

Apical distribution of HFE- β 2-microglobulin is associated with inhibition of apical iron uptake in intestinal epithelia cells

Miguel Arredondo^{1,*}, Victoria Tapia², Alejandro Rojas², Pabla Aguirre², Francisca Reyes², Maria Paz Marzolo² & Marco T. Núñez²

¹*Micronutrients Laboratory, Institute of Nutrition and Food Technology (INTA), Universidad de Chile, Macul 5540, Macul, Santiago, Chile;* ²*Department of Biology, Faculty of Sciences, and Millennium Institute for Advanced Studies in Cell Biology and Biotechnology, Universidad de Chile, Macul 5540, Macul, Santiago, Chile;* *Author for correspondence (Phone: 56-2-6781483; Fax: 56-2-2214030; E-mail: marredon@inta.cl)

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Abstract

Mutations in the *HFE* gene result in hereditary hemochromatosis, a disorder of iron metabolism characterized by increased intestinal iron absorption. Based on the observation that ectopic expression of HFE strongly inhibits apical iron uptake (Arredondo *et al.*, 2001, *FASEB J* **15**, 1276–1278), a negative regulation of HFE on the apical membrane transporter DMT1 was proposed as a mechanism by which HFE regulates iron absorption. To test this hypothesis, we investigated: (i) the effect of HFE antisense oligonucleotides on apical iron uptake by polarized Caco-2 cells; (ii) the apical/basolateral membrane distribution of HFE, β -2 microglobulin and DMT1; (iii) the putative molecular association between HFE and DMT1. We found that HFE antisense treatment reduced HFE expression and increased apical iron uptake, whereas transfection with wild-type HFE inhibited iron uptake. Thus, an inverse relationship was established between HFE levels and apical iron uptake activity. Selective apical or basolateral biotinylation indicated preferential localization of DMT1 to the apical membrane and of HFE and β -2 microglobulin (β 2m) to the basolateral membrane. Ectopic expression of HFE resulted in increased distribution of HFE- β 2m to the apical membrane. The amount of HFE- β 2m in the apical membrane inversely correlated with apical iron uptake rates. Immunoprecipitations of HFE or β 2m with specific antibodies resulted in the co-precipitation of DMT1. These results sustain a model by which direct interaction between DMT1 and HFE- β 2m in the apical membrane of Caco-2 cells result in down-regulation of apical iron uptake activity.

Introduction

Hereditary hemochromatosis (HH), one of the most common genetic disorders in individuals of Northern European descent, is characterized by increased intestinal iron absorption, which leads to progressive iron overload (reviewed in Pietrangeli, 2003). Intestinal iron absorption is operationally divided into three phases. The apical uptake phase sustained by the ferric reductase Dcytb and the iron import transporter DMT1, the intracellular phase, in which part of the incoming iron is

retained by ferritin, and the basolateral transfer, sustained by the export transporter Iregl (also called Ferroportin 1 and MTP1) and the ferroxidase hephaestin. The expressions of DMT1, Dcytb and Iregl are increased under iron deficiency conditions (Gunshin *et al.* 1997; Abboud and Haile 2000; McKie *et al.* 2001; Frazer *et al.* 2001), while the expression of hephaestin does not seem to be regulated by body iron requirements (Frazer *et al.* 2001). Which component of the iron absorption machinery is modified in HH is a subject of active research.

Hepcidin, a small liver-produced peptide with antibacterial activity originally identified as LEAP-1, has been recognized as an important regulator of body iron homeostasis. Mice with a target disruption of the *Usp2* gene, leading to the knockout of the hepcidin gene, showed marked iron overload (Nicolas *et al.* 2001). Iron overload in hepcidin knockout mice was remarkably similar to that observed in human HH, with increased circulating iron and increased liver iron loading. In contrast, transgenic mice overexpressing hepcidin presented postnatal mortality due to severe microcytic hypochromic anemia (Nicolas *et al.* 2002). A reduction in transferrin saturation decreases hepcidin production and *vice versa*, suggesting that hepcidin is a signaling molecule produced by the liver in response to elevated body iron stores (Frazer and Anderson, 2003). Interestingly, it was noted that HH patients presented decreased liver hepcidin production (Bridle *et al.* 2003). Moreover, decreased hepcidin production correlated with increased intestinal Ireg1. Indeed, increased Ireg1 activity in HH could generate the false 'iron deficiency' signal in HH enterocytes, evidenced by their low ferritin levels (Pietrangelo *et al.* 1995).

The mechanism by which hepcidin regulates the levels of the intestinal iron transporters is not well understood. Apparently, circulating hepcidin levels adjust the amount of iron absorbed by the duodenum by regulating the expression of both Ireg1 and Dcytb (Frazer *et al.* 2002; Yeh *et al.* 2004). Recently, hepcidin-induced Ireg1 internalization and down-regulation was reported in HEK293 and Hela cells (Nemeth *et al.* 2004). This effect has not been demonstrated in intestinal epithelia cells, where inhibition of DMT1 expression by Hepcidin was reported (Yamaji *et al.* 2004). Thus, more research is needed to understand Hepcidin action.

