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Iron and copper homeostasis and intestinal absorption using the Caco2 cell model

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Abstract

Whole body homeostasis can be viewed as the balance between absorption and excretion, which can be regulated independently. Present evidence suggests that for iron, intestinal absorption is the main site for homeostatic regulation, while for copper it is biliary excretion. There are connections between iron and copper in intestinal absorption and transport. The blue copper plasma protein, ceruloplasmin, and its intracellular homologue, hephaestin, play a role in cellular iron release. The studies reviewed here compare effects of Fe(II) and Cu(II) on their uptake and overall transport by monolayers of polarized Caco2 cells, which model intestinal mucosa. In the physiological range of concentrations, depletion of cellular iron or copper (by half) increased uptake of both metal ions. Depletion of iron or copper also enhanced overall transport of iron from the apical to the basal chamber. Copper depletion enhanced overall copper transport, but iron depletion did not. Pretreatment with excess copper also stimulated copper absorption. Plasma ceruloplasmin (added to the basal chamber) failed to enhance basolateral iron release, and Zn(II) failed to compete with Cu(II) for uptake. Neither copper nor iron deficiency altered expression of IREG1 or DMT1 (-IRE form) at the mRNA level. Thus, in the low-normal range of iron and copper availability, intestinal absorption of both metals appears to be positively related to the need for these elements by the whole organism. The two metal ions also influenced each other's transport; but with copper excess, other mechanisms come into play.

Introduction

Homeostasis of iron and copper

There is a tendency for living organisms to maintain a constant chemical and osmotic composition. For mammals, this is particularly evident in the composition of blood and extracellular fluids, the main substituents of which are held within a limited concentration range (Linder 1991a). Iron and copper offer interesting and contrasting examples of how the tendency for constancy in realized in different ways and to different degrees.

In the blood of humans and related mammals, concentrations of iron and copper proteins (and carriers for these metals) remain quite constant in the normal state. Average amounts of both metal ions absorbed daily by humans from the diet are very similar (about 1 mg). Total amounts in the average adult human are quite different, averaging about 3500 mg for Fe and only about 110 mg for Cu. The total copper content of the human adult tends to be quite constant; while that of iron and particularly of iron stores has a wide variation (Linder 1991a). There are several reasons for this difference in variability. First, iron is relatively difficult to absorb from the diet, in part because of a chemistry that resists its solubilization in neutral aqueous solution in the absence of chelating agents (like ascorbate or amino acids). [Only about 10% of dietary iron is absorbed.] The excretion of iron (mainly through the bile) is also very limited, making it difficult for the organism to rid itself of an excess, except

by bleeding. In addition, a large number of other factors, which are highly individual (pregnancy, menstruation, sweating, etc.), contribute the development of large individual differences in body iron content (Linder 1991a). Both absorption and excretion of iron are thus problematic.

The situation is quite different for copper (Linder 1991b, 1996). It is relatively easy to absorb. About 70% of dietary copper (about 1 mg) is absorbed daily by the human adult, and larger amounts are recycled from digestive tract secretions (perhaps an additional 3 mg Cu per day). In most mammals, copper is also easy to excrete. It has become clear that when excess copper is administered, or alternatively when there is a lack in the diet, the body adjusts its net excretion (Turnlund *et al.* 1989, 1998; Linder, 1991b; Dunn *et al.* 1991). Generally, also, excess copper is not 'stored'. As a result, the total in the body tends to be constant. It has thus been generally accepted that copper homeostasis is mainly controlled by regulation of excretion. Iron is different. Excess iron is stored (in ferritin), and the levels of iron stored vary widely among individuals (Linder 1991a). It is well documented that intestinal iron absorption responds to the needs of the body; while little is known about the mechanism and potential regulation of its excretion (also via the bile). Thus, the main regulation of iron homeostasis is thought to be at the level of intestinal absorption.

Intestinal iron absorption

Intestinal iron absorption varies in relation to need but is influenced also by other factors, including hypoxia, and inflammation. The individual steps by which iron enters and crosses the enterocyte and then its basolateral membrane, and the mechanisms by which changes in transport are brought about, are still poorly understood. Most studies agree that ferrous iron is preferentially absorbed (Linder *et al.* 1975; Linder & Munro 1977; Andrews 2000; Roy & Enns 2000; Wessling-Resnick 2000). However, iron administered as Fe(III) is absorbed as well (Nunez *et al.* 1994; Han *et al.* 1995; Andrews 2000; Roy & Enns 2000). A ferrireductase (DCYTB) that may mediate Fe(III) uptake has been identified and cloned (Riedel *et al.* 1995; Ekmekciaglu *et al.* 1996; McKie *et al.* 2001). The divalent metal transporter (DMT1/Nramp2/DCT1), also in the brush border, is likely to be responsible for most (or all) of the Fe(II) uptake (Gruenheid *et al.* 1995; Fleming *et al.* 1997; Gunshin *et al.* 1997). Protein expression of both DMT1 and the ferrireductase is inversely related to levels of iron within the enterocyte, accounting for the increased uptake of iron observed in deficiency (Linder & Munro 1977; Trinder *et al.* 2000; Yeh *et al.* 2000; Andrews 2000; Roy & Enns 2000; Wessling-Resnick 2000; Rolfs & Hediger 2001). A 561 amino acid protein with 12 transmembrane segments, DMT1 is expressed by most mammalian tissues. Its transport of Fe(II) is proton-coupled and dependent upon the membrane potential, as shown by the large inward current evoked in transfected oocytes upon their exposure to Fe(II), at pH 5.5; and loss of most of this current at pH 7.5 (Gunshin *et al.* 1997). Cu(II), and ions of Zn, Mn, Cd and Co, were also effective in evoking current, suggesting these are also transported.

The mechanisms controlling expression and functioning of these two brush border proteins are unknown and probably complex. In the case of DMT1, there are at least 4 different mRNA transcripts, two of which contain an iron responsive element (IRE) in the 3′UTR (Hentze *et al.* 2001). (One form of +IRE mRNA is the main one expressed by the duodenum.) Small portions of the corresponding polypeptides also differ in amino acid sequence (M. Hentze, this conference). As in the case of several other iron-regulated proteins, the presence of a 3′UTR-IRE might allow control of expression through stabilization of the mRNA by iron-responsive element binding proteins (IRPs) (Liebold & Guo 1992; Hentze & Kuhn 1996). This is the case for transferrin receptor, which has 5 IREs in the 3′UTR of its mRNA (Leibold & Guo 1992; Eisenstein 2000). Whether stabilization of DMT1 mRNA by IRPs actually occurs, and/or whether transcriptional regulation is involved, has not as yet been determined. New evidence also suggests that iron influences the deployment (rather than expression) of DMT1 at the brush border versus in endocytic vesicles (Ma *et al.* 2001). In addition (as already mentioned), this transporter appears to be involved in uptake of other metal ions.

Although uptake of iron across the brush border of the intestinal mucosa is at least partly regulated according to need, the main site for regulation of overall absorption may be at the basolateral end of the enterocyte (Linder & Munro 1977). Iron entering the mucosa is not necessarily passed on and can accumulate. Entry may be facilitated by residual DMT1, which stays at the brush border to aid the absorption of other metal ions. Thus, entry of iron into the enterocyte may occur even when it is not required. Control of basolateral

iron transfer is also far from perfect; and continuous exposure to excess dietary iron can lead to iron overload (Linder & Munro 1977; Oates *et al.* 2000a; Roy & Enns 2000).

