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Decreased Hephaestin Expression and Activity Leads to Decreased Iron Efflux From Differentiated Caco2 Cells

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ABSTRACT

Iron is transported across intestinal brush border cells into the circulation in at least two distinct steps. Iron can enter the enterocyte via the apical surface through several paths. However, iron egress from the basolateral side of enterocytes converges on a single export pathway requiring the iron transporter, ferroportin1, and hephaestin, a ferroxidase. Copper deficiency leads to reduced hephaestin protein expression and activity in mouse enterocytes and intestinal cell lines. We tested the effect of copper deficiency on differentiated Caco2 cells grown in transwells and found decreased hephaestin protein expression and activity as well as reduced ferroportin1 protein levels. Furthermore, the decrease in hephaestin levels correlates with a decrease of ^{55}Fe release from the basolateral side of Caco2 cells. Presence of ceruloplasmin, apo-transferrin or holo-transferrin did not significantly alter the results observed. Repletion of copper in Caco2 cells leads to reconstitution of hephaestin protein expression, activity, and transepithelial iron transport. J. Cell. Biochem. 107: 803–808, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: CERULOPLASMIN (Cp); FERROPORTIN1 (Fpn1); HEPHAESTIN (Hp); apo-TRANSFERRIN (apoTf); holo-TRANSFERRIN (holoTf)

Dietary iron is absorbed in various forms including heme [Lynch et al., 1989], ferritin [Theil, 2004] as well as "free" iron bound to low molecular weight chelates [Hunt and Roughead, 1999]. DMT1 is responsible for apical uptake of non-heme, non-ferritin bound iron [Gunshin and Hediger, 2002]. A ferrireductase, Dcytb, may also be involved in this process as well as members of the STEAP family of proteins [Latunde-Dada et al., 2002; Gleeson et al., 2005; Ludwiczek et al., 2008]. HCP1, expressed on the apical surface of intestinal enterocytes may represent a heme transporter [Latunde-Dada et al., 2006] although other studies suggest it represents a folate transporter [Inoue et al., 2008]. Finally, several studies have recently demonstrated that iron bound to ferritin can also be utilized [Theil, 2004] although the mechanism remains undefined. All of the iron from the various apical uptake processes converge on a single basolateral export process mediated by ferroportin1 (Fpn1) [McKie et al., 2000], an iron transporter and hephaestin (Hp), a ferroxidase [Vulpe et al., 1999].

Current evidence supports a role for Hp in facilitating iron release from duodenal enterocytes to circulation through oxidizing iron exported by Fpn1 [Vulpe et al., 1999]. Truncation of the protein in sex-linked anemia (*sla*) mice results in accumulation of iron in enterocytes and affected animals develop iron deficiency anemia [Vulpe et al., 1999]. While Hp retains partial ferroxidase activity there is reduced basolateral Hp levels [Kuo et al., 2004]. Copper-deficient rats have lower Hp levels and reduced systemic iron, which is reversed by copper repletion [Reeves and Demars, 2005]. Similarly, copper deficiency leads to lower Hp protein level and activity in mice and cultured cells [Chen et al., 2006].

A closely related ferroxidase, ceruloplasmin (Cp) plays a similar role in iron transport in tissues besides the duodenum. Cp is expressed both as a circulating plasma protein and in some tissues such as the brain as a GPI-linked protein [Salzer et al., 1998]. The disruption of the ceruloplasmin gene in people leads to accumulation of iron in several tissues due to the lack of its mobilization from its stores [Harris et al., 1995]. Furthermore, targeted disruption of the

Abbreviations used: CC, copper reconstituted; CD, copper deficient; CR, copper replete; D, differentiated; ND, non-differentiated.

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cp gene in mice led to similar defects in iron utilization [Harris et al., 1999]. Cell culture studies have also shown a role for Cp in iron release from some cell types [Young et al., 1997; Danzeisen et al., 2000]. Mutant ferroxidase-deficient Cp fails to facilitate iron export from U937 cells [Sarkar et al., 2003]. Interestingly, a recent report in Rat C6 glioma cells shows that Fpn1 is internalized and specifically degraded [De Domenico et al., 2007a] in the absence of GPI-linked ceruloplasmin.