Due to the importance of body iron homeostasis, it is possible that regulation of iron absorption does not rely on one single inhibitory mechanism. Evidence indicating that HFE may regulate the apical iron uptake phase comes from the observation that HFE-knockout mice have increased DMT1-mediated intestinal iron uptake (Fleming *et al.* 1999; Garrick *et al.* 2003), and that enterocytes from HH patients show increased intestinal DMT1 expression (Zoller *et al.* 2001; Rolfs *et al.* 2002). In line with these studies, we recently reported that HFE over expression in

intestinal Caco-2 cells result in inhibition of apical iron uptake despite increased DMT1 expression (Arredondo 2001). The decrease in apical iron uptake produced a decreased labile iron pool, with the concomitant activation of the IRE/IRP system (Arredondo *et al.* 2001). We speculated that wild type HFE could be a negative regulator of apical iron uptake by the enterocyte. Since HFE reportedly has a basolateral membrane location while DMT1 is an apical membrane protein, it is not readily apparent how HFE could down-regulate DMT1. In the present study, we tested the hypothesis that HFE reaches the apical membrane, reasoning that such an occurrence would open the possibility for a direct HFE/DMT1 interaction. We found that HFE forms a molecular complex with β 2-microglobulin (β 2m) and DMT1, that a fraction of HFE distributes to the apical membrane, and that the amount of HFE in the apical membrane inversely correlates with apical iron uptake. These findings support a model by which HFE directly inhibits apical iron uptake in intestinal epithelia.

Experimental procedures

Cells

Caco-2 cells (American Type Culture Collection #HTB37, Rockville, MD) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Grand Island, NY). Caco-2 cells grown in bicameral systems have become the choice cellular model to study human intestinal absorption and transport. In culture, the cells express a polarized small intestine phenotype (Peterson and Mooseker, 1993) with high levels of GLUT5, a glucose transporter found in the brush border of fetal and adult small intestine but absent in colon cells (Mahraoui *et al.*, 1992). Caco-2 cells have an active IRE/IRP system that regulates apical iron uptake and transepithelial iron transport as a function of intracellular iron levels (Tapia *et al.* 1996; Gárate & Núñez 2000).

Antibodies and immunodetection

Anti-HFE antibody, prepared against the C-terminal segment of human HFE, and anti-DMT1

antibody, prepared against the C-terminal segment of human DMT1 IRE-isoform were used as described previously (Arredondo *et al.* 2001). For Immunoprecipitation experiments, HFE antibody was purified by absorption chromatography against the immunizing peptide bound to Sepharose beads. $\beta 2m$ was detected with a rabbit polyclonal antibody (Sigma Chem. Co. M 2583). The antibody recognized a protein band of 11.7 kDa apparent mass. Human transferrin receptor 1 (TfR1) was recognized with OKT9 monoclonal antibody obtained from a mouse hybridome (American Type Culture Collection CRL-8021). For Western blot analysis, peroxidase-bound anti-rabbit IgG was used as a secondary antibody and a chemiluminescence assay kit (SuperSignal, Pierce Chem. Co., Rockford, IL) was used for detection. Chemiluminescence was detected using a Molecular Imager FX device (Bio-Rad, Hercules, CA).

Vector construction and transfection of Caco-2 cells

Cloning and transfection of HFE cDNA was done as previously described (Arredondo *et al.*, 2001). Throughout the text, cells will be named as control cells: transfected with the pcDNA3 vector and HFE cells: transfected with the wild type HFE gene. A full-length $\beta 2m$ cDNA was obtained from Caco-2 cells mRNA by reverse transcription reaction using 5'-ttcggtcgcagatgtctc-3' and 5'-cctccatgatctagattacatg-3' as forward and reverse primers, respectively. The cDNA was used as a template for PCR amplification using the following cycles: 94 °C for 1 min; 47 °C for 1 min; and 72 °C for 1 min. Samples were amplified for 30 cycles. Sequencing of the 0.35 kbp PCR product indicated a full-length $\beta 2m$ cDNA. The generated PCR product was cut with XhoI and XbaI. The fragment was purified by agarose gel electrophoresis and ligated into the pcDNA3 plasmid. Single isolates were selected and subjected to DNA sequencing to confirm the insertion. The construct was named pcDNA3- $\beta 2m$. HFE cells grown to half confluence (3 days after plating) were transfected with pcDNA3- $\beta 2m$. Lipofectamine (Gibco) at 5 μ l/ μ g of DNA was used for the transfection. The DNA-Lipofectamine mixture was removed after 36 h of incubation at 37 °C. After 4 days in culture, the cells were tested for $\beta 2m$ expression and iron transport.