How iron crosses the basolateral surface of the mucosal cell into the blood, and how this may be regulated is the subject of much current research. At least two mechanisms may exist, one involving a specific transporter (IREG1/ferroportin/MTP1), the other vesicular transport. IREG1 was identified in zebrafish, hypotransferrinemic mice and human tissues, and was found to be expressed in the basolateral membrane of intestinal epithelium (Donovan *et al.* 2000; McKie *et al.* 2000; Abboud & Haile 2000). An endo/exocytic cycling mechanism, involving transferrin, appears to be present as well, based on studies with Caco2 cells (Alvarez-Hernandez *et al.* 1994, 1998; Nunez *et al.* 1999). The laboratories of Glass and Nunez have shown that apotransferrin is endocytosed from the blood/interstitial fluid and travels to a compartment above the nucleus, before returning to the basolateral surface (Nunez *et al.* 1999; Alvarez-Hernandez *et al.* 1998). During its cycling (and perhaps in the trans-Golgi network), the apotransferrin may pick up iron, to form diferric-transferrin, which is then released into the blood. Hephaestin, a copper-containing protein homologous to ceruloplasmin and with ferroxidase activity (Vulpe *et al.* 1999, 2001; Frazer *et al.* 2001) may aid the binding of iron to apotransferrin, since only Fe(III) binds. *Iron-containing* transferrin in the blood can also enter enterocytes from the blood. However, it goes primarily to non-absorptive cells in the crypts, perhaps helping to inform them of the body's iron status (Wessling-Resnick 2000; Oates *et al.* 2000b). In Caco2 cells, Fe_2-transferrin also goes to a different vesicular compartment from that of apotransferrin (Nunez *et al.* 1999; Alvarez-Hernandez *et al.* 1998). HFE, the product of the gene defective in hemochromatosis (genetic iron overload; Feder *et al.* 1996, 1998) may also contribute information to crypt cells about the status of body iron stores, by influencing the rate of endo/exocytosis of Fe_2 transferrin (Oates *et al.* 2000b; Waheed *et al.* 1999). It might also, however, slow the cycling of apotransferrin into and out of absorptive cells to reduce iron absorption. In the studies presented here, only absorption of Fe(II) in the presence of apotransferrin was examined.

Intestinal copper absorption

Even more so than with iron, the overall pathway(s) and individual steps of intestinal copper absorption remain to be delineated. Important progress has been made in identifying transporters and intracellular proteins, termed 'copper chaperones', that may be involved. Much earlier work, mainly with rodents, indicated that uptake of Cu(II) by intestinal mucosa involved a non energy-dependent saturable carrier, active at lower copper concentrations, and diffusion at higher concentrations (Linder 1991b; Linder & Hazegh-Azam 1996). In contrast, transfer across the basolateral membrane into the blood was energy-dependent and more rate limiting for overall absorption. Thus, copper could enter the mucosa and accumulate, at least in some conditions. At high concentrations, there was evidence for increased uptake as well as overall absorption, suggesting that additional carrier systems and/or diffusion come into play to mediate additional transfer across the basolateral membrane. Additional carriers might be those specializing in other metal ions, particularly Zn(II) or Fe(II/III), as high doses inhibit copper absorption (and vice versa), particularly at the basolateral membrane (Linder 1991b; Yu *et al.* 1994). In rats, rates of copper absorption increase in pregnancy or cancer, and decrease with repeated estrogen treatment (Linder 1991b; Cohen *et al.* 1979; Karp *et al.* 1986). Thus, it appears that expression and/or deployment of copper transporters/transport systems can be regulated.

Whether and how nutritional copper status affects intestinal copper absorption has hardly been studied. Existing data suggest there is a biphasic response. In the polarized Caco2 cell model, pretreatment with excess copper was found to *enhance* uptake and overall transport of ^{64}Cu (Arredondo *et al.* 2000), opposite to what was expected for homeostasis. [We have confirmed those results (Zerounian & Linder 2002; see later).] Some potential transporters and carrier systems that may contribute to intestinal absorption have recently emerged. This has come from the cloning of genes responsible for genetic copper deficiency and overload (MNK and WND, respectively, altered in Menkes and Wilson diseases) (Mercer *et al.* 1993; Bull *et al.* 1993; Vulpe & Packman 1995; Kaler 1998; Camakaris *et al.* 1999), and for homologues of yeast genes for copper chaperones (Pena *et al.* 1999; Eide 1998; Labbe & Thiele 1999). The major plasma membrane transporter identified so far is CTR1, homologous to crt1 of yeast (Dancis *et al.* 1994; Zhou

& Gitschier 1997), cloned in humans, mice and rats (Lee *et al.* 2000; Kuo *et al.* 2001). It is an obvious candidate for uptake of copper by the brush border of intestinal mucosa. However, since copper also enters enterocytes from the blood (Linder 1991b), it may also be deployed at the basolateral membrane. hCTR1 promotes copper uptake into mammalian cells and substitutes for its homologue in yeast (Zhou & Gitschier 1997; Kuo *et al.* 2001; Moller *et al.* 2001; Lee *et al.* 2001). At least in yeast, efflux of 2 HK^+ ions accompanies Cu(I) influx (Pena *et al.* 1999; DeRome & Gadd 1987). Since Cu(I) is transported, a copper reductase may also be deployed at the brush border. Possibly, CTR1 can work in both directions, and this might account for the fact that copper is released into the gut lumen by intestinal juices (Linder & Roboz 1986; Linder 2002).

The other candidate transporter in the brush border (DMT1/Nramp2/DCT1) has already been described in connection with iron absorption. Whether and/or when this protein is involved in normal copper transport has not been fully established, but data of Arredondo *et al.* (2001) indicate it does play a role. Several other relevant genes/gene products have been identified, most of which are ubiquitously expressed. These include the new class of copper 'chaperones', cytoplasmic proteins first identified in yeast. They bind and transport incoming (and probably endogenous) copper ions, handing them (protein-to-protein) to specific enzymes or transporters, including superoxide dismutase, mitochondria, and the MNK and WND ATPases. Assuming that these chaperones have the same functions in enterocytes, one can imagine one or more pathways by which copper travels from the apical to the basolateral membrane, and into the blood. Copper entering via CTR1 (and/or DMT1) would be carried by the specific chaperone, HAH1/ATOX1, to the copper pumping MNK ATPase (ATP7A) in the trans-Golgi network (TGN) (Klomp *et al.* 1997; Larin *et al.* 1999; Pena *et al.* 1999). (This protein is defective in Menkes disease.) The copper might then enter exocytic vesicles and be released from the cell. Alternatively, since MNK can cycle between the TGN and plasma membrane (Petris *et al.* 1999), HAH1 might deliver copper directly to the basolateral membrane, for exit to the blood. Additional pathways for basolateral transport may also exist. However, the one involving MNK must be the most important, as Menkes disease is accompanied by severe copper deficiency. Alternative chaperones (perhaps still to be identified), as well as glutathione or metallothioneins, may also contribute to basolateral transport, perhaps carrying copper to CTR1 in the basolateral membrane, for transfer down a chemical gradient to the blood.

