REGULATION OF CELLULAR IRON HOMEOSTASIS BY HFE, A NON-CLASSICAL MHC CLASS I PROTEIN

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

Hereditary hemochromatosis is a well-described autosomal recessive disease of iron overload. Only recently has the mutated gene, *HFE*, been isolated. The pathology of hereditary hemochromatosis iron loading results from a defect in the regulated transport of iron through the intestine. Individuals with the disease are able to regulate iron uptake in response to changes in iron reserves, but they absorb more iron over their lifetime than unaffected individuals. This phenotype indicates that the hereditary hemochromatosis defect establishes an elevated "set point" for the maintenance of iron homeostasis.

HFE does not appear to be an iron transporter, rather it associates with the transferrin receptor which is a well characterized protein of iron uptake. The primary focus of this thesis is to describe the interaction of HFE with the transferrin receptor and the resulting effects on cellular iron homeostasis. HFE's association with the transferrin receptor prompted us to develop and test several hypotheses for the regulation of cellular iron homeostasis by HFE. HFE was expressed in a nonpolar HeLa cell line under the control of the tetracycline-responsive promoter. This system allows the use of the same clonal cell line to assay for changes in iron metabolic pathways in the presence or absence of HFE. A strong interaction between HFE and the transferrin receptor was observed that is evidenced by their co-immunoprecipitation within 30 minutes of their synthesis. The co-trafficking of HFE and the transferrin receptor continues throughout the transferrin-mediated iron uptake pathway and is required for HFE function.

HFE-expressing HeLa and HEK 293 cells have a low iron phenotype that is evidenced by a reduction in ferritin levels up to 10 fold and a 52% increase in transferrin receptor levels. This low iron phenotype persists at steady state, and suggests that HFE establishes a new, reduced, set point for cellular iron homeostasis. HFE does not affect the cycling kinetics of the transferrin receptor, but HFE does reduce the uptake of iron from transferrin by 33%. These results implicate a specific effect of HFE on transferrin-

mediated iron uptake that is limited to its interaction with the transferrin receptor. In contrast, HFE does not affect non-transferrin mediated iron transport pathways.

We have identified an HFE-specific reduction in the labile iron pool that is achieved through the transferrin-mediated pathway of iron uptake. This decrease in the labile iron pool increases IRP RNA binding activity almost 5 fold through the activation of both IRP isoforms. We speculate that HFE may regulate dietary iron absorption through its influence on the labile iron pool of intestinal epithelial cells specialized for iron transport. In the case of hereditary hemochromatosis, HFE function is severely diminished. We speculate that this prevents the establishment of a labile iron pool in equilibrium with body iron needs. Without the proper set point established, a chronic increase in iron absorption would occur through the intestinal epithelium as a result of the misregulation of iron dependent transcription, translation, degradation, and protein trafficking events that can modulate the activity of iron metabolic proteins.

ABBREVIATIONS

2-ME	Beta-mercaptoethanol
BSA	Bovine serum albumin
Df/Dsf	Desferrioxamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DMT1	Divalen Metal Transporter 1 (formerly Nramp2 and DCT1)
endo H	Endo β-N-acetylglucosaminidase H
ER	Endoplasmic reticulum
FcRn	Neonatal Fc Receptor
Fe_CTf	monoferric transferrin with iron bound in the carboxy terminal site
Fe_NTf	monoferric transferrin with iron bound in the amino terminal site
fHFE	FLAG-epitope tagged HFE
FITC	Fluoroscein isothiocyanate
Ft	Ferritin
HFE	Hereditary Hemochromatosis gene product
HH	Hereditary Hemochromatosis
HLA	Human Leukocyte Antigen
HRP	Horse-radish peroxidase
IRE	Iron Responsive Element
IRP	Iron Regulatory Protein
kDa	Kilodaltons
MHC	Major Histocompatability Complex
mRNA	messenger RNA
NTA	Nitrilotriacetic acid
PBS	Phosphate Buffered Saline
RIPA	Radioimmune Precipitation Assay
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SFT	Stimulator of Iron Transport
tet	Tetracycline
Tf	Transferrin
TfR	Transferrin Receptor
TfR2	Transferrin Receptor 2
TRITC	Tetramethyl rhodamine isothiocyanate
tTA	Tetracycline Transactivatable
UTR	Un-Translated Region

INTRODUCTION

HH Results from HFE Deficiency

Hereditary hemochromatosis (HH) is the most common genetic disease of individuals of Northern European Caucasian descent (Barton and Bertoli, 1996). One in ten individuals carry the disease-associated allele (Feder et al., 1996). HH is an autosomal recessive disorder of iron overload caused by the inheritance of a founder mutation in the HFE gene which is located near the HLA region of chromosome 6p (Feder et al., 1996). The HFE protein product is a nonclassical major histocompatability complex (MHC) class I type molecule. MHC class I type molecules require beta-2 microglobulin binding for proper folding and cell surface expression. The predominant HH mutation converts HFE amino acid 282[1] from a cysteine to a tyrosine and prevents the formation of a critical disulfide bond (Figure 0.1). The C282Y mutation prevents the formation of the beta-2 microglobulin binding pocket (Feder et al., 1996; Lebron et al., 1998) and therefore significantly reduces the expression of functional HFE (Feder et al., 1997).

The H63D mutation is a second HH mutation that occurs frequently in individuals heterozygous for the C282Y mutation (Feder et al., 1996). This mutation converts a histidine that forms a salt bridge with the aspartic acid at position 95 to another aspartic acid (Lebron et al., 1998). Because the H63D mutation is expressed at the cell surface in transfected cells and its function is similar to the wild type HFE (Feder et al., 1997), its exact contribution to the disease state is not as clear as the C282Y mutation. However, this mutation could decrease the stability of the HFE protein which might significantly decrease the amount of functional HFE, especially in conjunction with the inheritance of the C282Y HFE allele.

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[1] The numbering system reported herein for the HFE protein is in accordance with that published by Feder and colleagues (Feder et al., 1996) which includes the 22 amino acids contained in the signal sequence.

Humans and mice deficient in functional HFE experience chronic iron absorption from the duodenum leading to progressive iron overload of several organs including the liver, pancreas, pituitary, and heart (Levy et al., 1999; Rothenberg and Voland, 1996; Santos et al., 1996; Zhou et al., 1998). While increased iron saturation in humans can be detected in the blood within the first two decades of life, the progression of the disease is relatively slow. Often, damage to organs such as the liver or pancreas does not occur until the fifth or sixth decade of life and is the result of the inability to balance the increased iron uptake with efflux, since no efficient mechanism for iron excretion exists (Bothwell and MacPhail, 1998). Because HH results from the lack of functional HFE protein, we have designed our experiments with the intent of characterizing the function of the wild type HFE protein. Because the increase in iron absorption is the result of increased iron transport across a specialized epithelial cell layer, we have tested the hypothesis that HFE regulates cellular iron homeostasis.