HFE antisense treatment

Antisense oligonucleotides were obtained from Biosynthesis Inc. (www.biosyn.com) in the phosphorothioate form. The effect of HFE antisense 1 (5'-cagtcctctctccaggta-3'), HFE antisense 2 (5'-caggtaggtccctcccca-3') and antisense 3 (5'-cgtatatctctgctcttccc-3') are reported here. Caco-2 cells grown for 10 days in 0.33 cm² polycarbonate cell culture inserts (Transwells, Corning-Costar) were transfected with 5–20 μ g/ml of antisense oligonucleotides in the presence of Lipofectamine following the instructions of the manufacturer (Gibco Life Sciences). The antisense/Lipofectamine mixture was renewed every 24 h of incubation for 3 days after which ⁵⁵Fe uptake experiments were performed. HFE protein expression in the treated cells was checked by Western immunodetection.

Iron uptake and transport by Caco-2 cells

Control, HFE, or HFE/ $\beta 2m$ Caco-2 cells were grown for 12 days in 0.33 cm² polycarbonate cell culture inserts (Transwells, Costar, Cambridge, MA) in DMEM supplemented with 10% FBS. The cells were then washed with MOPS buffer (20 mM 3-(N-morpholino) propane sulfonic acid-Na, 94 mM NaCl, 7.4 mM KCl, 0.74 mM MgCl₂ and 1.5 mM CaCl₂, pH 6.75), and incubated at 37 °C for 1–60 min in MOPS buffer supplemented with 5 μ M ⁵⁵Fe²⁺ supplied as an ⁵⁵Fe-ascorbate complex (1:50, mol:mol) in the apical medium. Iron uptake was stopped by washing the inserts three times with ice-cold PBS supplemented with 1 mM EDTA. EDTA was included to eliminate non-specific ⁵⁵Fe binding. We ascertained that this treatment effectively removed ⁵⁵Fe radioactivity loosely bound to the cells. A cell extract was prepared as described (Arredondo *et al.* 2001) and the extracts were evaluated for protein by the BCA method, and for ⁵⁵Fe radioactivity in a gamma radioactivity counter. Iron uptake was expressed as pmol of iron per mg of protein.

HFE - DMT1 co-immunoprecipitation

Co-immunoprecipitations were performed in control Caco-2 cells. Briefly, Caco-2 cell extracts were pre-adsorbed overnight at 4 °C with protein A-Sepharose. An appropriated dilution of anti-HFE, anti-HFE incubated with HFE synthetic peptide

(QGSRGAMGHYVLAERE) that corresponds to the C-terminal of HFE (Arredondo *et al.*, 2001) or anti- β 2m antibody was also incubated overnight at 4 °C with protein A-Sepharose. After centrifugation to eliminate either the Sepharose beads or the not bound antibody, the extract supernatant was incubated with the protein A-Sepharose-antibody mix for 4 h at 4 °C. The beads were then washed three times by centrifugation (20 mM Hepes; 150 mM NaCl, 10% Glycerol, 1% Triton X-100; 500 $\times$ g for 1 min) and subjected to SDS-polyacrylamide gel electrophoresis and Western immunodetection using either anti-DMT1 or anti-TfR as detecting antibodies.

Determination of HFE apical/ basolateral distribution

The plasma membrane localization of HFE and DMT1 was studied by selective biotinylation of the apical or basolateral membrane domain (Zurzolo *et al.*, 1994). Control and HFE cells were grown for 14 days in 4.5 cm² polycarbonate cell culture inserts (Transwells, Corning-Costar, Cambridge, MA) in DMEM supplemented with 10% FBS. The cells were washed three times with saline – 0.5 mM CaCl₂, 1.0 mM MgCl₂ and incubated from either the apical or the basal side for 30 min at 4 °C with 0.5 mg/ml NHS-imino biotin (Pierce Chem. Co., No. 21217). After washing, the filters were cut out and cellular extracts were prepared incubating the cells with lysis buffer (Arredondo *et al.* 2001) for 1 h at 4 °C. After centrifugation, the extracts were incubated at 4 °C overnight with 50% immobilized streptavidin (Pierce Chem. Co., Rockford, IL), sedimented for 1 min at 1,300 rpm and washed first with TPII buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA; 0.2% BSA; 0.1% SDS), then with TPIII buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA; 0.2% BSA) and finally with TPIV buffer (20 mM Tris-HCl, pH 8.0). The streptavidin-pellet was dissolved in 30 μ l of 5 $\times$ loading buffer, boiled for 5 min and resolved by 8% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes for immunodetection.