In this study, we have tested the effect of copper deficiency and the resulting lower Hp and Fpn1 protein and Hp activity levels on iron transport from monolayers of differentiated Caco2 cells, a model intestinal cell line for iron transport. Our results indicate that the abrogation of Hp ferroxidase activity and lower Fpn1 protein levels lead to markedly reduced basolateral efflux of iron, whereas repletion of copper levels restores Hp and Fpn1 expression and iron efflux. The presence of soluble Cp, apoTf or holoTf in the basolateral medium does not restore the observed reduced transport. We conclude that basolateral egress of iron from Caco2 cells depends upon proper Hp expression and activity.

MATERIALS AND METHODS

REAGENTS

All reagents were from Sigma (St. Louis, MI) unless otherwise noted. The radioisotope $^{55}FeCl_3$ was purchased from Perkin–Elmer (Billerica, MA). Ceruloplasmin was from Vital Products (Boynton Beach, FL).

Caco2 CELL CULTURE

Caco2 cells were a kind gift from Dr. Paul Sharp (King's College London) at passage 28 and used for experiments at passages 30–35. Stock cultures were maintained in minimum essential medium (MEM) with Earle's salts and L-glutamine supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids solution, 100 U/L penicillin G, and 100 mg/L streptomycin at 37°C in a humidified atmosphere of 95% air 5% CO_2. The growth medium was changed every second day. Cells were split at 80% confluence using 0.5 g/L trypsin with 0.5 mmol/L EDTA in Dulbecco's phosphate-buffered saline (PBS). Prior to the experiments, 5×10^4 cells in 1.0 ml of supplemented MEM were seeded on 0.4 μm microporous polycarbonate membrane inserts (1 cm^2 Transwell inserts; Corning, Acton, MA). The basolateral chamber contained 1.5 ml of supplemented MEM. The medium on both sides of the filter insert was changed every 2 days. Transepithelial electrical resistance (TEER) was monitored to assess the formation of a tight cell monolayer, and the formation of a fully differentiated cell monolayer was typically established by days 18–21 with TEER $>250\ \Omega/cm^2$. For copper depletion studies (CD), Caco2 cell monolayers were differentiated (D) for 1, 7, 14, or 21 days in the presence of 40 μM bathocuproine disulfonic acid (BCS). For copper reconstitution (CC) studies, differentiated Caco2 cell monolayers for 18 days in the presence of 40 μM BCS were washed and re-incubated with normal media for 3 days.

Hp AND Fpn1 ANTIBODIES

Polyclonal rabbit anti-mouse Hp IgG was raised to the domain 4 amino acids (LDSRLLSEDVEGFQDSNRM) of Hp as described in Chen et al. [2003]. Rabbit anti-Fpn1 (CGKQLTSPKDTEPKPLEGTH) was made using the same protocol. Peroxidase-labeled anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

IMMUNOBLOT ANALYSIS

Caco2 cells were lysed as described [Zerounian and Linder, 2002]. For all studies, samples containing 100–200 μg protein were denatured by boiling for 5 min in 2× SDS sample buffer. The proteins were separated by SDS–PAGE (7.5% acrylamide running gel) and transferred to nitrocellulose membranes. Blots were first incubated for 1 h with blocking buffer (containing PBS, 0.1% Tween-20 and 10% nonfat dry milk), and then incubated with primary antibodies for 1 h at room temperature. Primary antibodies were used at the following concentrations: 1:3,000 for rabbit anti-Hp, 1:1,000 for rabbit anti-Fpn1. Blots were then washed three times in 0.1% PBS-T, incubated for 1 h at room temperature with 1:40,000 diluted peroxidase-labeled anti-rabbit secondary antibodies, and signals were visualized by enhanced chemiluminescence.

p-PHENYLENE DIAMINE OXIDASE ACTIVITY ASSAY

The oxidase activity of Hp was determined in Caco2 cells. Cells were washed and lysed as described above. Cell homogenates were centrifuged at 10,000*g* for 30 min to remove unlysed cells and nuclei. The clear lysate (50–100 mg of protein) was applied to a native nonreducing, nondenaturing 4–20% Tris-glycine PAGE gel (Invitrogen) and separated electrophoretically in native Tris-glycine electrophoresis buffer (25 mmol/L Tris, 250 mmol/L glycine). The gels were then incubated with 0.1% pPD in 0.1 mol/L acetate buffer, pH 5.45, for 2 h and air-dried in the dark. Purified human Cp was used as a positive control.