Cellular Iron Homeostasis

The most basic of biological functions (DNA synthesis, oxygen transport, and respiration) require iron. While life is nearly impossible without it, the amount of iron present in a biological system must be tightly regulated. If iron is not bound to a protein or another chelator, it can generate free radicals. In particular, reaction of ferrous iron with molecular oxygen or peroxides can lead to the generation of the hydroxyl radical which is highly reactive and unselective. Reaction of the hydroxyl radical with cellular components leads to the oxidation and cleavage of lipids, proteins, nucleic acids and even carbohydrates. Iron homeostasis maintains a balance that keeps iron stores replete and available for use, but prevents the saturation of protective machinery. Key to establishing homeostasis then, is the balance of pathways for iron uptake, storage, and efflux. We have tested for the ability of HFE to modulate some of these pathways. Therefore several

proteins that are shared in both cellular and organismal iron metabolic pathways are reviewed, briefly, below.

Transferrin-Mediated Iron Uptake

The primary iron source for most cells is derived from the serum protein, transferrin (Tf). Tf is an 80 kDa glycosylated protein that binds up to two ferric ions with a dissociation constant ~10^{-23} M (Aisen, 1998; Aisen et al., 1978; Leibman and Aisen, 1979; Lin et al., 1994). The Tf protein serves multiple purposes in the blood. First, Tf protects organs from the oxidative damage that freely circulating ferrous ion might cause by chelating blood iron stores. Additionally, Tf binds the insoluble ferric ion, keeping it in solution in the blood plasma and available to tissues. Finally, whereas Tf sequesters iron from most microorganisms, it plays an important part in the efficient delivery of iron to proliferating cells of the body, because it is recognized at the cell surface by the transferrin receptor (TfR) (Mason et al., 1997; Zak et al., 1994). The TfR is a glycosylated, single membrane spanning protein of approximately 94 kDa that dimerizes and is expressed at the cell surface [(Enns et al., 1996; Lok and Loh, 1998), and references therein]. At the slightly basic pH of 7.4, the TfR binds diferric Tf with a dissociation constant in the nanomolar range, but binds apo-Tf with a dissociation constant only in the micromolar range (Dautry-Varsat et al., 1983; Klausner et al., 1983; Tsunoo and Sussman, 1983). This difference in affinity provides for almost exclusive binding of the iron saturated Tf species to the TfR and allows for efficient delivery of iron to the cell.

The TfR cycles through the cell in a constitutive endocytic pathway (Ajioka and Kaplan, 1986) (Figure 0.2). After clustering of the receptor in clathrin-coated pits at the plasma membrane, the vesicle endocytoses, or pinches off, and traffics toward the microtubule organizing center (Geuze et al., 1983; Gruenberg et al., 1989; Schmid, 1992). The clathrin coat is lost and the intravesicular pH drops in an ATP-dependent manner (Yamashiro et al., 1983). Iron is transported into the cell in a two step manner. First, it

must be released from Tf. The change in vesicular pH causes conformational changes in both the TfR and Tf that greatly increases the dissociation constant of Tf for iron to 10^{-7} M, releasing it from Tf (Bali et al., 1991; Sipe and Murphy, 1991). The second step of the Tf-mediated iron uptake pathway occurs as the released ferric ion is converted to the ferrous ion and transported across the endosomal membrane (Harding and Stahl, 1983; Paterson et al., 1984) for use in cytoplasmic and mitochondrial iron-containing enzymes or for storage in ferritin (Ft), the primary cellular iron store. Apo-Tf remains bound to the TfR at low pH, but upon reaching the cell surface, the increased pH of the extracellular environment releases apo-Tf from the TfR. Diferric Tf replaces apo-Tf on the TfR and the cycle is repeated.

The kinetics of TfR internalization and recycling are closely regulated by the cell. A characteristic YTRF amino acid sequence in the cytoplasmic domain is known to be responsible for the efficient endocytosis of the receptor (Collawn et al., 1993; Jing et al., 1990; McGraw and Maxfield, 1990; McGraw et al., 1991). The TfR traffics to the recycling compartment in approximately 7 minutes and takes approximately 10 minutes to return to the cell surface. The combined kinetics of endocytosis and exocytosis lead to a distribution of the TfR with approximately 80% of the receptor in internal compartments and 20% on the cell surface [(Mukherjee et al., 1997) and references therein]. Changes in receptor cycling kinetics can be reflected as changes in this steady state distribution.

The observation that HFE stably associates with the TfR prompted the hypothesis that HFE might alter the binding affinity of the TfR for Tf or alter the cycling kinetics of the TfR through the cell. We investigated these possibilities in chapters 1, 3 and 4 and found that HFE had no physiologically relevant effect on the affinity of the TfR for Tf (chapters 1 and 4), nor did it significantly effect the cycling of the TfR (chapter 3). We have observed, however, that HFE reduces the efficiency of iron absorption from Tf (chapter 3), which returned our attention to the interaction between HFE and the TfR. We investigated several

potential mechanisms by which HFE might reduce the assimilation of iron from Tf, but did not conclusively identify such a mechanism (chapter 5).

A second TfR (TfR2) was recently described (Kawabata et al., 1999). It shares only 66% similarity with the extracellular domain of the TfR, but also binds diferric Tf. While TfR2 is involved in iron uptake from Tf, it may not have a completely overlapping function. TfR2 has a different internalization sequence than the YTRF of the TfR. The SQTVYQRV signal present in the TfR2 cytoplasmic domain, may target this protein to a different intracellular compartment for a different purpose. Unlike the TfR, TfR2 mRNA and protein are not likely to be iron regulated at the mRNA level because the TfR2 mRNA lacks stem loop structures that facilitate iron-dependent regulation (this relationship is discussed in more depth below). Additionally, TfR2 mRNA levels remain relatively constant under conditions of iron loading and iron deficiency (Fleming et al., 2000). To date, there is no known association of the TfR2 with HFE [West, Enns, and Bjorkman. (in press) JBC, 2000].

Non-transferrin Mediated Iron Uptake

While the Tf-mediated iron uptake pathway is very efficient, it is not the only means for iron uptake. At least two classes of iron transporters have been described. The first class is active at low pH. Divalent metal transporter 1 (DMT1, previously identified as Nramp2 and DCT1) is a putative 12 transmembrane domain ion transport protein. It contains a consensus cation transport motif and transports the ferrous ion and other divalent metals such as zinc, cadmium, manganese, copper, and cobalt (Fleming et al., 1997; Gunshin et al., 1997). DMT1 requires an acidic environment, because it is a symporter of a proton with the divalent metal (Gunshin et al., 1997). DMT1 is of utmost importance in the intestine. It is the primary transporter for iron uptake from the lumen of the duodenum (Bowen and Morgan, 1987; Canonne-Hergaux et al., 1999; Oates and Morgan, 1996; Trinder et al., 2000), but also overlaps with endosomal compartments where it may be

necessary for iron uptake from Tf in developing erythrocytes (Edwards et al., 1980; Garrick et al., 1999; Garrick et al., 1993; Gruenheid et al., 1999; Su et al., 1998). Regardless of its location at the intestinal apical membrane or in vesicles, DMT1 is likely to work in conjunction with an unidentified ferrireductase that converts the ferric ion to the divalent ferrous ion before it is transported (Inman et al., 1994; Jordan and Kaplan, 1994; Riedel et al., 1995).

