The hereditary hemochromatosis protein, HFE, lowers intracellular iron levels independently of transferrin receptor 1 in TRVb cells

Hanqian Carlson, An-Sheng Zhang, William H. Fleming, and Caroline A. Enns

Hereditary hemochromatosis (HH) is an autosomal recessive disease that leads to parenchymal iron accumulation. The most common form of HH is caused by a single amino acid substitution in the HH protein, HFE, but the mechanism by which HFE regulates iron homeostasis is not known. In the absence of transferrin (Tf), HFE interacts with transferrin receptor 1 (TfR1) and the 2 proteins co-internalize, and in vitro studies have shown that HFE and Tf compete for TfR1 binding. Using a cell line lacking endogenous transferrin receptors (TRVb cells) transfected with different forms of HFE and TfR1, we demonstrate that even at low concentrations Tf competes effectively with HFE for binding to TfR1 on living cells. Transfection of TRVb cells or the derivative line TRVb1 (which stably expresses human TfR1) with HFE resulted in lower ferritin levels and decreased Fe²⁺ uptake. These data indicate that HFE can regulate intracellular iron storage independently of its interaction with TfR1. Earlier studies found that in HeLa cells, HFE expression lowers Tf-mediated iron uptake; here we show that HFE lowers non–Tf-bound iron in TRVb cells and add to a growing body of evidence that HFE may play different roles in different cell types. (Blood. 2005;105:2564-2570)

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Introduction

Hereditary hemochromatosis (HH) is a common autosomal recessive disease of iron metabolism characterized by gradual accumulation of excess iron in organs such as the liver, heart, pancreas, and thyroid, resulting in symptoms, including hepatic cirrhosis and hepatocellular carcinoma, cardiomyopathy and arrhythmias, diabetes, and hypogonadotropic hypogonadism. The most common form of HH is caused by a single base pair mutation in the HH gene that results in the substitution of tyrosine for cysteine at amino acid position 260 (C260Y). The numbering system for both HFE and low-density lipoprotein receptor [LDLR] is based on the mature protein and does not include the signal sequence) in the mature protein, HFE. HFE is an atypical major histocompatibility complex (MHC) class I-related protein. The C260Y mutation disrupts a disulfide bond in its α3 domain, which abrogates its association with the β2-microglobulin (β2M) light chain and subsequent trafficking to the cell surface. Although the importance of functional HFE-β2M heterodimers in the maintenance of iron homeostasis has been further documented in studies using HFE knock-out mice, the mechanism by which HFE regulates iron metabolism in cells is still unknown.

In vitro and in vivo studies show that HFE associates with transferrin receptor 1 (TfR1) at neutral pH and that the binding sites for HFE and Tf overlap in TfR1. In vitro experiments demonstrate that HFE competes with Tf for binding to TfR1 at concentrations of diferric Tf lower than 100 nM. These experiments explain the early observation that HFE appears to lower the binding affinity of TfR to diferric Tf. Indeed, in HeLa, HEK293, and H1299 cells, expression of exogenous HFE results in the lowering of intracellular iron levels. However, HeLa and H1299 cells expressing HFE have about 30% lower rates of Tf-mediated iron uptake than parental controls, even in the presence of sufficiently high concentrations of diferric Tf that HFE does not affect the uptake of Tf. In addition, the W81A HFE mutant regulates Tf-mediated iron uptake to the same extent as wild-type HFE in HeLa cells, despite showing a 5000-fold lower affinity for TfR1 binding. These studies show that the effect of HFE on iron homeostasis does not depend solely on the interaction with TfR1 and is more complex than simple competition between HFE and Tf for binding to TfR1.

In this study we took advantage of a Chinese hamster ovary cell line, TRVb cells, which lacks endogenous transferrin receptors. These cells were stably transfected with different combinations of human HFE, β2M, and TfR cDNAs, and flow cytometry was used to study the competition between Tf and HFE for binding to TfR1 in intact cells. Our results show that this competition occurs even at concentrations of Tf well below those found in the blood. We went on to examine the role of HFE in iron homeostasis in cells lacking TfR1 and found that HFE can lower intracellular iron levels independently of TfR1.