Iron absorption and copper

It has long been known that copper plays a role in iron transport (Linder 1991b, 1993; Harris 1995, 2000). When copper deficiency is severe, and there is little or no copper in plasma ceruloplasmin, iron tends to accumulate in many tissues. Usually (but not always) (Prohaska *et al.* 1985), there also is a hypochromic, microcytic anemia similar to that produced by iron deficiency (Linder 1991b). Plasma ceruloplasmin has been implicated in releasing iron from the liver. This α_2-globulin normally binds 6 copper atoms in non-dialysable form (Zaitseva *et al.* 1996), and this copper accounts for two thirds of that in rat and human plasma (Linder 1991b; Wirth & Linder 1985). Ceruloplasmin-copper is required for its characteristic ferroxidase activity (Frieden 1970; Linder 1991b). Most of the protein is still made and secreted by the liver in copper deficiency but has little or no ferroxidase activity (Holtzman & Gaumnitz 1970). In copper deficiency and genetic aceruloplasminemia, humans and animals accumulate iron in liver and other tissues (Linder 1991b; Yoshida *et al.* 1995; Harris *et al.* 1999). Perfusion of such livers with ferroxidase-active ceruloplasmin results in an immediate release of iron into the circulation (Osaki & Johnson 1969; Ragan *et al.* 1969; Roeser *et al.* 1970). It is thought this reflects an enhanced oxidation of Fe(II), to allow it to bind to its plasma carrier, transferrin (Frieden 1970; Linder 1991b).

With regard to intestinal absorption, one report (Wollenberg *et al.* 1985) indicated that ceruloplasmin can promote this process, while another failed to find an effect (Coppen & Davies 1988). Wollenberg *et al.* found that the rate of appearance of ^{59}Fe in the portal blood, after intubation as Fe(III) into the intestinal lumen of copper deficient rats, was promoted by i.v. infusion of ceruloplasmin. As with the liver studies already cited, the enhanced release of iron was not promoted by instead infusing ionic copper or a copper-albumin complex. These results suggested that the intestine might at least partly depend upon plasma ceruloplasmin to release iron from the enterocyte into the blood, although there was other evidence inconsistent with this concept (Chase *et al.* 1952; Lee *et al.* 1968; Brittin & Chee 1969; Owen 1973; Williams *et al.* 1983). As summarized in this article, we did not

find that ceruloplasmin enhanced the release of iron from Caco2 cells when it was added to the basolateral medium (Zerounian & Linder 2002).

As already mentioned, an additional involvement of copper in iron absorption was indicated by the discovery of the ceruloplasmin homologue, hephaestin (see earlier), the gene product responsible for a sex linked anemia in mice (Vulpe *et al.* 1999). Actually, since it is a membrane protein, it is more reminiscent of FET3, the copper protein required for iron uptake by yeast (Askwith *et al.* 1994; Yuan *et al.* 1995). On the basis of the iron absorption model described earlier, we hypothesized that hephaestin was needed by the enterocyte to oxidize Fe(II) within endo/exocytic vesicles, so that it would be able to bind to apotransferrin and deliver iron to the blood via exocytosis. Recent studies in rats indicate that iron status has little or no effect on expression of this protein by the gastrointestinal tract (Frazer *et al.* 2001). However, we expected that copper deficiency would impair its function and thus reduce basolateral iron transfer.

Materials and methods

Caco2 cell culturing and measurements of iron and copper absorption

Caco2 cells were cultured and used for measurements of iron absorption as previously described (Zerounian & Linder 2002), based on the procedures developed by Jon Glass and his colleagues (Alvarez-Hernandez 1991) at the Feist-Weiller Cancer Center, LSU Medical Center (Shreveport, LA) to whom we are grateful for advice and guidance. Briefly, cells cultured in DMEM/20% fetal bovine serum/0.1 mM non-essential amino acids/1 mM Na pyruvate, and antibiotics (100 units/ml penicillin-G and streptomycin, and 250 units/ml Fungisone) were transferred to collagen-coated Transwells (CoStar, Corning, MA). Cells were used when trans epithelial electrical resistance reached about 250 Ohms/cm^2. Iron deficiency was induced by overnight exposure to 100 μM desferrioxamine, copper deficiency with triethylenetetraamine (Teta; 1 mM; Fluka, Milwaukee, WI), bathocuproine sulfonate (BCS; 40 mM μM; Sigma Chemical Corp., St. Louis, MO), or N,N-bis(2-aminoethyl)-1,3-propanediamine (tet; 20 μM; Acros Oganics, Geel, Belgium). Iron uptake and overall transport were followed with $^{59}FeSO_4$ (1 μM) added to the apical (brush border) chamber in Hepes-buffered saline (130 mM NaCl, 10 mM KCl, 50 mM

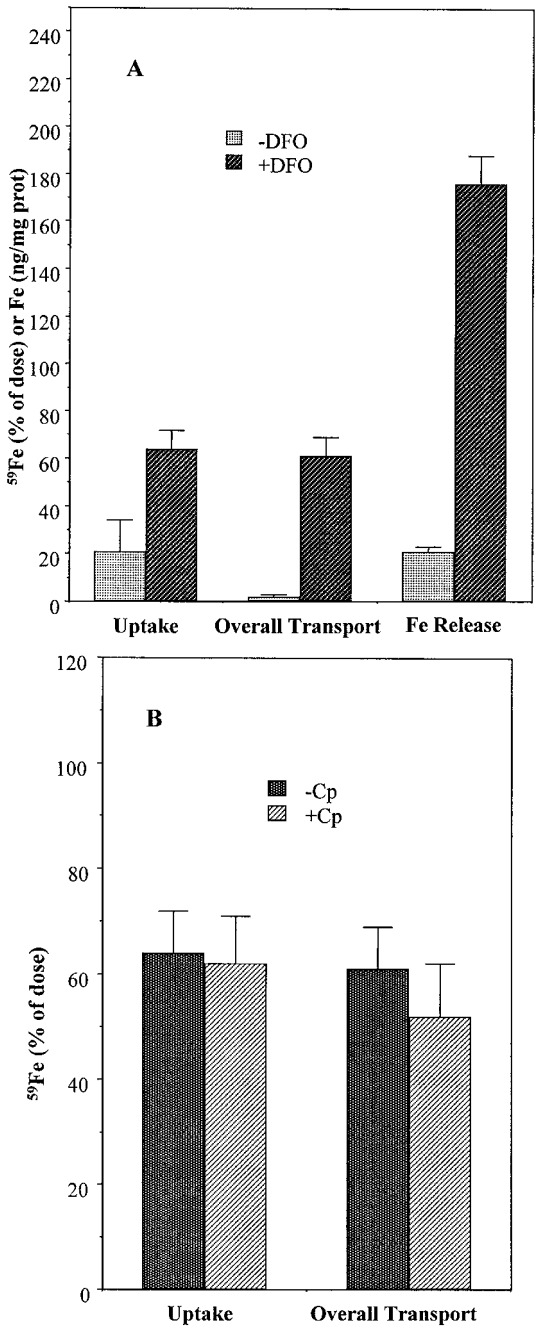

Fig. 1. Uptake and overall transport of Fe by Caco2 cell monolayers. Values are percent of ^{59}Fe dose taken up (Uptake) or transported to the basolateral chamber (Overall Transport) (Mean $\pm$ SD; $n = 6$) for two sets of experiments. 'Fe Release' is actual Fe (ng/ml cell protein) released into the basal chamber, measured with bathophenanthroline. A. Effect of iron deficiency induced by desferrioxamine (DF0). Light bars are without, and dark bars with, DFO pretreatment. B. Effect of ceruloplasmin (Cp), added to the basal medium during the transport phase. Dark bars are with, and light bars, without, Cp addition. Summarized from Zerounian and Linder 2002.