FERROXIDASE ACTIVITY ASSAY

The ferroxidase-specific assay differs from the pPD gel assay only in the final assay step. The gels were placed for 2 h at 37°C in a fresh solution of 0.00784% $Fe(NH_4)_2(SO_4)_2(H_2O)_6$ in 100 mmol/L sodium acetate, pH 5.0. Gels were then rehydrated with 15 mmol/L ferrozine solution in the dark. Color development was then monitored continuously and quantified by scanning densitometry. Cp activity was detected with this in-gel assay and served as a positive control.

ASSAY FOR CELLULAR ^{55}Fe RETENTION AND TRANSFER ACROSS MONOLAYERS

Fresh supplemented MEM was provided to the cells 1 day prior to the retention and transfer assays. To study Fe(II) and Fe(III) retention and transepithelial transfer by Caco2 cells, sample solutions were freshly prepared in uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, 50 mmol/L HEPES, pH 7), according to Han et al. [1995]. The uptake buffer was adjusted to pH 7 with 1 mol/L NaOH before addition of $^{55}FeIII(NTA)_4$ (nitrilotriacetic acid) to a final concentration of 1 μmol/L in the presence of 0.2 mM ascorbate. Sample solutions in volumes of 0.5 ml

were placed on the apical side of Caco2 cells, while the basolateral chamber contained 1.5 ml of MEM. Cells were incubated at 37°C in a humidified atmosphere of 95% air 5% CO_2. Transfer of iron across the cell monolayer was monitored by withdrawing a 0.2 ml aliquot from the basolateral chamber every 30 min, and this volume was immediately replaced by an equivalent amount of MEM. All transfer rates were found to be linear from 1 to 3 h on the basis of linear regression analysis ($P < 0.05$). After a 3 h incubation, the cells were washed three times with ice-cold wash buffer (150 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA, pH 7) and homogenized in PBS containing 2% Np-40 and 1 mmol/L EDTA. ^{55}Fe transferred to the basolateral chamber or associated with the Caco2 cell lysates was measured by liquid scintillation counting; protein determinations were done by the Bradford assay (Bio-Rad, Hercules, CA).

RESULTS

EFFECT OF COPPER DEFICIENCY AND REPLETION ON ^{55}Fe TRANSEPITHELIAL TRANSPORT IN Caco2 CELLS MONOLAYERS

Transepithelial ^{55}Fe transport in differentiated Caco2 cell monolayers was tested to monitor changes due to copper deficiency and repletion. Copper replete (CR) (closed triangles), copper depleted (CD) (open squares) or copper reconstituted (CC) (open triangles) Caco2 cell monolayers were incubated with uptake solutions containing 1 μM ^{55}Fe(NTA)$_4$ and 0.2 mM ascorbic acid for 3 h. 0.2 ml samples were collected from basolateral chambers at 30 min intervals for up to 3 h and ^{55}Fe determined by liquid scintillation. As shown in Figure 1, there is a marked decrease in ^{55}Fe transepithelial transport in copper depleted (CD) differentiated Caco2 cell monolayers compared to copper replete (CR) ones. Copper reconstitution (CC) restored ^{55}Fe transport. No difference was observed in ^{55}Fe uptake at the apical surface between copper replete (CR) and copper deficient (CD) Caco2 cell monolayers (data not shown).