A second class of iron transporters function at near neutral pH and may transport either ferric or ferrous ions. The stimulator of iron transport (SFT) was cloned from K562 cells, a human erythroid cell line, and has been shown to stimulate uptake of iron in a xenopus expression system at pH 7.4. SFT differs from DMT1 in that it modulates a pathway that is highly selective for iron, with cadmium being the only transition metal effective at inhibiting iron transport (Gutierrez et al., 1997). Additionally, it does not require a low pH environment to function. Other iron transporters that function at the slightly basic pH of the blood plasma are hypothesized to exist in parenchymal tissues such as the hepatocytes of the liver (Craven et al., 1987), and have been observed in cell culture (Kaplan et al., 1991; Sturrock et al., 1990) but have not yet been cloned.

We tested the hypothesis that HFE reduces iron uptake from Tf by reducing the efficiency of iron uptake through these transporters (chapter 5). However, we found no significant effect of HFE on non-transferrin mediated iron uptake.

Iron Storage

The primary protein for cellular iron storage is Ft. The Ft protein shell is a 430-460 kDa complex made up of a mixture of 24 Ft heavy (H) chain protein subunits (~21 kDa) and/or Ft light (L) chain protein (~19 kDa) subunits, that surrounds up to 4500 ferric ions in a semi-crystalline core (Mann et al., 1986). Ft has two basic roles within the cell. First, Ft provides a mobilizable iron source when cellular iron needs increase. Second, Ft provides protection for the cell against the oxidative capacity of the iron contained within it.

The ratio of H to L chains in the Ft shell changes depending upon specific cellular needs. The H chain bears oxidase activity important for the conversion of the ferrous ion to ferric, which is the oxidative state of iron in the Ft core (Lawson et al., 1989). Ft with more H chain takes up iron faster, suggesting it may be more important for protection against iron toxicity than efficient storage of iron (Levi et al., 1988). The L chain is directly involved with the formation of the iron core (Levi et al., 1992), suggesting Ft with more L chain is important for long term storage.

In addition to the flexibility of the ratios of H and L chains within the Ft complex, the amount of Ft H and L chains are regulated transcriptionally and translationally. Ft H chain is transcriptionally controlled, and is induced in response to *c-myc*, denervation, cytokines and heme (Ponka et al., 1998). Both Ft chains are sensitive to the iron needs of the cell. When intracellular iron levels increase, more Ft protein is translated. Alternatively, when intracellular iron levels drop, the amount of Ft protein decreases. This relationship is discussed in more depth below. Thus, the amount and composition of Ft species is regulated at two levels. First, by the number of Ft messages available for translation. Second, by the translation efficiency of the message.

We observed a reduction in Ft levels in cells in culture upon expression of HFE (chapters 1 and 2). We tested the hypothesis that this reduction in steady state Ft was the result of an HFE-dependent decrease in intracellular iron (chapters 3 and 6). The observation that HFE reduces iron uptake from Tf (chapter 3) and regulates the activity of proteins that respond to changes in intracellular iron status (chapter 6) support this hypothesis.

Iron Efflux

Efflux pathways for iron have not been easily identified in cultured cells, probably due to the high proliferative capacity of cultured cells and their great need for iron. However, two types of transporters involved in the efflux of iron from cells have been

described. Ferroportin1/Ireg1/MTP1 is the best-described protein of iron export (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). It is hypothesized to be a multiple membrane spanning protein and it is found in two specific cell types that are responsible for iron transport: the intestinal enterocyte that transports iron into the blood, and the macrophage that recycles iron from senescent red blood cells, delivering the remaining iron to hepatocytes.

Another iron export protein has been identified in spleen microsomal membranes enriched for heme oxygenase activity. In the spleen, macrophages use heme oxygenase in concert with cytochrome P450 reductase and biliverdin reductase to break down the heme porphyrin ring and to recycle the iron contained within it (Yoshinaga et al., 1982; Yoshinaga et al., 1982; Yoshinaga et al., 1982). An Fe-ATPase has been described that is hypothesized to transport iron recycled by the heme oxygenase complex into the lumen of the macrophage endoplasmic reticulum (Baranano et al., 2000). The observed iron transport activity is time, temperature, and magnesium dependent, but not much more is known concerning its exact physiological role or specificity.

Iron regulatory Proteins

Cellular iron homeostasis requires the concerted regulation of uptake, efflux and utilization pathways. This is largely achieved through the action of iron regulatory proteins (IRPs) [for review, see (Eisenstein and Blemings, 1998; Hentze and Kuhn, 1996)]. IRP1 acts either as an iron regulatory protein or the metabolic enzyme, cytoplasmic aconitase. These two mutually exclusive activities depend upon an elusive cytoplasmic pool of iron referred to as "the labile iron pool". When the labile iron pool is high, the protein is found in the cytoplasmic aconitase form with a [4Fe-4S] cluster in the active site. When the labile iron pool decreases, the protein loses the [4Fe-4S] cluster and is capable of binding conserved stem loop structures in target mRNAs (Haile et al., 1992; Rouault et al., 1991; Tang et al., 1992). These iron responsive elements (IREs) are found in either the 3' or 5'

untranslated region (UTR) of mRNAs that code for proteins involved in iron uptake, storage and metabolism (Aziz and Munro, 1987; Casey et al., 1988; Casey et al., 1988; Leibold and Munro, 1988). Unlike IRP1, IRP2 does not function as cytoplasmic aconitase. But IRP2 RNA binding is similarly regulated by intracellular iron levels. When the labile iron pool is high, IRP2 is degraded and is no longer available for RNA binding (Guo et al., 1995; Guo et al., 1994; Iwai et al., 1998; Iwai et al., 1995). Two well characterized proteins whose expression is regulated by IRPs are the TfR and Ft (Figure 0.3).

The TfR message contains a rapid turnover determinant in the 3' UTR which is highly susceptible to nuclease activity (Casey et al., 1989; Mullner and Kuhn, 1988). However, under low intracellular iron conditions, IRPs bind IREs near the instability element in the 3' UTR of the TfR message and preclude nuclease access to the mRNA (Koeller et al., 1989). With IRPs bound, the TfR message is stabilized approximately 10 fold (Casey et al., 1989; Mullner and Kuhn, 1988), leading to an increase in steady state TfR levels. With more receptors present on the cell surface there is an increase in Tf uptake that results in increased iron uptake. TfR is regulated in this manner, but not TfR2 because the message does not contain IREs, nor does it appear to be iron regulated (Fleming et al., 2000).