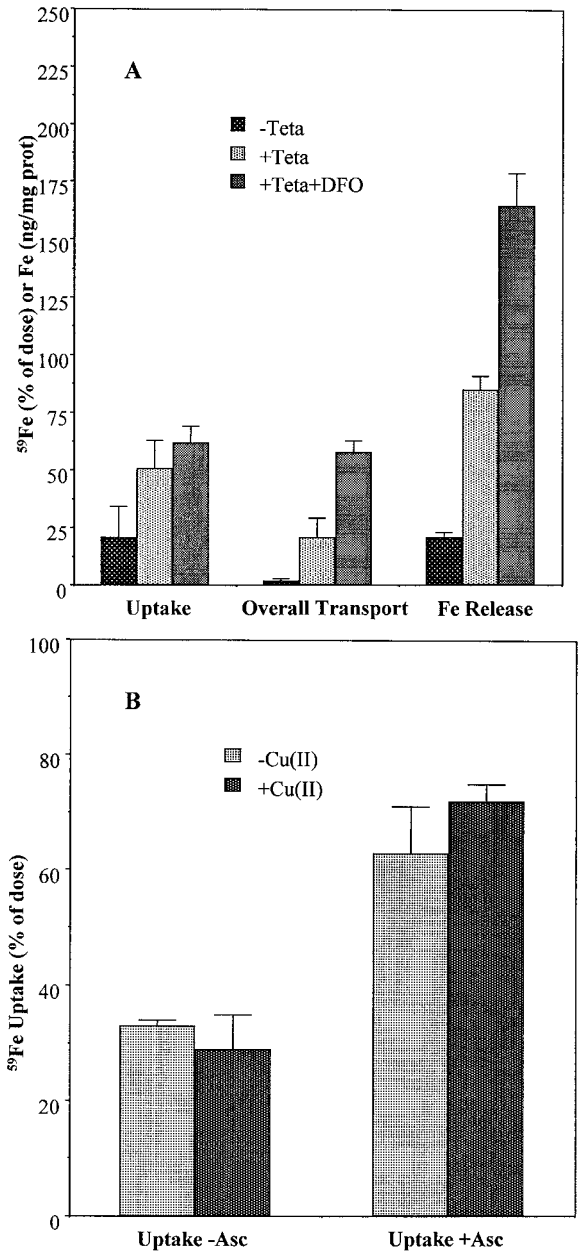

Fig. 2. Effect of copper and copper depletion on iron absorption. Values are percent of ^{59}Fe dose taken up (Uptake) or transported to the basolateral chamber (Overall Transport) (Mean ± SD; $n = 6$) for two sets of experiments. 'Fe Release' is actual Fe (ng/ml cell protein) released into the basal chamber, measured with bathophenanthroline. A. Effect of copper depletion induced by treatment with tetraethylenetetraamine (+Teta), with (grey bars; +Teta +DFO) and without (light bars; +Teta) iron depletion, as compared with controls (dark bars) not pretreated with chelator. B. Effect on ^{59}Fe uptake of the addition of equimolar non-radioactive Cu(II) (darker bars), in the absence (−Asc) and presence (+Asc) of ascorbate. Summarized from Zerounian and Linder 2002.

Hepes, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 7.4), with fresh 1 mM ascorbate. Human apotransferrin (38 μM; Sigma) was in the basal medium, which consisted of the same Hepes-buffered saline. In some cases, freshly purified human ceruloplasmin (about 144 μg/ml) was added as well. For copper transport, $^{64}CuCl_2$ (1 μM) was in the apical chamber, and human albumin (1%, w/v) in the basal medium. Samples were removed from the basolateral chamber at 30, 60 and 90 min, for determination of overall transport (as percent of ^{59}Fe or ^{64}Cu dose). At the 90 min time point, overall uptake was calculated from (a) the radioactivity remaining in the apical chamber (including washes – with Hepes-buffered saline/1 mM ascorbate - collected from the cells during harvest), and (b) from the recovery of ^{59}Fe or ^{64}Cu in cells and basal medium, which was also determined. $^{59}FeSO_4$ was obtained from Perkin Elmer/New England Nuclear; Boston, MA), the $^{64}CuCl_2$ from the MIR facility at Washington University (St. Louis, MO), courtesy of Dr Debra McCarthy.

Purification of ceruloplasmin

As already described (Zerounian & Linder 2002), portions of previously frozen human serum were purified by chromatography on DEAE-Sepharose CL6B (Middleton & Linder 1993). The three peak fractions containing enzymatically-active ceruloplasmin (about 25% yield) were pooled and immediately used in absorption studies.

Copper and iron analyses

Copper in the cells (after wet-ashing) and basal media was determined by furnace atomic absorption spectrometry, as previously described (Zerounian & Linder 2002). Iron in the same samples and media was assayed with bathophenanthroline disulfonate (Sigma) (James & Zak 1958).

Cell protein determinations

Cells in each Transwell were dissolved in 0.2M KOH and assayed for protein by the Bradford dye binding method, using reagents from BioRad (Richmond, CA), and bovine serum albumin as the standard (Zerounian & Linder 2002).

Real Time PCR

This was performed on cDNA prepared from total RNA of Caco2 cells, extracted with RNAzol B (Tel-Test 'B', Inc.; Iso-Tex Diagnostics, Friendswood, TX), as previously described (Tran *et al.* 2002). Reverse transcription with oligodT primers was with 1 μg portions of RNA. One μl portions of each 20 μl reaction were used for Real Time PCR with specific primers and fluorescent/quencher-labeled probes for human DMT1 (the non-IRE-containing mRNA), IREG1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The same standards (made of pooled and aliquotted cDNA) were used undiluted, diluted 10-fold and diluted 25-fold, to construct a standard curve for each run. Real Time PCR was performed with the Bio-Rad iCycler iQ Real Time Detection System (Hercules, CA). Specific primers and probes were as follows.

Non-IRE-DMT1 (GenBank Accession No. AF064 484): Product length 127 bp;
Forward primer: 5′ GTGCTGAGATTATAGGCGTG 3′
Reverse primer: 5′ GCTGAAAGGGGAAAGTGATG 3′
Probe: 5′6-FAM/CCACTGCATCCAGCTCACTCCT CATTTCTTTC/BHQ-1 3′
Cycle 1, 2 min 50°; cycle 2, 2 min 95°; cycles 3–53, 15 sec 95°, 1 min 60°.

IREG1 (GenBank Accession No. AF215636): product length 84 bp;
Forward primer: 5′ GTGGCTTTATTTCGGGATG 3′
Reverse primer: 5′ GAGCTGGGGTTTTCTGG 3′
Probe: 5′6-FAM/CCATGTGCGTGGAGTACGTCTT GCTC/BHQ-1 3′
Cycle 1, 2 min 50°; cycle 2, 2 min 95°; cycles 3–53, 15 sec 95°, 1 min 57.9°.