Statistical analysis

Variables were tested in triplicates, and the experiments were repeated at least twice. Results

were expressed as a mean $\pm$ SD. Variability among experiments was less than 20%. One-way ANOVA was used to test differences in means, and Bonferroni's post-hoc *t*-test was used for comparisons (GraphPad InStat software). Differences were considered significant if $p < 0.05$.

Results

HFE antisense treatment increases apical and basolateral ⁵⁵Fe transport

Since HFE over expression strongly inhibits apical iron uptake in Caco-2 cells (Arredondo *et al.* 2001), it was of interest to test for the effect of reduced HFE expression on iron uptake by Caco-2 cells. To that end, HFE expression and iron uptake rates were analyzed in cells treated with HFE-antisense oligonucleotides (Figure 1). Of three HFE antisense tested, antisense-1 inhibited HFE expression in a dose-response way (Figure 1A). Densitometry analysis revealed a 4.2 ± 0.49 -fold and a 3.0 ± 0.45 -fold decrease in HFE protein expression in cells treated with 20 and 5 μ g/ml of antisense-1 compared to control cells (Figure 1A, line 1 versus line 4 and 5, respectively) ($p < 0.05$). Twenty μ g/ml of antisense-2 (Figure 1A, lane 3), and 2.5 μ g/ml of antisense-1 (data not shown) produced just a marginal decrease in HFE expression. Up to 20 μ g/ml of antisense-3 did not inhibit HFE expression (data not shown). Cells treated with 20 μ g/ml of antisense-1, which produced a marked decrease in HFE expression, showed 3.5- and 8.9-fold increases in ⁵⁵Fe uptake compared to control and HFE cells, respectively (Figure 1B). In agreement with previously reported data (Arredondo *et al.* 2001), we found that cells over-expressing HFE presented a marked inhibition of apical iron uptake (Figure 1B). Thus, apical iron uptake by Caco-2 cells inversely correlated with HFE protein levels.

Analysis of transepithelial ⁵⁵Fe fluxes indicated that cells treated with antisense-1 presented a 2.4-fold increase in iron transport when compared to control cells, whereas HFE cells showed a 0.46-fold decreased transport (Figure 1C). The fraction of total Fe transported to the basolateral side was 8.0%, 5.6% and 13.7%, for control cells, HFE cells and control cells incubated with antisense-1, respectively. Although there is no

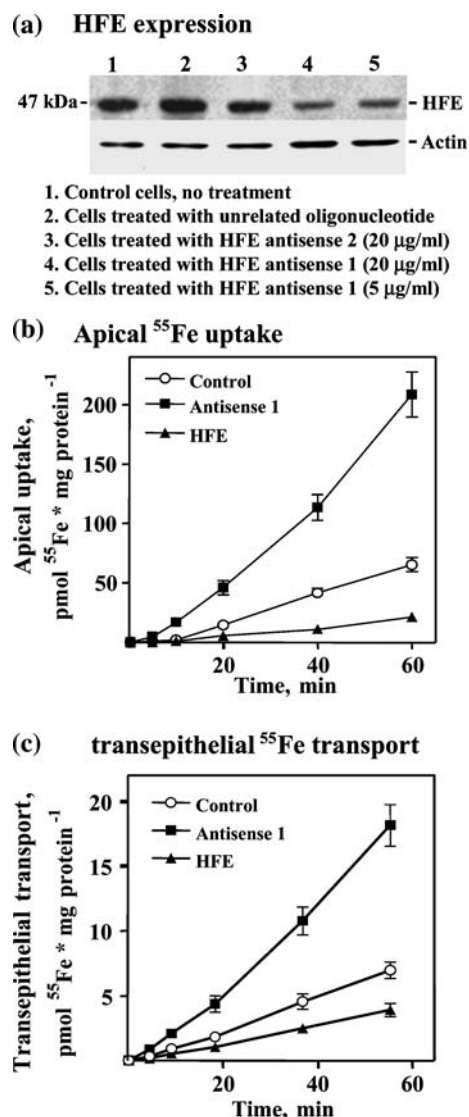


Figure 1. Characterization of Intracellular Iron metabolism in Caco-2 transfected with antisense oligonucleotides against HFE. (a) HFE protein expression. Cells were treated with HFE antisense oligonucleotides for 3 days after which Western immunodetection of HFE was performed. Strong inhibition was observed with 5 and 20 µg/ml of antisense 1 while antisense 2 and unrelated oligonucleotide had little or no effect. (b) Apical ^{55}Fe uptake. Control cells grown in bicameral inserts were treated for 3 days with 20 µg/ml of antisense 1, after which apical ^{55}Fe uptake experiments were performed. As a reference, ^{55}Fe uptake was also performed in HFE cells. Results show an inverse correlation between HFE expression and apical iron uptake. (c) Transepithelial ^{55}Fe transport. Transepithelial ^{55}Fe transport was evaluated by determining ^{55}Fe radioactivity in the basolateral media from cells grown in bicameral inserts as described in (b). Transepithelial iron transport inversely correlated with HFE expression ($n = 3$ independent experiments).

unequivocal interpretation to these later results, it is possible that HFE also inhibits iron transport through the basolateral membrane, since HFE overexpression inhibits, while HFE underexpression stimulates basolateral iron transport.