The Ft H and L chain messages contain an IRE in the 5' UTR (Casey et al., 1988; Leibold and Munro, 1988). Under low iron conditions IRPs bind this IRE and preclude access of the small ribosomal subunit to the translation initiation factor eIF4F (Muckenthaler et al., 1998). As a result, Ft protein synthesis decreases as do steady state Ft levels. When the labile iron pool increases, IRPs cannot bind the Ft IRE. This results in increased translation of the Ft protein and increased storage of iron.

Our observation that HFE inversely regulates Ft and TfR expression in cells in culture (chapters 1 and 2) suggested that HFE reduces the labile iron pool that regulates the IRE RNA binding activity of IRPs. We tested the hypothesis that HFE increases the IRE

RNA binding activity of IRP1 and IRP2 and found that HFE-expression increased the RNA binding activity of both IRP isoforms (chapter 6).

Recent findings suggest DMT1 activity may also be IRP regulated. DMT1 has two mRNA splice products that code for DMT1 isoforms with different carboxy termini (Canonne-Hergaux et al., 1999; Lee et al., 1998). The mRNA encoding one of these has a single stem loop structure in the 3' untranslated region of the mRNA, which is reminiscent of IRP-dependent regulation like that of the TfR. While DMT1 mRNA binds IRPs and is negatively regulated by iron (Han et al., 1999; Lee et al., 1998; Tandy et al., 2000; Trinder et al., 2000; Wardrop and Richardson, 1999), the exact mode of regulation (transcriptional or message stability) has not been rigorously evaluated. In addition, evidence for iron dependent post-translational regulation, possibly through trafficking or compartmentalization of the transporter was reported recently (Trinder et al., 2000). While iron dependent transcriptional and post-translational regulatory mechanisms may be independent of IRPs, they still require regulation through the labile iron pool, reflecting its importance in the maintenance of iron homeostasis.

By increasing iron storage and decreasing iron uptake when intracellular iron levels increase, IRPs protect the cell from iron overload and oxidative damage. Alternatively, by increasing iron uptake and decreasing iron storage, IRPs facilitate rapid mobilization of iron when intracellular levels drop. Though the target concentration may vary among cell types (Kovar et al., 1997), this exquisite system maintains intracellular iron at a fixed concentration or "set point" that remains in equilibrium with cellular iron stores.

Organismal Iron Homeostasis and HH

Iron uptake, storage, and efflux processes all occur within an organism, but iron homeostasis for the entire organism is achieved by regulating dietary iron uptake. A single layer of specialized intestinal cells, called enterocytes, transport iron across the epithelial

barrier (Daniele and D'Agostino, 1994; Hocker and Weidenmann, 1998). The cells that make up this layer are polarized such that the apical membrane faces the intestinal lumen and is specialized for transport of heme and other iron chelates into the cell. The remainder of the enterocyte plasma membrane is separated from the apical membrane by tight junctions. Iron is also transported across this basolateral membrane, from the enterocyte to the blood plasma for utilization by the organism.

The basolateral membrane of the intestinal epithelium is in indirect contact with the blood plasma, receiving signals concerning iron adequacy from humoral regulators. In the face of depleted iron stores, an unknown regulator modifies the iron transport machinery of the enterocyte and increases the transfer of dietary iron from the enterocyte to the blood (Cox and Peters, 1980; Gavin et al., 1994; Hunt and Roughead, 2000; Nathanson et al., 1985). This pathway is hypothesized to be affected in individuals with HH because a chronic increase in the transfer of iron from the enterocyte to the blood is observed in individuals with HH. McLaren and colleagues observed that iron transport occurs at a higher rate in HH patients than in controls (McLaren et al., 1991). Over time, this constant increase in the transfer of iron to the blood leads to iron overload.

With the identification of the gene mutated in HH (Feder et al., 1996) came the localization of the protein product, HFE, to multiple organs. However, HFE's localization to the specific cells of the duodenal crypt that give rise to enterocytes indicated a role for HFE in iron transport (Parkkila et al., 1997). Because HFE has no recognizable iron transport properties, it is expected to be a part of the mechanism that regulates the rate of iron transport across the mature enterocyte. The multi-organ pathology of HH is likely to result from the misregulation of iron homeostasis and transport across this single cell type (McLaren et al., 1991). We have tested HFE-specific regulation of cellular iron uptake and homeostasis in a nonpolar cell system.

We have expressed the wild type HFE protein under the control of the tetracycline (tet) responsive promoter (tet-off) in HeLa cells (Figure 0.4). The tet responsive system

allows us to compare the regulation of iron metabolic proteins in cells that express HFE against the same clonal cell line that does not express HFE. It has given us the opportunity to compare small differences in protein expression and iron metabolism in the presence or absence of HFE without the complication of clonal variation. Utilizing this system, we have been able to distinguish between the receptor-mediated and transporter-mediated iron uptake pathways. We have been able to "map" the specificity of HFE's effect on cellular iron homeostasis to the TfR mediated pathway of iron uptake, and to test the mechanism by which this pathway is regulated by HFE. This simple system has provided significant insight into HFE's regulation of iron homeostasis. Future experiments must test the hypotheses we develop herein in a more complex polarized cell system, as well as in an animal model.