Materials and methods

Plasmids and subcloning

The pCB6 plasmids encoding HFE-GFP-LDLR and HFE-GFP were gifts from Dr Pamela Bjorkman (California Institute of Technology, Pasadena).
The HFE constructs were subcloned via Asp718/HindIII digestion into the pCDNA3.1+ hygro plasmid (Invitrogen, BV Carlsvad, CA). The HFE-GFP-LDLR construct contains the entire coding sequence of HFE, a Leu/Gln linker region, enhanced green fluorescent protein (EGFP), and the cytoplasmic domain of the LDLR (lys 790-Ala 839).

**Cell culture**

TRVb cells (which express no endogenous TfR1), TRVb cells (TRVb transfected with human TfR1),19 and TRVb32-8 cells (TRVb transfected with a mutated TfR1 lacking amino acids 3-59 of the cytoplasmic domain)20 were gifts from Dr Timothy McGraw (Cornell Medical College, Ithaca, NY). The TRVb1/HFE/β2M and TRVb1/HFE-GFP/β2M cell lines were generated by cotransfection of TRVb1 cells with pCDNA3 3.1 hygro+ HFE and pBA encoding β2M (gift from Dr John Feder, Bristol Meyers-Squibb, Princeton, NJ), or with pCDNA3.1 hygro+ HFE-GFP and pBA β2M. The TRVb32-8/HFE-LDLR-GFP/β2M cell line was generated by cotransfection of TRVb32-8 cells with pCDNA3.1 hygro+ HFE-LDLR-GFP and pBA β2M. TRVb2/HFE/β2M cells were generated by cotransfection of TRVb cells with pCDNA3.1 hygro+ HFE and pBA β2M. Cells were selected with hygromycin (300 μg/mL) and subcloned once. TRVb cells were maintained in F12 medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mg/mL glucose, and 10 μM ferric Fe3+ nitritotriacetate (Fe-NTA). TRVb1 cells and TRVb/HFE/β2M were maintained in the same growth medium with 400 μg/mL G418 (Geneticin; Calbiochem, San Diego, CA) and 300 μg/mL hygromycin (Sigma), respectively. TRVb32-8/HFE-LDLR-GFP/β2M, TRVb1/HFE-GFP/β2M, and TRVb/HFE/β2M cell lines were maintained in growth medium with both G418 and hygromycin.

For Fe or NTA treatments, cells were incubated with 1 to 3 mg/mL (12.5-37.5 μM) human diferric Tf (Intergen, Chicago, IL) or 150 μM Fe-NTA for 24 hours to load cells to induce similar levels of ferritin, then analyzed as described in the following analysis. Fe-NTA was always freshly prepared at a ratio of 1:40 as described previously.17 At this ratio of Fe to NTA the iron remains in solution longer. Treatment of cells over this time period did not alter their growth or viability (data not shown). The concentrations given in the text refer to the concentration of Fe in the solution. Tf saturation was confirmed by measuring the solution absorbance at 465 and 280 nm (100% saturation OD465/OD280 = 0.045).

**Immunodetection**

Subconfluent cells grown in 35-mm dishes were washed twice with phosphate-buffered saline (PBS; pH 7.4) and solubilized with lysis buffer (20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) pH 7.4, 1 mM sodium citrate, 0.5% (vol/vol) NP-40, 10 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM EDTA (ethylenediaminetetraacetic acid), and 1 mM sodium orthovanadate plus 2 mM benzamidine, 40 μg/mL leupeptin, 40 μg/mL soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 4 μg/mL pepstatin on ice. Lysates were centrifuged at 13 000 g for 5 minutes at 4°C to remove nuclei and assayed for total protein by bicinchoninic acid protein assay (Pierce, Rockford, IL). Lysates (normal- or transfected-condition) were prepared 55Fe using the freshly prepared 55FeCl3 (Perkin Elmer Life Sciences, Piscataway, NJ) using IP Lab Gel 1.5 software.

**5Fe**2+ uptake assay

Reduction of 55Fe3+ to 55Fe2+ was performed according to an established method26 with some modification. Briefly, 55FeCl3 (Perkin Elmer Life Sciences, Boston, MA) was incubated with ascorbate at a ratio of 1:100 for about 20 minutes to allow complete reduction of 55Fe3+ to 55Fe2+. Incubation buffer (25 mM Tris (tris(hydroxymethyl)aminomethane) HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 10% 2-mercaptoethanol)24 for 5 minutes at 94°C prior to electrophoresis on 12% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose, checked for protein loading by PonceauS staining, and blocked with 5% nonfat milk in 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.05% Tween-20 buffer. Immunoblot analysis was performed using sheep anti-TfR1 serum (1:5000 dilution),22 mouse monoclonal anti-β-actin (1:2000; Chemicon, Temecula, CA), rabbit anti-HFE serum (1:1000 dilution; Dr Pamela Bjorkman, Caltech), sheep anti-human ferritin (1:100 dilution; The Binding Site Ltd, Birmingham, United Kingdom), 1:10 000 no. 137 rabbit anti-HFE serum (Dr John Feder, Bristow Meyers-Squibb, Princeton, NJ), and mouse anti-β2M (1:10 000 dilution; Immunotech, Cedex, France) followed by the appropriate secondary antibody conjugated to horseradish peroxidase and chemiluminescence (SuperSignal; Pierce, Rockford, IL).