^{55}Fe uptake in HFE and HFE/ $\beta 2\text{m}$ transfected Caco-2 cells

Adequate levels of $\beta 2\text{m}$ are necessary for the normal trafficking of HFE in COS cells (Waheed *et al.*, 1997). Thus, the decreased iron uptake in HFE cells could be secondary to $\beta 2\text{m}$ deficiency. To investigate if Caco-2 cells express sufficient $\beta 2\text{m}$ to support normal HFE traffic, we studied its expression in control and HFE cells. Control Caco-2 cells displayed basal $\beta 2\text{m}$ expression that increased with HFE transfection (Figure 2a, compare first and second lanes). Co-transfection of HFE and $\beta 2\text{m}$ resulted in increased expression of $\beta 2\text{m}$ (Figure 2a, third lane). Similar levels of HFE expression were found in HFE and HFE/ $\beta 2\text{m}$ cells (not shown). To test for the adequacy of $\beta 2\text{m}$ levels to support iron uptake in these cells, $^{55}\text{Fe}^{2+}$ uptake in control, HFE and HFE/ $\beta 2\text{m}$ cells was determined (Figure 2B). The ^{55}Fe uptake rates of HFE and HFE/ $\beta 2\text{m}$ cells were undistinguishable and lower than the rate of control cells (34.4 ± 1.2 ; 35.9 ± 2.5 and 73.8 ± 0.3 pmoles $\times$ mg protein⁻¹, respectively) ($p < 0.001$). These results indicate that Caco-2 HFE cells express enough $\beta 2\text{m}$ to support the undisturbed traffic of the HFE protein.

Apical/basolateral membrane distribution of DMT1, HFE, $\beta 2\text{m}$ and TfR

We next explored the possibility that transfection with HFE induced redistribution of DMT1, HFE or $\beta 2\text{m}$ that could explain the reduced apical iron uptake. For this purpose, the distribution of these proteins in control and HFE cells was studied by a biotinylation assay, which unequivocally differentiates between apical and basolateral membrane domains in polarized cells (Figure 3a). In control cells, DMT1 showed the expected apical distribution. HFE transfection increased the levels of DMT1 in the basolateral membrane (Figure 3b), while the levels in the apical membrane stayed relatively constant. Because DMT1 levels in the

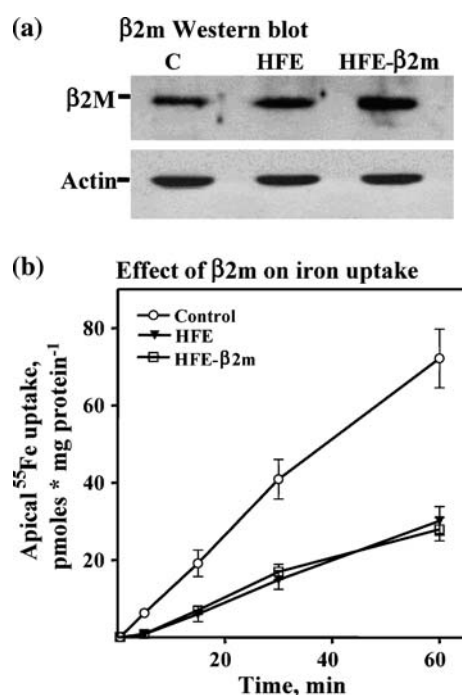


Figure 2. Apical ^{55}Fe uptake by control, HFE, and HFE/ $\beta 2\text{m}$ cells. (a) $\beta 2\text{-microglobulin}$ levels. HFE cells were transiently transfected with pcDNA3- $\beta 2\text{m}$. After 4 days, cells were tested for $\beta 2\text{m}$ protein levels by Western blot. Note that HFE and HFE/ $\beta 2\text{m}$ cells showed increased levels of $\beta 2\text{m}$ compared to control. (b) Apical ^{55}Fe uptake of HFE/ $\beta 2\text{m}$ cells. Control, HFE and HFE/ $\beta 2\text{m}$ cells were tested for apical ^{55}Fe uptake. HFE and HFE/ $\beta 2\text{m}$ cells presented similar ^{55}Fe uptake rates, which were significantly lower than ^{55}Fe uptake from control cells ($n = 3$ independent experiments).

apical membrane were similar in control and HFE cells, the marked decrease in apical iron uptake activity observed in these cells is not explained by decreased apical distribution of DMT1.