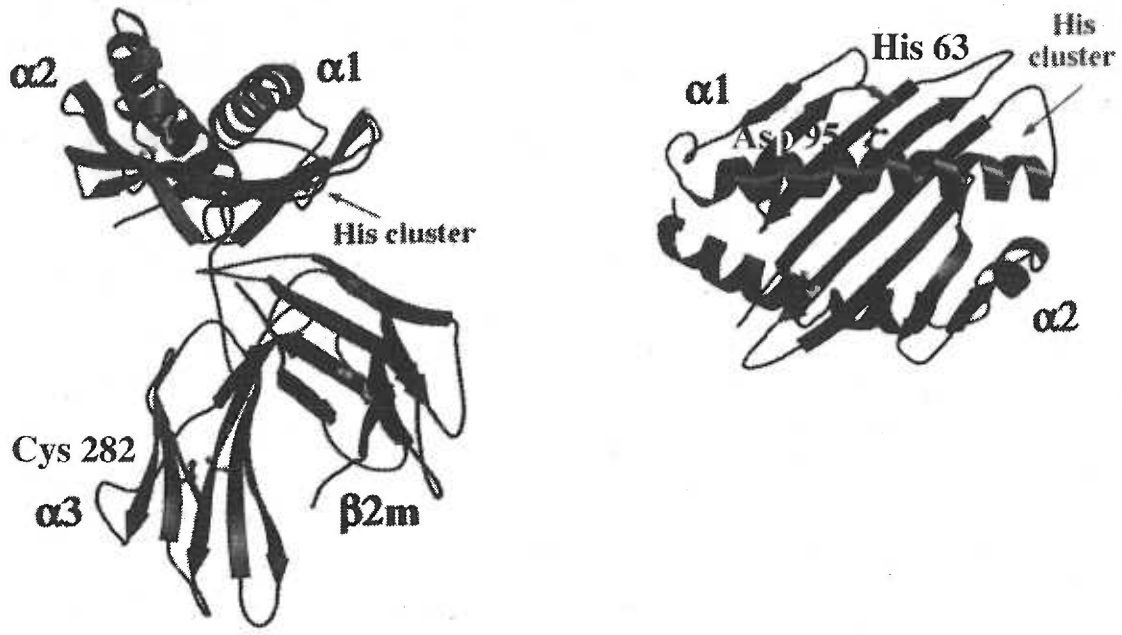

Figure 0.1. HFE crystal structure from Lebron, Bennett et al. (1998) Cell 93 (1). The crystal structure of HFE confirms the hypothesized structural instabilities resulting from the amino acid conversions in the HH disease alleles. The C282Y mutation (left) disrupts formation of the disulfide bond that anchors the beta-2 microglobulin binding pocket. Without beta-2 microglobulin, HFE cannot fold properly, nor is it efficiently expressed at the cell surface. The H63D mutation (right) prevents the formation of a salt bridge with aspartic acid 95. While this mutation does not prevent folding and cell surface expression of HFE, it is likely to reduce the stability of HFE.

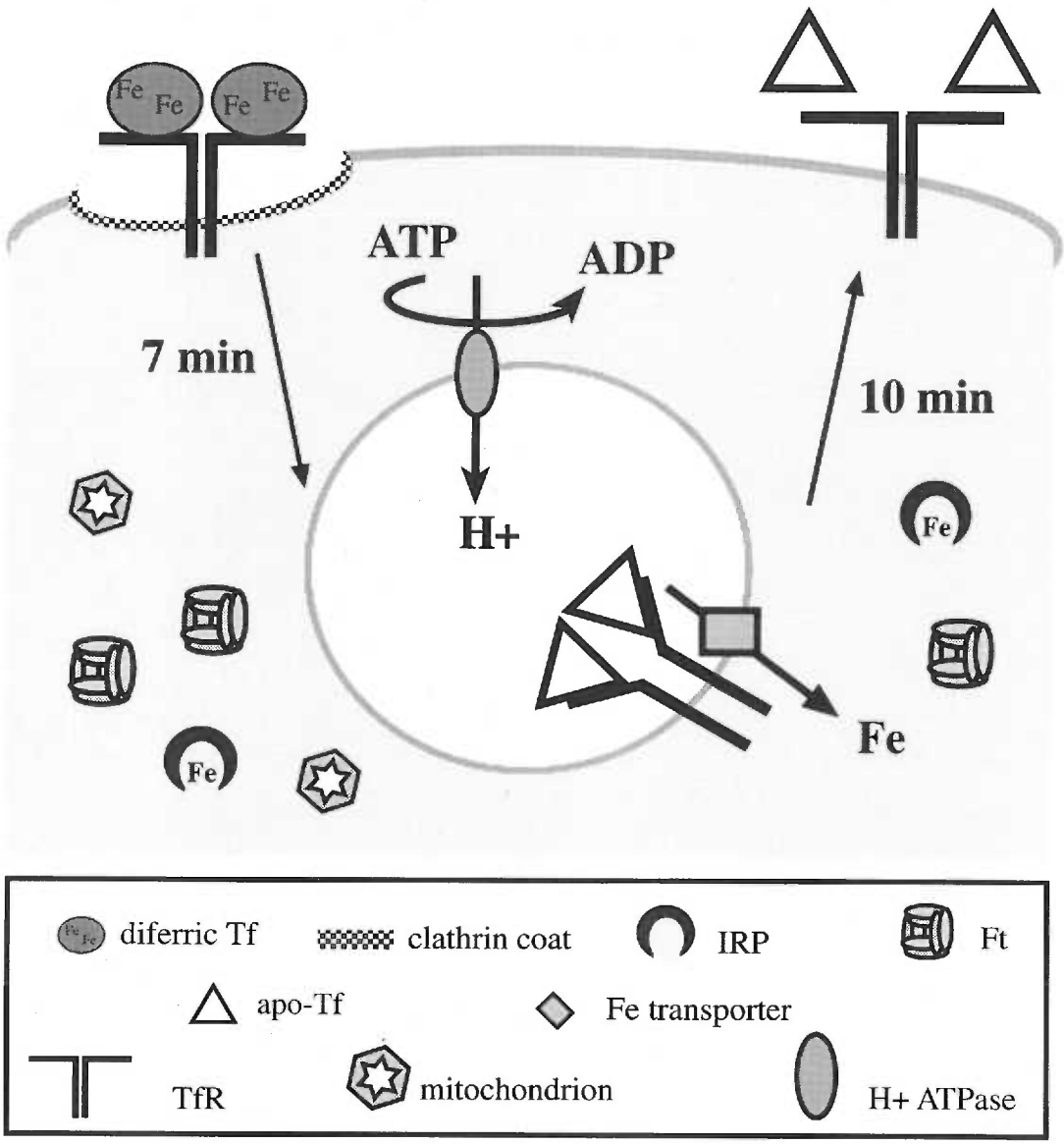

Figure 0.2. Clathrin mediated constitutive internalization and recycling of the TfR. The TfR internalizes in clathrin coated pits. After the vesicle pinches off the plasma membrane, the clathrin coat is lost and an ATP-dependent proton pump acidifies the vesicle. The change in pH induces conformational changes in Tf and the TfR that potentiate iron loss from Tf. Iron is transported across the endosomal membrane to be stored in Ft, incorporated into mitochondrial proteins, IRPs, and other iron-containing enzymes. Apo-Tf remains bound to the TfR at low pH. The vesicle cycles back to the cell surface where apo-Tf is replaced by diferric Tf at the cell surface.