GAPDH [GenBank Accession No. AF106860 (rat) and J04038 (human)]: product length 105 bp;
Forward Primer: 5′ ATGGCCTTCCGTGTTCC 3′
Reverse primer: 5′ CCTGCTTCACCACCTTCT 3′
Probe: 5′ FAM/TGACMTGCCGCCTGGAGAAACC TGC/BHQ-1 3′
Cycle 1, 2 min 50°; cycle 2, 2 min 95°; cycles 3–53, 15 sec 95°, 1 min 60°.

For quantitative validity, efficiencies of the PCR reactions were monitored. Only runs with efficiencies greater than 95% were considered acceptable.

Statistical analysis

Results are expressed as means ± standard deviation, for the number of determinations in the parentheses. Statistical analysis of the data was by one way ANOVA. Probability (P) values < 0.05 were considered significant.

Results

Iron homeostasis, copper and ceruloplasmin in intestinal iron absorption

To determine whether iron deficiency enhanced absorption of iron by the Caco2 cell monolayers as it does *in vivo*, the effect of pretreating cells overnight with desferrioxamine (DFO) was examined. This treatment lowered levels of cellular iron 55% (Zerounian & Linder 2002). As summarized in Figure 1A, uptake and overall transport of ^{59}Fe(II) were both markedly enhanced by the iron deficiency. The release of actual iron into the basal chamber, measured with bathophenanthroline, was enhanced 8-fold (Figure 1A), compared with a 30-fold increase based on radioactivity.

To assess whether ceruloplasmin would increase the rate of release of iron basolaterally, freshly isolated and enzymatically-active ceruloplasmin (from human serum) was added to the basal chamber during the absorption study. Effects on both iron-normal and iron-deficient cells were investigated. As shown by the data in Figure 1B for iron deficient cells, no stimulation by ceruloplasmin was observed; i.e. there was no stimulatory effect of adding the ferroxidase.

The possibility that copper deficiency would inhibit iron absorption was also examined. Cells were made copper deficient with three different specific chelators. Overnight treatment with tetraethylenetetraamine (Teta) lowered cellular copper concentrations 43% (Zerounian & Linder 2002). Contrary to expectations, copper depletion *enhanced* iron absorption (Figure 2A; +Teta). Uptake increased from 20 to 50% of dose; overall transport of ^{59}Fe increased from 2 to 22% of dose, or about 10-fold. Measurements of actual iron indicated that overall absorption increased about 4-fold. The same response was observed with the other copper chelators (Zerounian & Linder 2002). Additional pretreatment, to deplete cellular iron with desferrioxamine (+DFO), had the same effect as copper depletion on iron uptake but increased overall iron

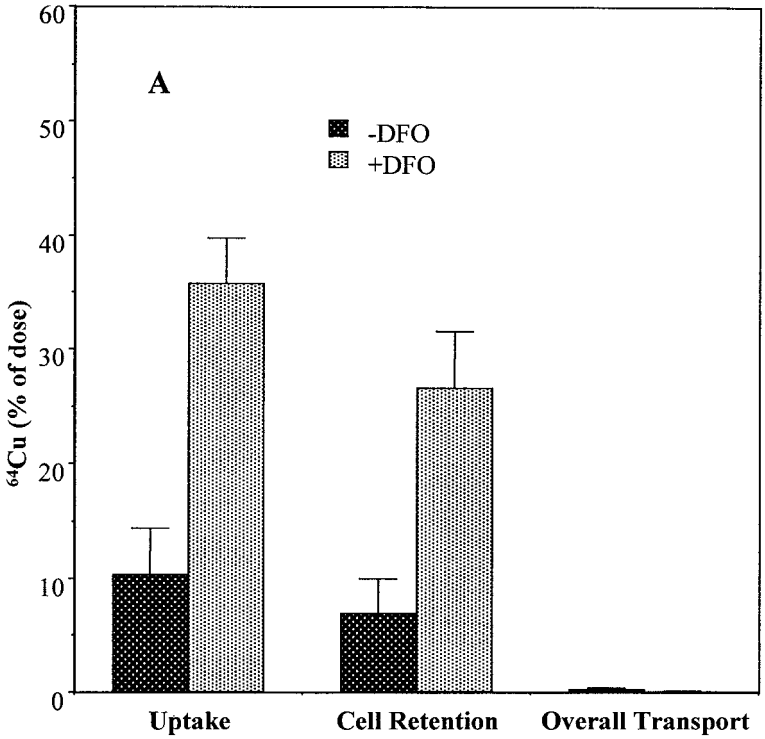

Fig. 3. Effects of iron and copper depletion, and zinc, on copper absorption by Caco2 cell monolayers. Values are percent of ^{64}Cu dose taken up (Uptake), retained (Cell Retention), or transported to the basolateral chamber (Overall Transport) (Mean ± SD; $n = 6$) for two sets of experiments. A. Effect of pretreatment with DFO (light bars) compared with controls (dark bars). B. Effect of pretreatment with Teta (light bars) as compared with controls (dark bars). C. Lack of inhibition of copper absorption by non-radioactive Zn(II) (Zn:Cu 10μM:1μM) in Cu-deficient cells. Dark bars are with, light bars without, added Zn.

transport over that observed with copper depletion alone (Figure 2A).

To begin to assess whether changes in DMT1 would explain the increased uptake of iron observed in copper depletion, ^{59}Fe(II) uptake was measured in the presence of an equimolar concentration of non-radioactive Cu(II). Studies were carried out with and without ascorbate in the apical medium. No inhibition by copper was observed (Figure 2B).

Copper homeostasis, iron, zinc and copper absorption

The effect of iron deficiency on *copper* absorption was also examined. As shown in Figure 3A, pretreatment with DFO significantly enhanced uptake of ^{64}Cu across the brush border membrane. However, it did not stimulate release of ^{64}Cu from the basolateral end of the enterocyte, increasing retention of the absorbed copper instead. DFO pretreatment had a small (13%) but significant lowering effect on cellular *copper* concentrations (Zerounian & Linder 2002).

The effects of copper depletion on copper absorption were more striking (Zerounian et al. 2001). Overnight pretreatment with Teta markedly enhanced both uptake and overall transport of ^{64}Cu, and lowered retention of the radioisotope (Figure 3B).

Since Zn(II) uptake by the intestinal mucosa was also thought to occur through DMT1, effects of Zn(II) competition on ^{64}Cu(II) uptake were assessed. Concentrations of Zn(II) 10-fold higher than Cu(II) (mol:mol) failed to inhibit uptake or overall transport of the radioactive copper (Figure 3C).