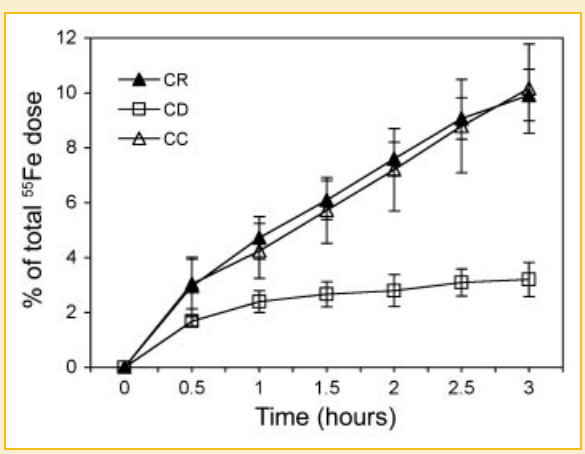

Fig. 1. Copper deficiency decreases transepithelial transport of ^{55}Fe across Caco2 cell monolayers. Iron transport was measured in copper replete (CR) (closed triangles), copper deficient (CD) (open squares), and copper reconstituted (CC) (open triangles) differentiated Caco2 cell monolayers. Transport was normalized to the total protein content measured for each insert. Data are means ± SD for three replicates wells.

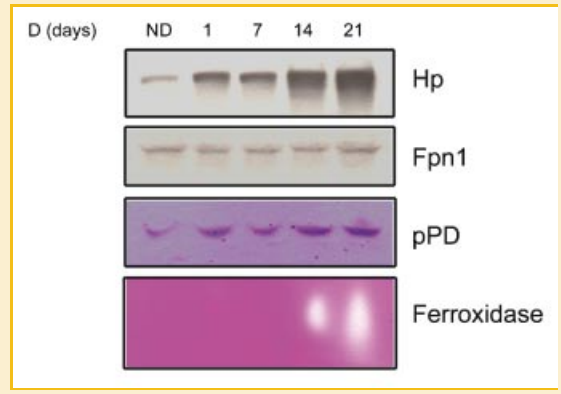

Fig. 2. Hp expression increases with Caco2 differentiation. Immunoblot analysis of Hp and Fpn1 in extracts of non-differentiated (ND) and differentiated Caco2 cell monolayers. Hp and Fpn1 protein levels were determined as described in Materials and Methods section. Hp activity was measured by the oxidation of pPD or ferroxidase activity measured by the ferrozine assay.

Hp AND Fpn1 EXPRESSION AND Hp ACTIVITY IN Caco2 CELLS

The expression of Hp and Fpn1 and Hp activity were tested in non-differentiated Caco2 cells and Caco2 cells differentiated on microporous membrane inserts for various times. As depicted in Figure 2, differentiated Caco2 cells exhibited a significant increase of Hp expression correlating with post-confluence growth as compared to non-differentiated (ND) cells. pPD oxidase and ferrozine activity levels showed a similar pattern whereas Fpn1 did not show any significant change.

Hp AND Fpn1 PROTEIN AND Hp ACTIVITY LEVELS IN COPPER REPLETE AND COPPER DEFICIENT Caco2 CELL MONOLAYERS

The effect of copper deficiency and reconstitution were studied in Caco2 cell monolayers. Figure 3A shows decreasing Hp and Fpn1 protein and Hp activity levels in Caco2 cell monolayers differentiated for 14 days and then copper depleted (CD) by treatment with increasing concentrations of BCS for 7 days as compared to copper replete (CR) 21-day differentiated Caco2 cell monolayers. Figure 3B shows lower Hp and Fpn1 protein and Hp activity levels in copper deficient (CD) Caco2 cell differentiated for 1, 7, 14, and 21 days as compared to copper replete (CR) 21-day differentiated Caco2 cell monolayers. Copper reconstitution (CC) restored Hp and Fpn1 protein and Hp activity levels.

EFFECT OF Cp, apo-Tf, AND holo-Tf ON ^{55}Fe TRANSEPITHELIAL TRANSPORT IN DIFFERENTIATED Caco2 CELL MONOLAYERS

Soluble ceruloplasmin facilitates iron export through loading of oxidized iron onto apo-Tf [Young et al., 1997]. In order to verify the role of Cp-apo-Tf interaction in facilitating iron transport in intestinal cells, copper-deficient (CD) and copper-replete (CR) differentiated Caco2 cell monolayers were loaded with ^{55}Fe(NTA)$_4$ as above and transepithelial transport was monitored. We first