The basolateral/apical distribution of HFE and $\beta 2\text{m}$ in control and HFE cells was next studied using a biotinylation protocol that specifically labels surface proteins (Figure 3a). In control cells, HFE was found in both the basolateral and the apical membrane, with a preferential basolateral location. HFE transfection resulted in a marked increase of HFE in the apical membrane. Similarly, $\beta 2\text{m}$ was barely detectable in the apical membrane of control cells (Figure 3b), while HFE transfection produced a dramatic increase of $\beta 2\text{m}$ in the apical membrane (Figure 3b). To ascertain that HFE transfection did not produce a general change in membrane polarity, we studied the distribution of TfR, a basolateral membrane marker. TfR distributed exclusively to the basolateral

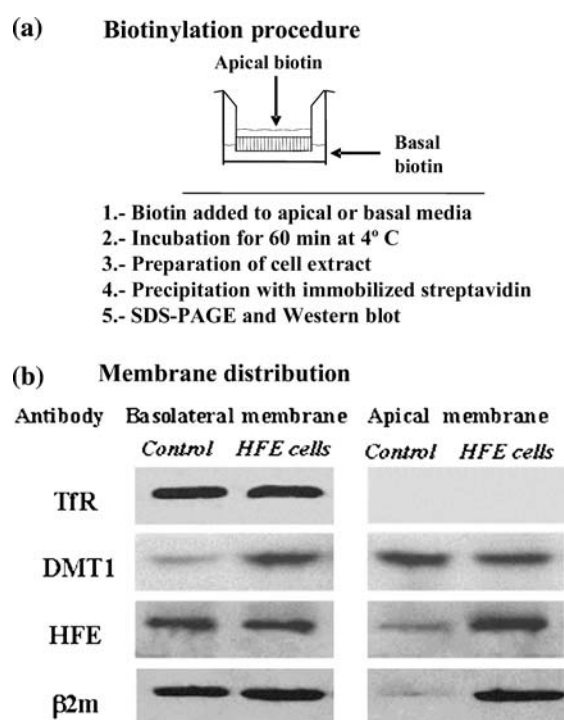


Figure 3. Apical/basolateral membrane distribution of DMT1, HFE, $\beta 2\text{m}$ and TfR. Insert-grown control and HFE cells were biotinylated from either the apical or the basal medium. Cells extracts were prepared and precipitated with immobilized streptavidin. The precipitates were resolved in 8–12% SDS-PAGE gel and TfR, DMT1, HFE and $\beta 2\text{m}$ were immunodepleted by Western blot. (a) scheme of selective biotinylation of the apical and basolateral membrane. (b) Apical/basolateral distribution of TfR, DMT1, HFE and $\beta 2\text{m}$ in control and HFE-expressing cells. Shown is one of three independent experiments for each blot.

membrane in control cells, and HFE transfection did not change this distribution pattern (Figure 3b). Therefore, the increased basolateral presence of DMT1, HFE and $\beta 2\text{m}$ in HFE cells was not due to a general shift in membrane polarization.

HFE-DMT1 and HFE-TfR co-immunoprecipitation

The presence of both DMT1 and HFE in the apical membrane raised the possibility of a direct interaction between HFE and DMT1. This putative interaction was assessed by co-immunoprecipitation assays using anti-HFE and anti- $\beta 2\text{m}$ antibodies (Figure 4). DMT1 was detected in the immunoprecipitates with HFE antibody (Figure 4a). Additionally, $\beta 2\text{m}$ antibody also

