transferrin receptor pathway or the transferrin receptor independent pathway (25). All nucleated cells tested also possessed the capability to transport iron independent of transferrin (3, 7, 15, 19, 30, 33, 39, 42). The physiological role of these pathways in nonintestinal cells is unclear in the absence of iron-overloaded states. The pathways for inorganic iron transport independent of transferrin into nucleated nonintestinal cells have been shown to be the same as used by intestinal cells (15). The relationship between inorganic ferrous and ferric iron transport has been unclear. The data in this communication indicate that cellular uptake of ferric and ferrous iron occurs via different pathways. Current data suggest that ferric iron utilizes the IMP whereas ferrous iron uses the DMT-1 pathway.

The existence of two pathways was shown by a combination of biochemical, immunologic, and genetic methods. Each method demonstrated two separate pathways. Biochemically, manganese inhibited the uptake of ferrous but not ferric iron. It is conceivable that ferrous iron could be catalytically oxidized to ferric iron before transport and that this step was inhibited by manganese. However, the ferric chelator Tiron inhibited ferric uptake, but not ferrous, so that this mechanism is not probable. Ferric iron was not converted to ferrous iron before transport, because in that case

Distribution of ^{59}Fe in Various Iron Binding Proteins in K562 Cells Effect of 2-Mercaptoethanol

Fig. 7. Schema for the biochemical isolation of iron-binding proteins of the β_3 -integrin-mobilferrin pathway (IMP) and DMT-1 is illustrated. These methods were undertaken after incubation of K562 cells with $^{59}Fe^{3+}$ citrate for 10 min before homogenization of the cells either with (solid bars) or without (hatched bars) prior addition of 2-mercaptoethanol to the tissue cell cultures. More radioiron was associated with DMT-1 in the K562 cultures containing 2-mercaptoethanol. Conversely, most of the radioiron was associated with the proteins of the IMP in the absence of the reducing agent. However, both pathways coexisted under both conditions of the experiment. This suggests that ferric iron uptake can occur via the IMP without ferrireduction.



ferric iron uptake would have been inhibited by manganese, which was not observed.

Immunologically distinct antibodies to key elements in the separate pathways inhibited specific pathways, but no antibody inhibited both pathways. The antibody to the integrin involved in the IMP inhibited ferric transport but not ferrous uptake. The antibody to DMT-1 inhibited ferrous transport but had no effect on ferric uptake by the cell. Because neither antibody inhibited uptake of both ferric and ferrous iron, there must be specific elements unique to each pathway making the pathways distinct. The lack of inhibition of ferric iron uptake with anti-DMT-1 and ferrous iron with anti- β_3 -integrin excludes nonspecific iron binding to cells as a significant variable in these studies.

Genetic methods showed that overexpression of the DMT-1 pathway did not increase ferric transport. However, it increased both ferrous iron and manganese uptake.

Individualized absorptive pathways for nutritional metals protect against deficiencies of other vital metals by not competing for the same transport pathway to enter the enterocyte. For example, the divalent cation zinc appeared to be transported by a pathway unshared with either ferrous or ferric iron. Separate pathways for zinc and iron would prevent zinc deficiency in mammals on an iron-replete diet (15). A shared pathway for ferrous iron and manganese would be less important because most dietary iron is ferric iron.

The relative physiological roles in the absorption of iron by the two nonheme iron uptake pathways, the IMP and the DMT-1, are not known. The following information is available. 1) DMT-1 missense mutations were associated with a disease in rodents (mk mouse and Belgrade rat) (20, 21), and the calreticulin/mobilferrin knockout mouse has not been viable (18, 31). 2) The DMT-1 protein was dramatically increased in intestinal cells in severe iron deficiency in mice (8), and the activity of the IMP was increased in iron-deficient rats (12). 3) The DMT-1 protein was immunologically undetectable in the microvilli of freshly obtained normal human duodenum at Whipple surgery (unpublished data) and in the duodenum of normal rodents (8), and the mobilferrin-integrin pathway was active in normal human duodenum (14). 4) The DMT-1 protein appeared to transport some nonferrous metals of nutritional importance, whereas the IMP was specific for import of ferric iron into the cell (23). 5) Lastly, a defect in the IMP was identified in a family with sideroblastic anemia, a genetic disorder with iron overloading (4).

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