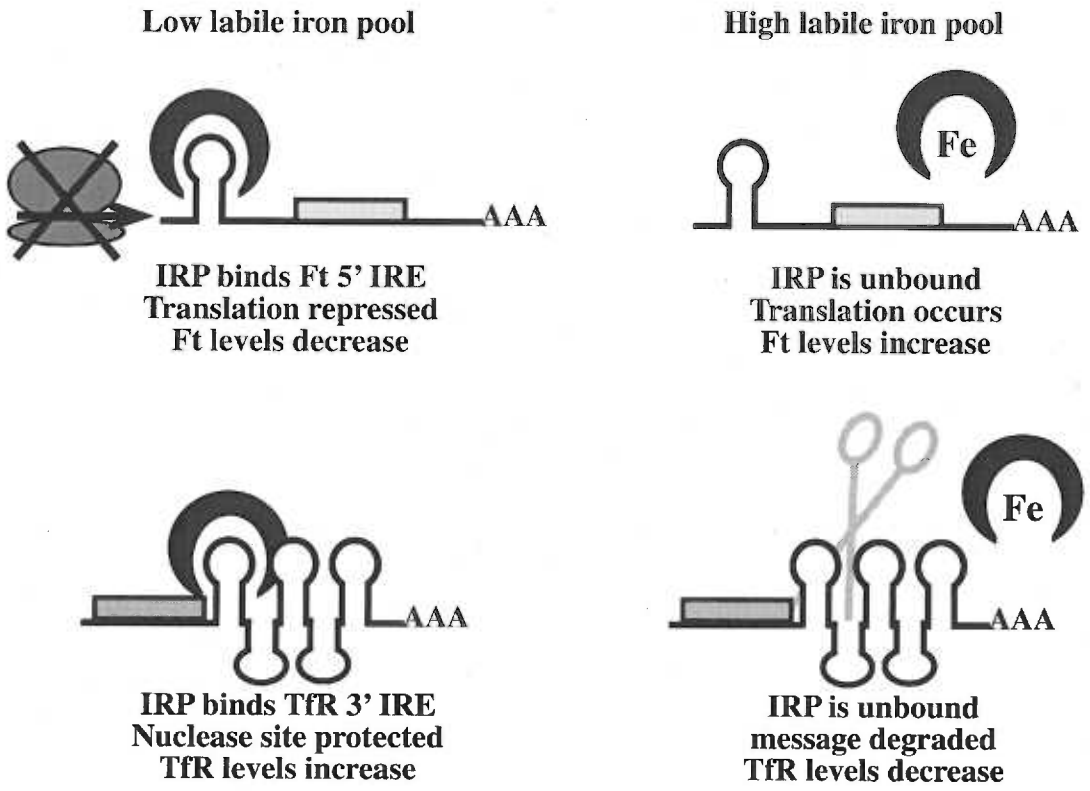

Figure 0.3. The regulation of iron uptake and storage by the labile iron pool. When the labile iron pool is low, IRP1 is not fully saturated with iron and IRP2 is stable. Both IRPs bind to IREs. Binding of IRPs to the IRE of the Ft message (top left) blocks translation of the mRNA. Binding of IRPs to the TfR message (bottom left) stabilizes the mRNA. When the labile iron pool is high, IRP1 binds iron, eliminating IRE binding activity. IRP2 is rapidly degraded by the proteosome. When IRP comes off the Ft mRNA, more Ft is translated (top right). The TfR mRNA is less stable without IRPs bound, decreasing the amount of TfR synthesized (bottom right).

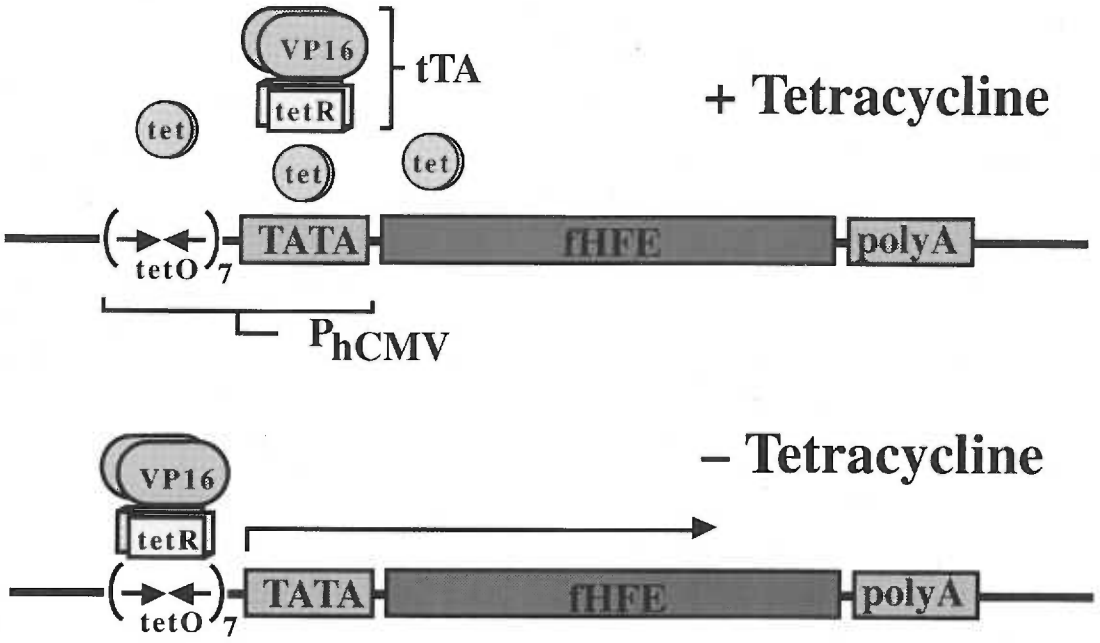

Figure 0.4. Expression of fHFE in fWTHFE/tTA HeLa cells. Flag epitope-tagged wild type HFE (fHFE) is under the control of the tetracycline (tet) responsive promoter in our fWTHFE/tTA HeLa cell line. In the presence of tet, the VP16 fusion protein binds tet which prevents association with the promoter. HFE is not expressed. When tet is withdrawn from the medium, the VP16 fusion protein binds the promoter and facilitates transcription of the HFE message.

CHAPTER 1

Co-Trafficking of HFE, a nonclassical MHC class I type Molecule, with The Transferrin Receptor Implies Role in Intracellular Iron Regulation

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SUMMARY

The mechanism by which a novel MHC class I protein, HFE, regulates iron uptake into the body is not known. HFE is the product of the gene which is mutated in over 80% of hereditary hemochromatosis patients. It was recently found to co-precipitate with the transferrin receptor and to decrease the affinity of the transferrin receptor for transferrin. In this study, HeLa cells were transfected with HFE under the control of the tetracycline repressible promoter. We demonstrate that HFE and the transferrin receptor are capable of associating with each other within 30 minutes of their synthesis with pulse-chase experiments. HFE and transferrin receptor co-immunoprecipitate throughout the biosynthetic pathway. Excess HFE is rapidly degraded whereas the HFE-transferrin receptor complex is stable. Immunofluorescence experiments indicate that they also endocytose into transferrin positive compartments. Combined, these results suggest a role for transferrin receptor in HFE trafficking. Cells expressing HFE have modestly increased levels of transferrin receptor and drastically reduced levels of ferritin. These results implicate HFE further in the modulation of iron levels in the cell.