**Soluble HFE treatment**

TRVb and TRVb cells were treated with 0.5 μM soluble HFE (shFE; a kind gift from Dr Pamela Bjorkman, Caltech) overnight as previously described23 with or without the addition of 150 μM Fe-NTA. The effects of shFE on iron homeostasis in these cells were evaluated by immunodetection of ferritin.

**Flow cytometry analysis**

Cells were washed twice with PBS (pH, 7.4) and released from the dish with dissociation buffer (Sigma). Cells (1 × 107/mL) were incubated with Ham F12 medium containing 10% PBS with or without 1 μg/mL to 10 mg/mL (12.5 mM-125 μM) diferric Tf for 30 minutes at 37°C in 5% CO2. After treatment, cells were centrifuged for 5 minutes at 1000g at 4°C, washed once with Hanks buffer supplemented with 3% FBS and 10 mM HEPES, and then incubated with monoclonal anti-TfR1 (1:200 dilution) or rabbit anti-HFE serum (1:200 dilution; Dr Pamela Bjorkman) for 30 minutes at 4°C to bind cell-surface TfR1 or HFE. After washing, the cells were incubated with phycoerythrin-conjugated anti-mouse immunoglobulin G (IgG; 1:200 dilution; Caltag, Burlingame, CA) or anti-rabbit (1:200 dilution) antibodies for 30 minutes at 4°C. Following a final wash with supplemented Hanks buffer, flow cytometric analysis was performed in a Becton Dickinson FACS Calibur flow cytometer. The median fluorescence of the IgG1 control at the same concentration as the monoclonal anti-TfR1 was less than one tenth of the anti-TfR1 signal. The median fluorescence of rabbit preimmune serum at the same dilution as the anti-HFE serum was subtracted from the HFE-positive signal.
Cells were incubated in complete medium containing 0, 0.001, 0.01, 0.1, 1, 3, or 10 mg/mL diferric Tf, and cell surface TfR1 was measured by flow cytometry (the values for 0.001 and 0.01 were nearly identical and appear as a single point in this particular experiment). These experiments were repeated 3 times using 3 different clones for each cell line.

Thus, if Tf effectively competes with HFE for binding to the TfR1, TfR1 for internalization some of the excess HFE will remain on the cell surface, causing a lower relative increase in fluorescence than would be expected. HFE does not, then incubation of cells with Tf would be expected to displace HFE from TfR1 and lead to the accumulation of HFE on the cell surface without affecting the distribution of TfR1. To test this case, TRVb1 cells expressing wild-type human TfR1 were transfected with human β2M, and an HFE chimera containing GFP on its cytoplasmic domain and GFP-positive cells were analyzed for cell surface HFE by incubating the cells at 4°C with an antibody to TfR1 and subjected to flow cytometry analysis as described in “Materials and methods,” using antibodies to detect TfR1 and HFE in separate experiments. (A) Increase in cell-surface TfR1 but not HFE in TRVb32-8/HFE-GFP-LDLR/β2M cells incubated with Tf. TRVb32-8 cells express a form of TfR1 lacking the endocytic signal, so internalization of this mutant TfR1 is dependent on interaction with HFE-GFP-LDLR, in which the cytoplasmic domain of LDLR mediates constitutive endocytosis. (B) Increase in cell-surface HFE but not TfR1 in TRVb1/HFE-GFP/β2M cells incubated with Tf. (C) Concentration dependence of the change in cell surface TfR1 in TRVb32-8/HFE-GFP-LDLR/β2M cells incubated with Tf. Cells were incubated in complete medium containing 0, 0.001, 0.01, 0.1, 1, 3, or 10 mg/mL diferric Tf, and cell surface TfR1 was measured by flow cytometry (the values for 0.001 and 0.01 were nearly identical and appear as a single point in this particular experiment). These experiments were repeated 3 times using 3 different clones for each cell line with similar results.