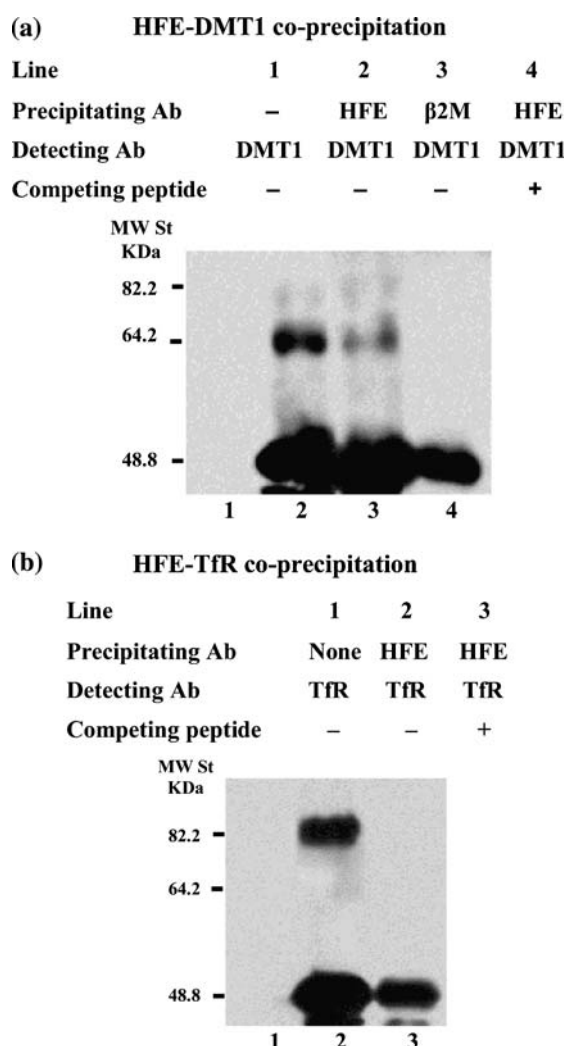


Figure 4. HFE-DMT1 and HFE-TfR immunoprecipitation. Cellular extracts from Caco-2 cells were precipitated with anti-HFE; anti-HFE incubated with HFE synthetic peptide or anti-β2m antibodies as described in the Experimental Procedures. The immunoprecipitates were resolved by 8% SDS-PAGE. DMT1 (a) or TfR (b) were immunodetected by Western blot. HFE antibodies precipitated DMT1 and TfR. β2m antibody also precipitated DMT1. The band detected at 50 kDa in (a) and (b) corresponds to rabbit IgG. The interference of this band with HFE detection made impractical the co-precipitation of HFE with anti-DMT1 antibodies. Shown is one of three independent experiments for each blot.

immunoprecipitated DMT1, suggesting the presence of a β2m-HFE-DMT1 complex. As a control, we studied the HFE-TfR interaction (Figure 4b). A complex between HFE and TfR was detected in the immunoprecipitate with HFE antibody. Thus, HFE seems to interact with DMT1 besides its known interaction with TfR.

Discussion

The precise mechanisms by which mutations in the HFE protein result in increased intestinal iron absorption are poorly understood. A current model states that HFE is necessary for hepcidin synthesis in the liver and the consequent hepcidin-mediated inhibition of Ireg1 synthesis in intestinal cells (Frazer and Anderson 2003). Although attractive, this model does not account for the observed inhibition by HFE of apical iron uptake observed in Caco-2 cells (Arredondo *et al.* 2001), or for the decreased transferrin-bound iron uptake observed in HFE expressing HeLa and H1299 cells (Roy *et al.* 1999; Wang *et al.* 2002). Thus, redundancy in the control of intestinal iron uptake may exist through mechanisms other than hepcidin inhibition. Based on the strong inhibition of apical iron uptake observed in HFE-overexpressing Caco-2 cells, we proposed that HFE is a negative regulator of DMT1 activity (Arredondo *et al.* 2001). In the present work, we studied the cellular mechanisms that underlie this inhibition.

A strong correlation was found between the amount of HFE expression and inhibition of apical iron uptake in Caco-2 cells. HFE-transfected cells had diminished apical iron uptake capacity while cells with decreased expression of HFE had an increased capacity. Since HFE overexpression decreased, and HFE underexpression increased ⁵⁵Fe uptake, it is apparent that HFE is a negative regulator of apical iron uptake in Caco-2 cells. Moreover, since the cells respond to both increases and decreases of HFE, HFE levels seems to be limiting for its negative control of iron uptake in Caco-2 cells.

HFE levels also inversely correlated with basolateral ⁵⁵Fe transport, purportedly mediated by Ireg1/ferroportin. The data is difficult to interpret since only 8% of the ⁵⁵Fe taken up from the apical side was transported to the basolateral side. In HFE cells this value decreased to 5.6%, while in antisense-1-treated cells increased to 13.7%. Certainly, a more *ad hoc* experimental setup is needed to test for the effect of HFE on Ireg1 activity in Caco-2 cells.

Co-expression of β2m and HFE had the same effect on apical Fe uptake than HFE transfection alone. Consequently, Caco-2 cells, at difference to COS cells, express adequate amounts of β2m to accomplish its function in HFE trafficking, and the

iron-deficient phenotype elicited by HFE transfection is not linked to $\beta 2m$ deficiency. Similar results were reported in H1299 cells (Wang *et al.* 2002). Concomitant with increased HFE expression, HFE cells underwent re-distribution of DMT1 to the basolateral membrane. This redistribution, however, did not prevent normal DMT1 levels in the apical membrane. Therefore, decreased levels of DMT1 in the apical membrane are not the cause of the diminished apical iron uptake in HFE cells, and other mechanisms must be invoked to explain this marked decrease.

A strong correlation was found between the inhibition of apical iron uptake and the amount of HFE and $\beta 2m$ present in the apical membrane. We suggest that the presence of HFE- $\beta 2m$ in the apical membrane would allow for increased *in situ* interaction between DMT1 and HFE- $\beta 2m$, and that this interaction would be necessary for the down-regulation of DMT1 activity. The co-precipitation of DMT1 with HFE and $\beta 2m$ reported here supports the likelihood of a direct interaction between these three proteins.