Time course of effects of copper depletion on copper and iron absorption

Since both iron and copper absorption were stimulated by copper deficiency, we examined the time course of the enhancements induced by Teta exposure. Figure 4A shows rates of copper and iron uptake as a function of time of pretreatment with the copper chelator. In the case of copper, a significant increase in rate of uptake occurred between 4 and 6 h, and

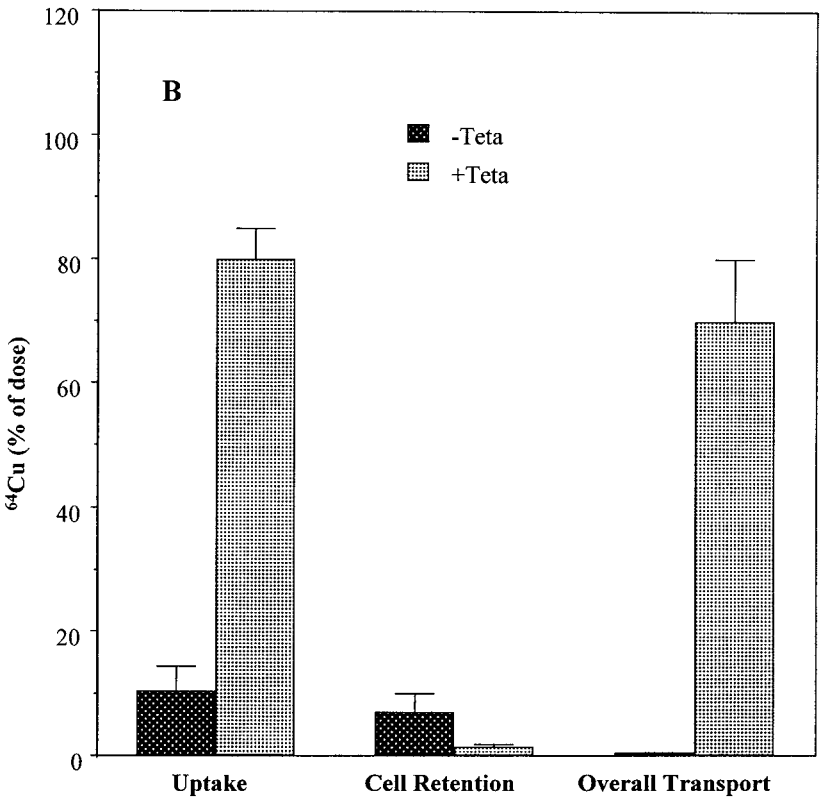

Fig. 3. Continued.

there were additional increases thereafter, maximum rates being achieved by 14 h. Increases in rates of *iron* uptake appeared to occur later than those for copper (Figure 4A), although maximum rates were reached at about the same time. As concerns overall transport of the two isotopes (Figure 4B), the time course of increase for copper paralleled that for copper uptake, showing the first significant change between 4 and 6 h of Teta treatment. The time course of changes in overall iron transport induced by copper depletion roughly paralleled those for copper (Figure 4B), although the degree of change was less dramatic.

Expression of transporter mRNAs in iron and copper deficiencies

To begin to determine whether and what changes in the expression of specific transporters might underlie the effects of copper deficiency on iron and copper absorption, levels of mRNA for the non-IRE form of DMT1 mRNA, and for IREG1 were measured, using Real Time PCR. As shown in Table 1, acute copper deficiency had little or no effect on the levels of these mRNAs, whether determined relative to samples from untreated cells in the same PCR run or relative to mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) measured in the same samples in the same run. The effects of DFO pretreatment were also examined (Table 1), and there, too, no acute differences in mRNA expression were observed.

Discussion

Figure 5 depicts potential pathways by which iron may be absorbed via the enterocyte. At the brush border, there may be at least two different transporters that ferry Fe(II) (DMT1) and Fe(III) (DCYTB) to the cytoplasmic side of the membrane. From there, iron somehow finds its way (a) to the vesicular compartment, or (b) perhaps more directly via the cytoplasm to the basolateral membrane. By the vesicular route, the Fe(II) may be oxidized by copper-containing hephaestin and bind to apotransferrin that has entered from the blood, via endocytosis, forming diferric transferrin that may exocytose back out. Alternatively or in addition, Fe(II) may be transferred across the basolateral membrane

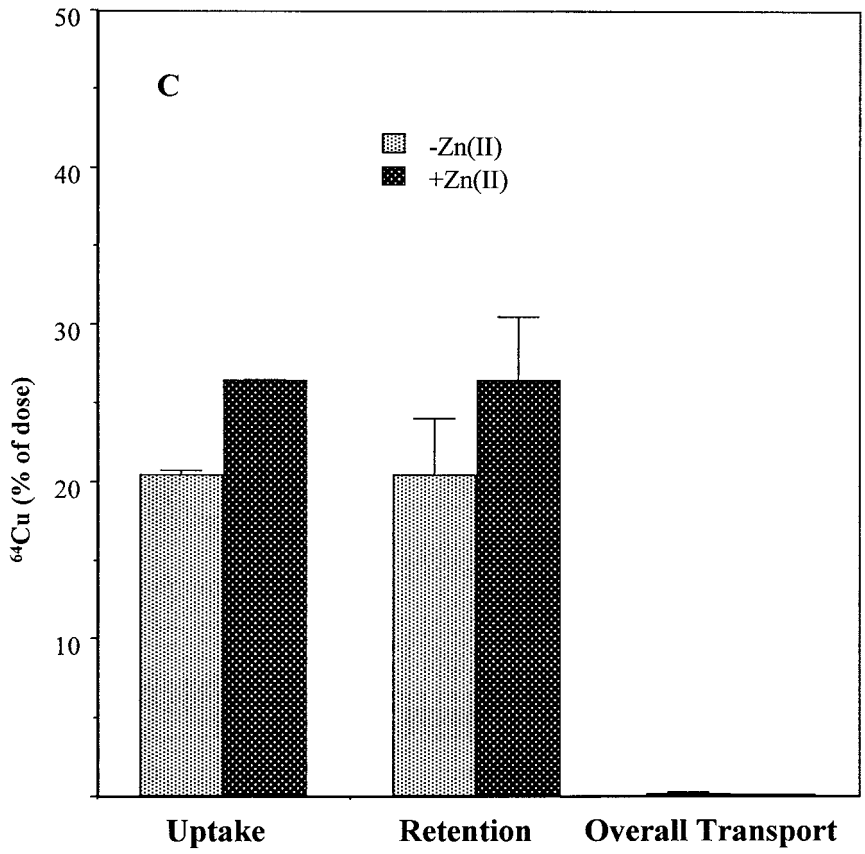

Fig. 3. Continued.

Table 1. Effects of copper and iron depletion on expression of transporter mRNAs in the Caco2 cells, using Real Time PCR. Shown are concentrations of mRNA for the non-IRE-DMT1 and IREG1 (as well as the GAPDH control), in units obtained from the cycle (CT) in which levels of specific PCR product, obtained from cDNA samples of cells treated and not treated with copper or iron chelators (Teta and DFO), respectively) and increasing in a logarithmic manner, rose above a 'noise-free' threshold. Values are CT (Mean ± SD, for N samples indicated) for several runs and experiments, or ratios of values for the iron transporter mRNA over that for GAPDH in the same run. None of the means differed significantly.