INTRODUCTION

HH is an autosomal recessive disease more common in people of Northern European descent than cystic fibrosis, phenylketonuria and muscular dystrophy combined (Barton and Bertoli, 1996). It is characterized by massive iron overload of the parenchymal tissue of many organs including the liver, pancreas, heart, and pituitary. Siderosis and cirrhosis of these organs causes liver failure, diabetes mellitus, cardiac arrhythmia, hypogonadism, and other complications leading to premature death (Bonkovsky, 1996; Bothwell et al., 1995; Powell et al., 1970). Over 80% of HH patients have the same mutation in the HFE gene (Feder et al., 1996).

Positional cloning of HFE from the HLA-linked region of chromosome 6p and its subsequent crystallization (Lebron et al., 1998) has revealed its significant sequence and structural homology (37% identity in the ectodomain) to MHC class I proteins. MHC class I molecules are normally involved in antigen peptide presentation. HFE has not been found to bind such peptides. Its crystal structure suggests the ancestral peptide binding groove is too narrow for such a function (Lebron et al., 1998). Emerging results suggest HFE's functional role may lie in its interaction with the TfR (Feder et al., 1998; Lebron et al., 1998; Parkkila et al., 1997) HFE is therefore described as a nonclassical MHC class I molecule. MHC class I molecules are capable of functions outside of antigen presentation. Some have been shown to interact with receptors such as the insulin receptor and the epidermal growth factor receptor (Due et al., 1986; Fehlmann et al., 1985; Phillips et al., 1986; Schreiber et al., 1984; Verland et al., 1989) Antibodies against these MHC class I proteins decrease surface binding of insulin or epidermal growth factor to their respective receptors suggesting these MHC class I proteins are involved in the formation of high affinity ligand binding sites. The neonatal Fc receptor (FcRn) is a nonclassical MHC class I molecule that acts as an antibody transporter. The current model of FcRn function proposes binding of ligand at acidic pH and transport in acidic vesicles through the

epithelial monolayer. Further evidence suggests the involvement of FcRn in IgG homeostasis in the blood plasma [see review (Ghetie and Ward, 1997) and references therein]. Likewise, HFE may be involved in regulated transport of molecules associated with iron homeostasis.

The molecular evidence concerning HFE's mediation of iron homeostasis has been converging on its close association with the TfR. Northern blot analysis indicated HFE is widely distributed throughout the body with more abundant message levels in organs which are major sites of iron metabolism, namely the liver and intestine (Feder et al., 1996). HFE's unique pattern of protein expression in the gastrointestinal tract [specifically in the crypt cells of the ileum, the putative site of iron absorption in the gut (Parkkila et al., 1997)] suggests a role for HFE in the sensing of bodily iron levels and the regulation of transport of iron across epithelial layers. HFE expression in the basolateral membrane of the gut epithelia and the identification of HFE in the placenta (Parkkila et al., 1997) are indicative of a more general association with the TfR in regulating iron transport across epithelial barriers. It has long been suspected that individuals with HH have a defect in the transport of iron across the mucosal barrier: that is, transport into the blood rather than uptake from the lumen (Powell et al., 1970). The perinuclear staining of HFE in the proliferating crypt cells is indicative of co-localization with recycling receptors, such as the TfR (Anderson et al., 1990) rather than with the hypothesized iron transporter on the apical surface of these cells. HFE's regulatory influence on iron transport therefore, may be either in internal cellular compartments or at the basolateral cell surface.

More specific evidence for HFE's interaction with the TfR is demonstrated by the co-immunoprecipitation of HFE with the TfR (Feder et al., 1998; Parkkila et al., 1997) and more significantly demonstrated by HFE's ability to increase the dissociation constant of Tf with the TfR (Feder et al., 1998; Lebron et al., 1998). The association of HFE with the TfR and its yet to be defined role in iron regulation led us to investigate more closely its trafficking in the cell. For this study, we have expressed a FLAG epitope-tagged HFE

(fHFE) under the control of the tetracycline repressible promoter. This system allows us to tightly regulate the expression level of fHFE within the same stable cell line. The results of the present study demonstrate that cells expressing fHFE have decreased Ft levels and increased TfR number, implying low intracellular iron levels. We also demonstrate that fHFE is able to associate with the TfR within 30 minutes of its synthesis and can associate with the TfR in both the biosynthetic and metabolic pathways. fHFE pools which are not associated with the TfR are rapidly degraded. Immunofluorescent detection of fHFE in HeLa cells indicates it co-localizes with the TfR and Tf on the cell surface and in intracellular compartments. Localization of fHFE on the cell surface and in endocytic compartments implicates HFE in the regulation of iron uptake, especially in light of indirect evidence that suggests cells expressing HFE have decreased intracellular iron stores.

MATERIALS AND METHODS

Plasmids- The plasmid pcDNA3.1+ containing the HFE cDNA clone 24 fused to the octapeptide FLAG sequence (Progenitor, Inc.) has been previously described (Feder et al., 1997). The tetracycline repressible plasmid, pUHD10-3, was a kind gift from Drs. M. Gossen and H. Bujard (Zentrum fur Molekulare Biologies, Universitat Heidelberg) (Gossen and Bujard, 1992).

Subcloning - The HFE coding sequence is contained within a, ~3.0 kb NheI/XbaI fragment, which was subsequently cloned into the XbaI site of pUHD10-3, resulting in the fWTHFE/pUHD10-3 construct.

Cell Culture - HeLa cells transfected with the tetracycline transactivatable plasmid, pUHD 15-1, were a gift from Dr. Sandra L. Schmid (Scripps Research Institute). They were co-transfected with the fWTHFE/pUHD10-3 plasmid and the pBSpac plasmid containing the puromycin resistance gene (Verland et al., 1989) using lipofectAMINE (Life Technologies) according to the manufacturer's directions. Colonies selected with puromycin (200 ng/ml) and recloned to ensure a pure cell line were screened by SDS-PAGE and Western blot for expression of fHFE using the 1868 rabbit anti-FLAG [previously described, (Warren et al., 1997)] and CT1 rabbit anti-HFE, [previously described, (Feder et al., 1997)] antibodies. The clone used for these experiments is named fWTHFE/tTA HeLa. The resulting fWTHFE/tTA HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (tet+) or without (tet-) 2 μg/ml tetracycline.

Iodination- Human diferric Tf was labeled with Na [^{125}I] using lactoperoxidase and used for Scatchard analysis as previously described (Warren et al., 1997).