**Tables**

**Table 1. Summary of flow cytometry data**

<table>
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<th>TfR1</th>
<th>TfR1 + 10 mg/mL Tf</th>
<th>HFE</th>
<th>HFE + 10 mg/mL Tf</th>
<th>Fold increase, TfR1</th>
<th>Fold increase, HFE</th>
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<tbody>
<tr>
<td>TRVb32-8/HFE-GFP-LDLR/β2M</td>
<td>376 ± 18</td>
<td>1028 ± 143</td>
<td>162 ± 16</td>
<td>222 ± 35</td>
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<td>TRVb1/HFE-GFP/β2M</td>
<td>1014 ± 84</td>
<td>1250 ± 73</td>
<td>208 ± 13</td>
<td>376 ± 25</td>
<td>1.2</td>
<td>1.8</td>
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</tbody>
</table>

Data are changes in cell surface TfR1 and HFE (mean fluorescence units). These results were determined from 3 independent experiments for each cell line.
HFE expression in TRVb1 cells decreases intracellular ferritin levels

Expression of exogenous HFE lowers intracellular iron stores and decreases ferritin levels in HeLa and HEK293 cells,15,23 whereas in some monocytic and a colonic cell line, HT29, it increases ferritin levels.31,32 We, therefore, used 3 methods to examine the effect of HFE expression on the iron status of TRVb1 cells. In the first method, qualitative observations were made on the effect of HFE/β2M on the ferritin levels of the cell lines examined. Ferritin levels in TRVb1 and TRVb1/HFE/β2M cells are very low, but when similar ferritin levels were achieved by the addition of either 3 mg/mL diferric Tf or 150 μM Fe-NTA to the medium, HFE expression significantly decreased ferritin levels relative to untransfected controls. Ferritin levels were not only lower in diferric Tf-treated TRVb1/HFE/β2M cells compared with TRVb1 cells but also in Fe-NTA–treated cells (Figure 2A). Ferritin levels increased in TRVb1 cells with both Tf and Fe-NTA treatment. To differentiate between the possibilities that the lower ferritin levels seen were due to clonal variation and that HFE/β2M expression was responsible for the lower ferritin levels detected in the TRVb1 HFE/β2M cells, TRVb1 cells were incubated with soluble HFE (sHFE) overnight. Treatment of cells with sHFE decreased ferritin levels in TRVb1 cells (Figure 2B), ruling out the chance that the variation in ferritin levels between clonal cell lines was the cause in the differences in ferritin levels. These results indicate that HFE/β2M expression can regulate the accumulation of iron derived from both Tf-mediated and non–Tf-mediated uptake mechanisms.

HFE expression in TRVb1 cells increases the binding of IRP to IRE

The lower ferritin levels in TRVb1 cells expressing HFE/β2M suggested that the cells had diminished intracellular iron. Ferritin synthesis is regulated by iron regulatory proteins (IRPs; reviewed by Hentze and Kuhn33). Electrophoretic mobility shift assays were used as a second method as an indicator of intracellular iron status. The amount of IRPs binding to the IREs of ferritin mRNA reflects intracellular iron status. We, therefore, evaluated the effect of HFE/β2M expression on IRP binding to IREs in TRVb1 cells. Cell extracts normalized to total protein were mixed with a 32P-labeled IRE probe containing the 5’ untranslated region of L-ferritin. The expression of HFE/β2M in TRVb1 cells increased the fraction of IRPs bound to IREs compared with TRVb1 cells, indicating a lower intracellular iron pool, relative to controls (Figure 3). Treatment of both TRVb1 and TRVb1/HFE/β2M cells with 1 mg/mL diferric Tf or 150 μM Fe-NTA resulted in decreased IRE/IRP binding relative to total IRPs detected when extracts were treated with 2-mercaptopropanol, but, even in the presence of high concentrations of Tf or Fe-NTA, IRE/IRP fraction was slightly higher in TRVb1/HFE/β2M than in TRVb1 cells. These results indicate that HFE can play a role in decreasing the iron pool in the presence of concentrations of Tf that would be expected to diminish the Tf1-HFE interaction and are consistent with our flow cytometry analysis showing competition between Tf and HFE over a wide range of Tf concentrations.