	Non-IRE DMT1	GAPDH	DMT1/GPDH
Normal (12-30)	29.4 ± 0.3	21.7 ± 0.6	1.35
Cu-Deficient (16-6)	29.6 ± 0.3	21.8 ± 1.1	1.35
Fe-Deficient (6)	29.3 ± 0.4	21.2 ± 0.7	1.35
	IREG1	GAPDH	IREG1/GPDH
Normal (6)	23.5 ± 0.3	20.2 ± 0.2	1.16
Cu-Deficient (4)	23.7 ± 0.4	20.5 ± 0.1	1.15
Fe-Deficient (6)	24.2 ± 0.1	20.2 ± 0.3	1.19

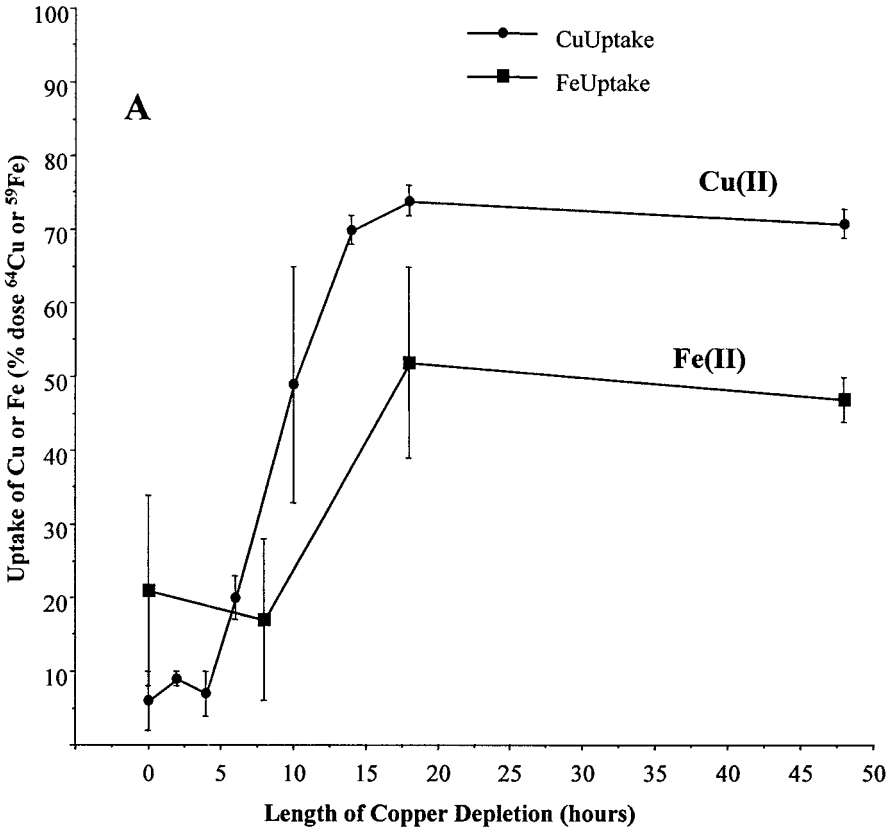

Fig. 4. Time course of the response of iron and copper absorption to copper depletion in Caco2 cell monolayers. Cells were pretreated with Teta for indicated lengths of time (Length of copper depletion; h) before uptake (A) and overall transport (B) of ^{64}Cu and ^{59}Fe were measured. Points and error bars reflect Mean values ± SD ($n = 6$ except for untreated cells, where $n = 12$). A. Changes in uptake; B. Concomitant changes in overall transport.

by ferroportin/IREG1/MTP1, for uptake by transferrin in the plasma. As concerns copper absorption (reviewed more extensively in Linder 2002) and described in the Introduction, uptake and might involve not just CTR1 but also DMT1 in the brush border, and a vesicular process dependent on the Menkes protein (MNK) which pumps copper into vesicles for exocytosis or cycles to the basolateral membrane, for direct pumping of copper into the blood.

The studies reviewed and reported here indicate that there are definite interactions between iron and copper with regard to intestinal absorption, at least as evident from the Caco2 cell model for this process. Our studies and those of others (Alvarez-Hernandez *et al.* 1991, 1994,1998; Nunez *et al.* 1994, 1999) have verified that polarized monolayers of these cells (with tight junctions) react in the same way to changes in iron availability as does the intestinal mucosa of the whole animal. Iron deficiency enhances both uptake of iron at the brush border and its overall transport to the 'blood' side of the monolayer, and iron treatment has the reverse effect. Unexpectedly, we found that depleting cells of copper had the same kind of effect as iron depletion, enhancing both uptake and overall transport. This suggests that copper availability in some way influences the expression of transporters associated with brush border and basolateral iron transport. Clearly, this needs to be further pursued.

Our initial measurements of the expression of potential transporters indicated that levels of mRNA for the non-IRE-containing form of DMT1 were not altered by acute copper depletion. Therefore, expression of the corresponding protein isoform of DMT1 may not be responsive to copper. [This isoform differs only marginally from DMT1 that results from translation of +IRE mRNAs (M. Hentze, this conference.).] Alternatively, expression of the protein could be regulated post-transcriptionally. Clearly, we would expect that the +IRE-containing form of the DMT1 mRNA to be the one most important for iron uptake and thus the

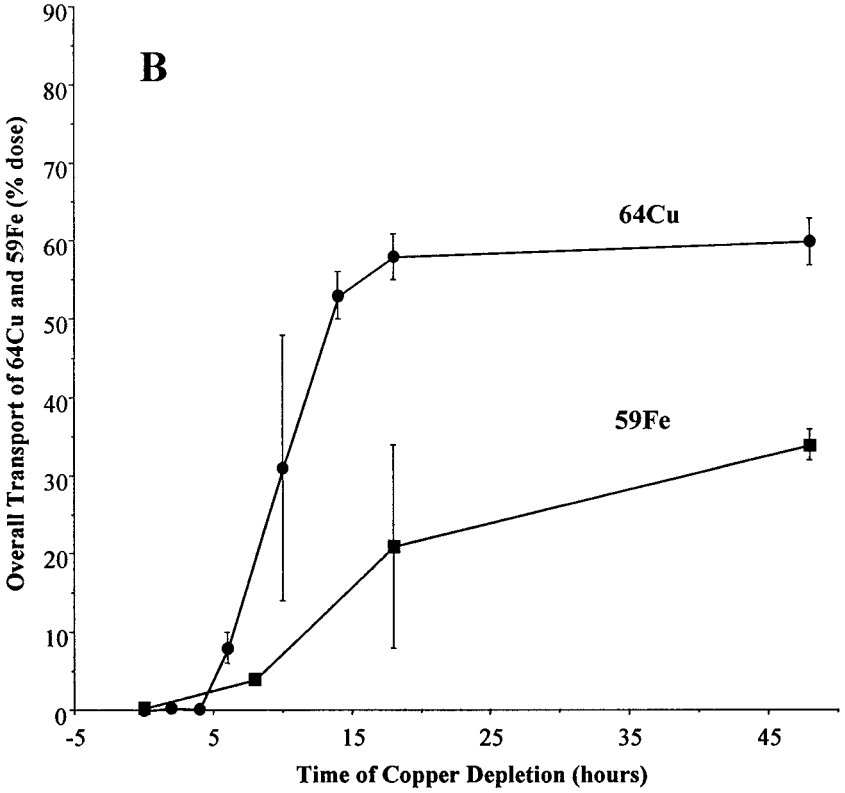

Fig. 4. Continued.

one to respond to copper depletion; and those studies are still in process. In agreement with others using placental cell models (Gambling *et al.* 2001), we found that iron deficiency did not change concentrations of the non-IRE-form of DMT1.