We found that HFE antisense treatment resulted in increased transfer of iron from the cell into the basolateral medium, a process mediated by the iron transporter Ireg1. This response may simply be a reflection of increased availability of iron for transport, given by increased apical uptake. Alternatively, it could be due to a decreased inhibitory effect of HFE on the basolateral transport process. This later interpretation is on line with recent evidence indicating inhibition of basolateral iron transport by HFE in HT29 cells (Davies and Enns, 2004).

Figure 5 illustrates a model for the regulation of intestinal iron absorption by HFE. The model proposes transcytotic traffic of HFE- $\beta 2m$ from the basolateral to the apical membrane. Such traffic was described in Caco-2 cells for aminopeptidase N (Le Bivic *et al.* 1990). In the apical membrane, HFE- $\beta 2m$ binds to DMT1 inhibiting its transport activity. Alternatively, HFE could inhibit either Ireg1 or hephaestin thus inhibiting the transfer step of iron absorption. Preliminary experiments of HFE transcytosis in our laboratory (Núñez and Tapia, unpubl. data) indicate that this is a relatively fast (2–3 h) mechanism of response, and it could correspond to a first line of defense against body iron overload, before the hepcidin system takes charge. A negative control of HFE- $\beta 2m$ on

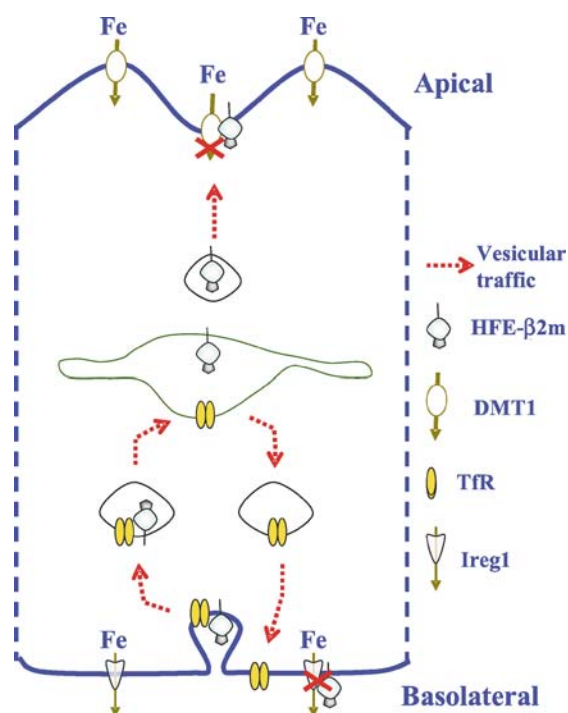


Figure 5. Proposed model for HFE inhibition of intestinal iron uptake. TfR-dependent HFE- $\beta 2m$ endocytosis brings the HFE- $\beta 2m$ complex into endosomal compartments where it dissociates from TfR and reaches the apical compartment by transcytosis. Once in the apical compartment the complex interacts with DMT1 inhibiting its transport activity. HFE may also inhibit basolateral iron transport, thus exerting a double control on transepithelial iron transport.

the activity of DMT1 agrees with most of the experimental evidence of intestinal iron fluxes in HH reported up to date. In particular, it agrees with the increased DMT1-mediated intestinal iron uptake found in HFE-knockout mice (Fleming *et al.* 1999; Levy *et al.* 2000) and the marked inhibition of iron uptake found in HFE-overexpressing cells (Roy *et al.* 1999; Arredondo *et al.* 2001; Wang *et al.* 2002). Certainly, additional factors are involved in the regulation of intestinal iron absorption. Increased expression of Ireg1 found in HH (Zoller *et al.*, 2001; Rolfs *et al.* 2002), and the inverse correlation found between hepcidin and Ireg1 traffic (Nemeth *et al.* 2004), could indeed account for a mechanism by which decreased liver hepcidin synthesis in HH would result in the enterocyte in augmented availability of Ireg1 and the consequent iron deficient phenotype.

In summary, we determined that apical iron uptake inversely relates to the levels of HFE and

$\beta 2m$ in the apical membrane. We also found evidence for the formation of a ternary complex between HFE, $\beta 2m$ and DMT1. These observations are in line with the proposal that the HFE- $\beta 2m$ complex directly inhibits DMT1 iron uptake activity in the apical membrane. We hypothesize that HFE interacts with a variety of proteins involved in iron homeostasis including TfR, DMT1 and, probably, Ireg1. Depending on its membrane distribution and relative affinities for competing ligands, HFE will down-modulate iron transport at the apical membrane or at the basolateral membrane with differential strength. Further experimentation should be done to determine the mechanisms by which HFE distributes to the apical and basolateral membrane.

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