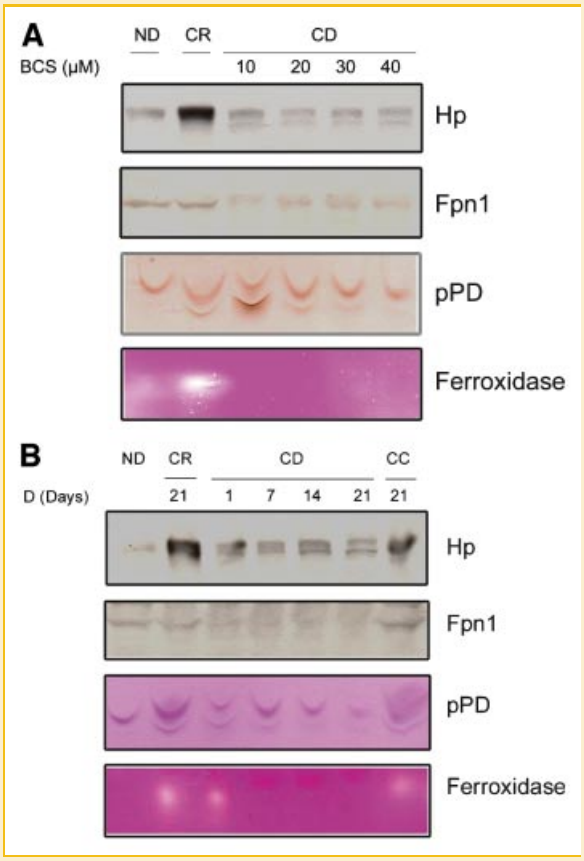

Fig. 3. Copper chelation decreases Hp and Fpn1 protein levels and Hp activity. A: Immunoblot of Hp and Fpn1 in extracts of non-differentiated (ND), copper replete (CR), and copper deficient (CD) differentiated Caco2 cell monolayers incubated with increasing amounts of BCS for 7 days. Hp and Fpn1 protein levels were determined as described in Materials and Methods. Hp activity was measured by the oxidation of pPD or Hp ferroxidase activity measured by the ferrozine assay. B: Immunoblot analysis of Hp and Fpn1 in extracts of non-differentiated (ND); copper replete (CR), copper deficient (CD) and copper reconstituted (CC) differentiated Caco2 cell monolayers. Hp and Fpn1 protein levels were determined as described in Materials and Methods section. Hp activity was measured by the oxidation of pPD or Hp ferroxidase activity measured by the ferrozine assay.