HFE acts independently of TfR1 to lower ferritin levels in TRVb cells

The lack of endogenous transferrin receptors in TRVb cells allowed us to determine whether HFE/β2M acted through association with TfR1 or independently of TfR1 in decreasing intracellular iron levels. Cells were transfected with HFE and β2M and treated with 3 mg/mL diferric Tf or 150 μM Fe-NTA for 24 hours (Figure 4A), and ferritin levels were used as a qualitative marker of intracellular iron levels. In the absence of TfR1, cells expressing HFE and β2M had lower ferritin levels than the parental TRVb cells. The effect was most pronounced in the Fe-NTA–treated cells. Since TRVb cells do not express endogenous TfR1, they are not expected to respond to Tf at all; the slight effect that was observed is probably due to nonspecific pinocytosis of Tf and release of iron at such a high concentration of Tf. Incubation of TRVb cells overnight with soluble HFE also resulted in lowered ferritin levels (Figure 4B), indicating that the lower ferritin levels were not a result of clonal variation of ferritin within cells.
Expression of HFE in TRVb cells decreases ferrous iron accumulation in cells

To determine the mechanism by which HFE/β2M mediates lower intracellular iron levels in cells, iron uptake studies were performed. Cells were incubated in buffer containing 10 μM 55Fe and 1 mM ascorbic acid at pH 5.5; which is the optimal pH for DMT1-mediated iron uptake) for 30 minutes before being washed and lysed; intracellular 55Fe was measured by liquid scintillation counting of the lysates. Expression of HFE significantly decreased ferrous iron accumulation in both TRVb and TRVb1 cells but not in HeLa cells (Figure 5). These results indicate that HFE/β2M regulates iron accumulation in TRVb cells differently than in HeLa cells even though both cell lines have lower intracellular iron levels in response to HFE expression. Quantitative polymerase chain reaction (PCR) analysis did not reveal any difference in DMT1 levels in response to HFE expression in TRVb or HeLa cells (data not shown), which implies that HFE might regulate iron uptake via a post-translational mechanism or act on an as yet unidentified protein involved in iron uptake. Because we measured iron uptake for 30 minutes, HFE could be increasing iron efflux from cells. No detectable iron efflux could be measured from these cells (results not shown).

Discussion

In vitro experiments using truncated forms of TIR and HFE11,12 or solubilized full-length HFE and TIR7 have demonstrated that Tf and HFE compete with each other for binding to TIR1. These studies also indicated that at concentrations of diferric Tf in the blood (~1–4 mg/mL; ~12.5–60 μM) no HFE would be expected to bind TIR1. Since HFE and TIR1 are integral membrane proteins, the possibility exists that the effective concentration of HFE in the vicinity of TIR1 is greater than that of soluble proteins such as Tf. We tested this possibility in living cells using CHO-derived cell lines expressing either wild-type or truncated TIR1 together with either wild-type HFE or a chimeric HFE fused to the LDLR cytoplasmic domain. In these experiments, Tf did not affect the cell-surface levels of whichever protein carried the internalization signal (wild-type TIR1 or HFE-LDLR), but caused a redistribution to the surface of its binding partner (TIR1-32/8 or wild-type HFE), demonstrating direct competition between soluble Tf and cell-surface HFE for binding to cell-surface TIR1. These observations are in agreement with previous in vitro data7,12,18 and imply that Tf can compete with HFE for binding to TIR1 even in tissues in which Tf levels could be orders of magnitude lower than the levels in serum. Both Kupffer cells and hepatocytes are exposed to serum concentrations of Tf. Such concentrations of Tf would be expected to reduce the association between HFE and TIR1, but as Figure 1C shows, it does not completely abrogate it: approximately 16% of the total change in fluorescence occurs between 0.1 and 3 mg/mL diferric Tf. Thus, even in the liver, competition between HFE and Tf for TIR1 binding may be relevant to iron homeostasis.

The results from the inhibition of ferrous iron uptake in cells expressing HFE/β2M but not TIR1 strongly indicate that HFE regulates ferritin and intracellular iron levels by a mechanism independent of its interaction with TIR1. They are in agreement with studies showing that the W81A HFE mutant (which has a greatly reduced affinity for TIR1) has the same effect as wild-type HFE on the iron phenotype of HeLa cells17 and with evidence for a TIR1-independent effect on macrophage iron phenotype.31 These data lead us to speculate that the target(s) of HFE action might be involved in iron import. We, therefore, examined whether HFE affects Fe2+ uptake, which is mediated by iron transporters such as DMT1. In the TRVb cells HFE/β2M expression inhibits ferrous iron accumulation over 30 minutes. These results differ from the results that we and other investigators observed in HeLa cells in which HFE inhibits Tf-mediated iron uptake,15,24,34,35 HEK293,36 and H129916 cells. The degree to which HFE expression inhibits Fe2+ accumulation is, however, comparable to the decrease in Tf-mediated iron uptake in HeLa cells.