The increased overall transport of iron observed in acute copper deficiency was not explained by changes in the mRNA for ferroportin, which is in the basolateral membrane (Figure 5). Again, this may not be surprising, in that we were studying primarily the particular form of iron absorption dependent upon apotransferrin (Alvarez-Hernandez *et al.* 1994, 1998; Nunez *et al.* 1999), and this pathway may not involve ferroportin/IREG1/MTP1. The presence of two separate pathways for iron transit across the enterocyte may also explain why, in our studies, ceruloplasmin failed to have an effect (Figure 5). If the iron binding to apotransferrin is oxidized by hephaestin, while in vesicles, then it has already bound to transferrin, and ceruloplasmin (added basolaterally) would not be needed. On the other hand, iron arriving at the basolateral cell surface via ferroportin/IREG1/MTP1 might require oxidation by ceruloplasmin, for binding to its carrier (transferrin) in the same extracellular fluid. This remains to be further explored. Copper deficiency also did not acutely change expression of IREG1/ferroportin, at the mRNA level, and the same was observed for acute iron deficiency. A lack of effect of iron availability on levels of this mRNA agrees with the report of Gambling *et al.* (2001) for BeWo cell monolayers that model the placenta. They disagree with the report of McKie *et al.* (2000) for intestinal cells, but this may reflect differences in timing and range of iron availabilities. Moreover, IREG/ferroportin/MTP1 mRNA also has a functional IRE in the 5′UTR (McKie *et al.* 2000). So iron is likely to regulate expression of the protein primarily at the post-transcriptional (translational) level.

The fact that copper depletion (to about half of normal) did not hinder iron transport also indicates that hephaestin, if needed for ferroxidation in the apotransferrin vesicles (Figure 5), holds copper more tightly (and/or turns over much more slowly) than other copper-binding cell substituents.

Another of our findings was that copper availability influences *copper* uptake and absorption. Perhaps

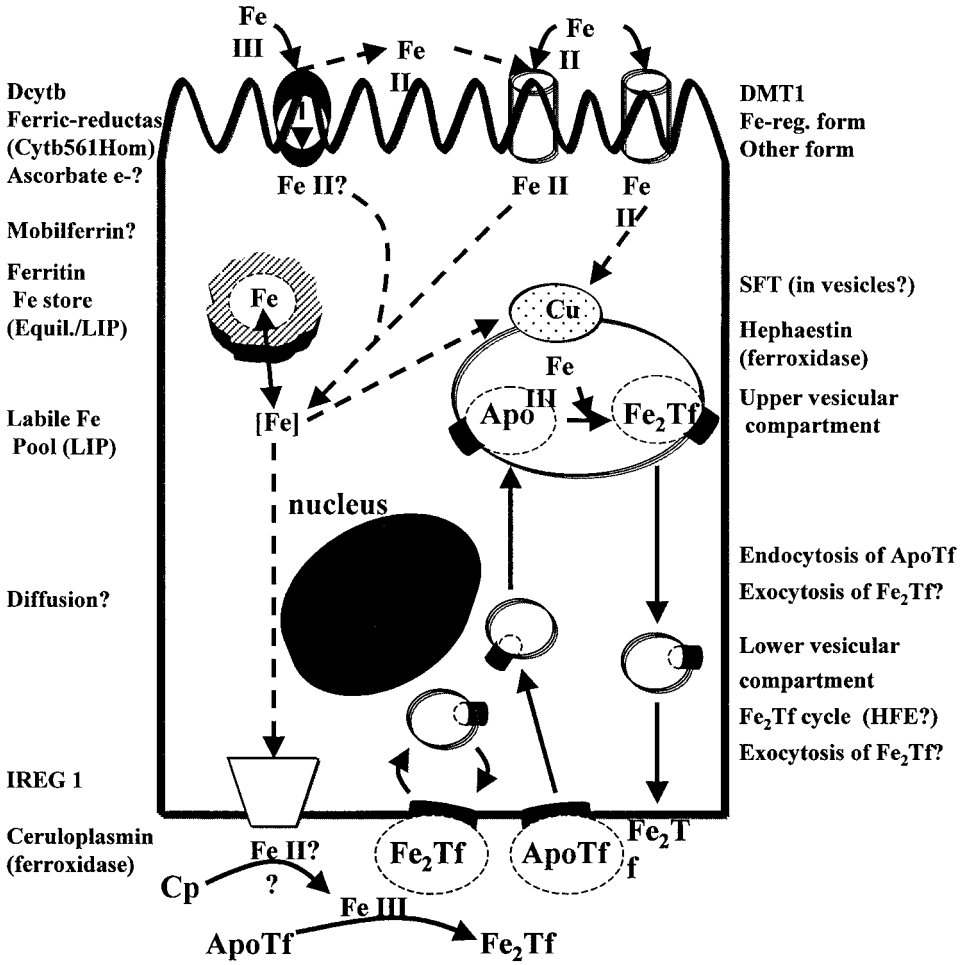

Fig. 5. Overview of potential pathways for absorption of Fe into and across the enterocyte and Caco2 cell monolayer. Potential transporters and related enzymes in the brush border (top) and at the basolateral membrane (bottom) are indicated, as well as factors, such as hephaestin (with Cu and ferroxidase activity), apo and diferric transferrins (ApoTf, Fe_2Tf), and ceruloplasmin (Cp) that might be involved in various steps. A storage role for ferritin, and equilibrium of ferritin iron with that in the labile iron pool (LIP), is also postulated. Ascorbate may provide the electrons needed for reduction of Fe(III) by DCYTB (Cytb561 homologue) (see McKie *et al.* 2001).

surprisingly, there have been very few studies of the potential effects of nutritional copper status on intestinal copper absorption. Our studies indicate that there can be stimulation of copper uptake by both a lack and by an excess of copper. The observations, summarized here, that copper depletion clearly increases copper uptake and overall transport (Zerounian et al. 2001) indicates for the first time that there can be homeostatic regulation of intestinal copper absorption. Our converse observation, namely that an adaptation to excess copper also enhances uptake and overall transport, agrees with recent studies by Arredondo *et al.* (2000), also in Caco2 cells.

The finding that DMT1 mRNA was not altered by copper deficiency suggests that this transporter is not the one whose expression is increased to enhance copper absorption. Indeed, our findings (a) that equimolar concentrations of copper did not hinder Fe(II) uptake (which must be via DMT1), and (b) that 10:1 Zn:Cu failed to hinder copper uptake, suggest that DMT1 may not be important for copper absorption. However, the concentrations of copper used to compete with iron for absorption may not have been sufficient; and in the case of zinc uptake, the role of DMT1 remains to be clarified (see Garrick, this issue). In favor of an involvement of DMT1 in copper absorption are (a) the report of Arredondo *et al.* (2001) that suppression of DMT1 expression reduces copper transport, and (b) our finding (summarized here) that iron deficiency enhances copper uptake. However, in the latter

case, iron depletion induced with desferrioxamine did cause a small but significant (18%) decrease in cellular copper (Zerounian & Linder 2002). This may have contributed to the enhancement of copper absorption observed. The contributions of DMT1 and CTR1 to intestinal copper absorption must thus still be clarified.

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