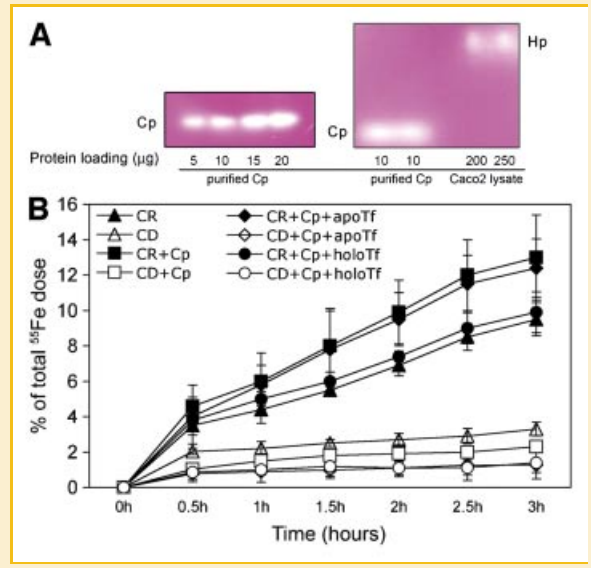

Fig. 4. No effect of Cp, apoTf and holoTf on transepithelial transport of ^{55}Fe across copper replete and copper deficient Caco2 cell monolayers. A, left panel: Ferroxidase activity of purified human Cp (5–20 μg protein) showing dose response and (right panel) ferroxidase activity of 10 μg protein of purified human Cp compared to ferroxidase activity of Hp in 200 and 250 μg of differentiated Caco2 cell extract protein. B: $^{55}FeCl_3$ transport was measured in copper replete (CR) (closed triangles) and copper deficient (CD) (open triangles); Copper replete (CR + Cp) (closed squares) and copper deficient (CD + Cp) (open squares) differentiated Caco2 cell monolayers incubated with media containing 30 μg/ml Cp; copper replete (CR + Cp + apoTf) (open diamonds) and copper deficient (CD + Cp + apoTf) (closed diamonds) Caco2 cell monolayers with media containing 30 μg/ml Cp and 30 μg/ml apo-Tf; copper replete (CR + Cp + holoTf) (closed circles) and copper deficient (CD + Cp + holoTf) (closed circles) cells with media containing 30 μg/ml Cp and 30 μg/ml holo-Tf. Transepithelial transported ^{55}Fe was monitored every 30 min up to 3 h as described in Materials and Methods section. Transport was normalized to the total protein content measured for each insert. Data are means ± SD for three replicates wells repeated for three times.

documented the ferroxidase activity of purified human ceruloplasmin (Fig. 4A, left panel) and compared the activity of purified Cp to the activity of Hp present in Caco2 extracts (Fig. 4A, right panel). We estimate that the activity in 10 μg of purified Cp is comparable to the Hp activity present in 200 μg of Caco2 extract. Each Caco2 monolayer transwell contains approximately 400 μg of total protein so we estimate that each Caco2 monolayer contains ferroxidase activity equivalent to that of 20 μg of purified Cp. We estimate that we are providing 2.5 times the level of ferroxidase activity provided by copper replete Caco2 cells (e.g., Hp) as Cp ferroxidase activity. We then investigated the effect of addition of exogenous Cp and Apo-Tf to iron transport. As shown in Figure 4B, no statistically significant differences were observed between cells incubated with media only (closed triangles), media containing 30 μg/ml active Cp (the well volume is 1.5 ml so a total of 45 μg of Cp was added) (closed squares), 30 μg/ml Cp and 30 μg/ml apo-Tf (closed diamonds) or 30 μg/ml Cp and 30 μg/ml holo-Tf (closed circles). These results indicate that Cp alone or in the presence of physiological concentrations of apoTf or holo-Tf does not further enhance iron export from Caco2 cell monolayers. The same concentrations of Cp alone (open squares), Cp with apo-Tf (open diamonds) or Cp with holoTf (open circles) did not enhance ^{55}Fe export in copper deficient (CD) Caco2 cell monolayers. These results suggest that exogenous ferroxidase activity provided by Cp cannot compensate for the loss of endogenous Hp activity in the Caco2 monolayer.

DISCUSSION

Current evidence indicates that mammalian iron transepithelial efflux requires the integral membrane Fe^{2+} transporter, ferroportin, and a ferroxidase. Hephaestin is a membrane bound ferroxidase which highly expressed in the intestinal brush border cells. At least some Hp protein is present on the basolateral surface in close proximity [Kuo et al., 2004] and possibly associated with the Fpn1 protein [Han and Kim, 2007]. The phenotype of the *sla* mice which

show a partial but not complete defect in intestinal iron transport has lead to the model that Hp facilitates iron efflux rather than being absolutely required [Chen et al., 2004]. Similarly the finding that aceruloplasmenia in people and mice leads to gradual defects in iron mobilization from several tissues rather than complete abrogation of iron transport suggests a similar accessory role for Cp [Harris et al., 1998]. However, the model of Hp as a facilitator is complicated by the finding that Hp in the *sla* mutation retains partial activity and may not represent a complete null and the residual iron transport activity in *sla* could be a consequence of this limited ferroxidase activity [Chen et al., 2004]. It still remains unclear why iron oxidation is required for iron exit since ferroportin transports Fe^{2+} [McKie et al., 2000]. It is possible but still not demonstrated that oxidation is required to release iron from ferroportin. While the plasma iron carrier Tf clearly binds Fe^{3+}, the role of Hp in loading Tf remains uncertain although some groups have found an association of Hp with apo-Tf in cell culture studies [Griffiths et al., 2005]. In this report, in order to address some of these outstanding questions we directly assessed the role of the Hp protein in iron export in a Caco2 in vitro cell culture system. We took advantage of the observation by us and others that copper deficiency in cell culture leads to rapid reduction of Hp protein and almost complete absence of assayed oxidase activity [Nittis and Gitlin, 2004; Chen et al., 2006]. We and others had previously found decreased Hp levels and activity in copper-deficient mice and rats with concurrent decrease in iron absorptive ability [Reeves et al., 2005; Chen et al., 2006]. In this study, we directly assessed the importance of Hp in iron transport in the Caco2 model and found a dramatic decrease in iron export in cell lines without Hp ferroxidase activity. Our results support a model in which Hp ferroxidase activity is required for iron export.