Our results differ from those of Waheed et al.37 who found that, while expression of wild-type HFE lowered intracellular iron levels in TRVb1 cells, simultaneous overexpression of β2M resulted in a higher iron phenotype. This led the investigators to conclude that the lower iron phenotype somehow resulted from a lack of β2M expression. More recent results, however, are consistent with our findings: cotransfection with β2M did not alter the effect of HFE on iron phenotype in H1299 cells.16 Further, our earlier studies show

![Figure 4. HFE lowers ferritin levels in TRVb cells.](image-url)
that in HeLa cells, which show a lower iron phenotype when overexpressing exogenous HFE, β2M levels increase as HFE expression increases.\textsuperscript{17}

A growing body of evidence indicates that the effect of HFE expression in a variety of cell lines and in different tissues is cell-type dependent. Expression of wild-type HFE lowers intracellular iron levels in TRVb, HEK293, HeLa, and H1299 cells.\textsuperscript{16} It does so in TRVb cells by lowering Fe\textsuperscript{2+} accumulation, whereas in HeLa cells it lowers Tf-mediated iron uptake.\textsuperscript{15,35} In contrast, in macrophages,\textsuperscript{38} in the macrophage-derived THP1 cell line\textsuperscript{31} and in a colonic carcinoma cell line, HT29,\textsuperscript{32} HFE expression results in the accumulation of iron resulting in higher ferritin levels within the cell. The accumulation of iron is the consequence of an inhibition of iron efflux from these cell types. Differences in response to HFE have been recorded even between cells of similar lineage, whereas macrophage-like THP-1 cells show increased ferritin and decreased TIR1 levels, monocyte-like U937 cells show the opposite response when treated with soluble HFE or infected with recombinant vaccinia expressing HFE.\textsuperscript{31} Cell-type specificity in vivo has been observed as well. In the early stages of HH due to the C260Y HFE mutation, hepatocytes accumulate excess iron, whereas Kupffer cells are iron-deficient (reviewed by Chorney et al\textsuperscript{10}). Both cell types normally express HFE.\textsuperscript{30,41}

Since HFE can result in either the accumulation or depletion of intracellular iron stores, we speculate that it can interact with either the proteins involved in the import or export of iron and that the differences seen between cell lines and in tissues depends on the ratios of exporters and importers and TIR1. HFE appears to cause accumulation of intracellular iron in cell lines that exhibit active iron efflux.\textsuperscript{11,38} In the absence of evidence for iron efflux in ovary-derived cells our present results showing that expression of HFE/β2M lowers intracellular iron stores in TRVb cells are consistent with this speculation. In this context it is important to note that TRVb cells were selected for their ability to survive without transferrin receptors, so that alternative mechanisms of iron acquisition may be up-regulated for cell survival. Such mechanisms may be relatively unimportant in HeLa cells, which take up about 5 times as much iron from Tf as from Fe-NTA,\textsuperscript{35} but they could be important in hepatocytes that have robust non-Tf mediated iron uptake. Both the relative contributions of the 2 pathways to iron acquisition and the potential for ferrous iron uptake to be up-regulated if Tf-mediated uptake drops may serve to mask any effect of HFE on non–Tf-mediated iron uptake in HeLa cells. In TRVb cells, the absence of Tf receptors may reveal HFE effects that cannot be observed in HeLa cells. This idea is consistent with the growing body of evidence indicating that HFE activity is cell-type specific and may depend on the relative levels and/or activities of other molecules involved in iron homeostasis. The challenge now becomes to identify the proteins responsible for the cell-type dependence of HFE effects on iron homeostasis.

**Acknowledgments**

We thank Pamela Bjorkman for the HFE constructs and C. William Hooker for careful reading of the manuscript and helpful discussions. We also thank Dr Anthony Bakke for training on the Becton Dickinson GACScan flow cytometer, Emily L. Anderson for excellent technical assistance, Dr Timothy McGraw for the TRVb cell lines, and Dr John Feder for the HFE and β2M plasmid and antibody no. 137 to HFE.

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