The coupled ferroxidase-transporter mechanism of iron transport was first identified in *S. cerevisiae*. The yeast ferroxidase Fet3p converts ferrous iron to the ferric form which is subsequently imported through Ftr1p [Stearman et al., 1996]. Interestingly, the structural integrity of Fet3p is not only necessary for Fet3p function but also for the stability of Ftr1p [Stearman et al., 1996]. In the absence of Fet3p, the Ftr1p does not properly localize to the plasma membrane in yeast. Recently a similar interdependence between iron transporter and ferroxidase was demonstrated in mammalian brain cells, proper Fpn1 expression and localization required GPI-CP expression in brain cells [De Domenico et al., 2007a]. This requirement can be substituted with the yeast Fet3p [De Domenico et al., 2007a] which suggests that the ferroxidase activity of Cp rather than some other interaction domain of Cp is important for the proper localization of Fpn1. In this study, we noted a correlation between Hp activity and Fpn1 protein levels and suggest that Fpn1 stability may depend on proper Hp expression, activity and localization in Caco2 cells. It is not clear yet if only the ferroxidase activity of Hp is needed for proper expression and stability of Fpn1 as in the other tissues [De Domenico et al., 2007a]. The Hp protein fails to localize to the basolateral membrane during copper-deficiency prior to its degradation in the proteasome [Nittis and Gitlin, 2004]. We speculate that in copper deficiency Hp does not localize to the basolateral membrane and does not interact with Fpn1 leading to the degradation of the latter protein. While the exact mechanism is not resolved in this report, it is still indicative of a structural-functional interrelationship between the oxidase and the transporter.

We investigated a possible role for Tf and Cp in facilitating iron export. Localization studies have suggested that Hp co-localizes with Fpn1 to the basolateral membrane in association with transferrin receptor (TfR) [Han and Kim, 2007] in differentiated Caco2 cells. In our study the addition of apo- or holo-Tf did not influence iron export which suggests that Tf does not play a direct role in iron export. Cp is a plasma ferroxidase and some studies have indicated that Cp may play a role in intestinal iron transport. We found that the addition of soluble active Cp in amounts previously reported to effect Fe release [Young et al., 1997] was not able to compensate for the absence of Hp and suggest a need for a membrane-bound ferroxidase expressed on the basolateral domain of intestinal cell membrane [Vulpe et al., 1999]. It is not known whether this represents the need for a greater local concentration provided by a membrane bound ferroxidase or some particular property of Hp.

We conclude that there is an absolute requirement for membrane bound Hp expression and basolateral localization for proper Fpn1 expression and localization and that the coupled ferroxidase-transporter iron transport is the mode of iron exit from intestinal cells similar to that of the brain [De Domenico et al., 2007b]. Our result agree with a previous study that reported no role of Cp in intestinal iron uptake, retention or transepithelial release [Zerounian and Linder, 2002]. One study suggested a role of Cp in iron transport in intestinal cells [Cherukuri et al., 2005] and demonstrated decreased iron absorption in $Cp^{-/-}$ mice after repeated bleeding. One possibility suggested by the authors was that Cp may only play a role in iron absorption when Hp is saturated. In our study, Cp was not able to compensate for the reduction of Hp levels and this finding is difficult to reconcile with the hypothesis that Cp can functionally substitute for Hp. It is possible that Cp may not play a direct role in iron export from the enterocyte but could play a different role in intestinal iron absorption. For example, iron must transit from the enterocyte across the mucosal layers and Cp could be important for the effective mobilization of iron across the intestinal mucosa after exit from the enterocyte.

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