Immunoprecipitation - fWTHFE/tTA HeLa cells were washed three times with 2 ml PBS (phosphate-buffered saline) pH 7.4 and lysed with NET-Triton (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4 with 1% Triton X-100). Cell lysates were preadsorbed at least 45 minutes at 4°C with 50 µl Pansorbin (Calbiochem) for ~10^6 cells to reduce precipitation of nonspecific protein. Preadsorbed lysates were incubated at least 45 minutes at 4°C with 30 µl rabbit anti-mouse (Miles Scientific) coated Pansorbin and 2 µg of either mouse anti-FLAG (M2, Kodak) or mouse anti-hTfR antibody (4091 and 4093, gifts from Vonnie Landt, University of Washington, St. Louis) or with 50 µl Pansorbin and 3 µl sheep anti-hTfR serum for ~10^6 cells. The Pansorbin pellet was resuspended in 200 µl NET-Triton and washed through 1 ml RIPA (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, 150 mM NaCl, 0.2% sodium azide) + 15% sucrose. Samples were eluted in 100 µl 2X Laemmli buffer (Laemmli, 1970) with 10% 2-mercaptoethanol and subjected to SDS-PAGE analysis on a 10% acrylamide denaturing gel.

Pulse-Chase Experiments - Uninduced (tet+) and induced (tet-) subconfluent fWTHFE/tTA HeLa cells in 35 mm dishes were washed three times with sterile PBS, pH 7.4 and preincubated 15 minutes at 37°C in RPMI minus methionine (RPMI -met) medium (GIBCO-BRL) prior to labeling. Cells were pulsed for ten minutes at various time points with 100 µCi or incubated overnight with 10 µCi of [^{35}S] methionine in RPMI -met medium with 10% fetal bovine serum. The cells were then washed three times with PBS, and chased at 30 minute intervals between 0 and 120 minutes with complete medium. At the indicated times, cells were lysed 5 minutes on ice in NET-Triton. Cell extracts were subjected to immunoprecipitation with M2 or 4093 as described above and analyzed by

SDS-PAGE on a 10% acrylamide denaturing gel under reducing conditions. Gels were fixed, treated with Amplify (Amersham) 30 minutes, dried and subjected to PhosphorImager analysis (Molecular Dynamics).

Endo β-N-Acetylglucosaminidase H treatment - Immunoprecipitated pellets were resuspended in 90 µl denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) and boiled for 10 minutes at 95°C. The samples were iced and 10 µl of 10X citrate buffer (500 mM sodium citrate, pH 5.5) was added to the pellet. Samples were incubated at least 2 hours at 37°C in the presence or absence of Endo β-N-acetylglucosaminidase H (endo H, 8U, New England Biolabs). Samples were eluted with 50 µl 2X Laemmli buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE analysis on a 10% acrylamide denaturing gel. Gels were fixed, treated with Amplify, dried and subjected to PhosphorImager analysis.

Half Life experiments - Subconfluent fWTHFE/tTA HeLa cells (tet+ and tet-) in 35 mm dishes were washed three times with sterile PBS, pH 7.4 and preincubated 15 minutes at 37°C in RPMI -met medium prior to labeling. Cells were pulsed for one hour at various time points with 100 µCi of [^{35}S] methionine in RPMI -met medium with 10% fetal bovine serum. The cells were washed three times with PBS, and chased in 2 hour increments from 0 to 8 hours with complete medium. At the completion of the chase time point, all cells were lysed 5 minutes on ice in NET-Triton. Cell extracts were subjected to immunoprecipitation with the M2 antibody to immunoprecipitate fHFE or with the 4091 antibody to immunoprecipitate the TfR as described above, and analyzed by SDS-PAGE on a 10% acrylamide denaturing gel under reducing conditions. Gels were fixed, treated with Amplify, dried and subjected to PhosphorImager analysis.

Western Immunodetection- Cell extracts from ~$3x10^5$ cells were diluted with 4X Laemmli buffer or immunoprecipitates were eluted with 2X Laemmli buffer (Laemmli, 1970) and subjected to electrophoresis on 10 or 12% polyacrylamide-SDS gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis was performed using sheep anti-hTfR serum [described previously (Warren et al., 1997) at 1:10,000 dilution], mouse anti-flag antibody, M2, (Kodak, 1:20,00 dilution) and/or sheep anti-hFt antibody (the Binding Site, 1:100 dilution) followed by the appropriate secondary antibody conjugated to horse-radish peroxidase and chemiluminescence (SuperSignal, Pierce) per manufacturer's directions.

Tunicamycin Treatments - Subconfluent fWTHFE/tTA HeLa cells (tet+ and tet-) in 35 mm dishes were washed three times with sterile PBS, pH 7.4 and preincubated one hour at 37°C with 5 µg/ml tunicamycin (Calbiochem) or mock treated with dimethylsulfoxide (DMSO, final concentration 0.05%). Cells were washed three times with sterile PBS, pH 7.4 and incubated overnight at 37°C with complete medium. The next day, cells were lysed for 5 minutes on ice in NET-Triton and subjected to immunoprecipitation with M2 or sheep anti-hTfR serum as described above. Immunoprecipitates were eluted with 100 µl 2X Laemmli buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE on a 10% acrylamide denaturing gel. Gels were subjected to Western blot with monoclonal anti-hTfR antibody [H68.4, Zymed, as described in (Warren et al., 1997)].

Tf-agarose precipitation - Lysates of 10^6 tet+ or tet- fWTHFE/tTA HeLa cells were preadsorbed for 1 hour at 4°C with 100 µl bovine serum albumin (BSA) covalently linked to agarose (50% suspension in PBS). Supernatants were incubated for 1 hour at 4°C with 200 µl Tf covalently linked to agarose (50% suspension in PBS). The pellet was washed two times with NET-Triton and eluted twice with 75 µl 2X Laemmli buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE on a 12% acrylamide denaturing gel as

described above. Gels were transferred to nitrocellulose and subjected to Western blot as described above.

PhosphorImager Quantitation - IP Lab Gel 1.5 (Molecular Dynamics) was used to quantitate images by determining the volume within a region of fixed pixel number at each band of interest within the gel. Bands were normalized for methionine numbers within the protein (10 for fHFE and 14 for the TfR monomer).

Immunocytochemistry - Subconfluent fWTHFE/tTA HeLa cells (tet+ and tet-) grown on coverslips were washed three times with 2 ml PBS pH 7.4 and, if applicable, incubated 30 minutes at 37°C with 4 μg/ml Texas-Red labeled Tf (Molecular Probes) in DMEM with 20 mM HEPES, pH 7.4 and 2 mg/ml ovalbumin. All cells were washed three times with 2 ml PBS and fixed 15 minutes in 3% paraformaldehyde at room temperature and washed an additional two times in PBS. Cells were blocked in 10% newborn calf serum in PBS with 0.5% Triton X-100 to permeabilize cells for at least 30 minutes at room temperature. The fixed cells were incubated for at least one hour at room temperature in 1:50 diluted sheep anti-hTfR, 100 μg/ml 4093 anti-TfR antibody, 25 μg/ml M2 anti-FLAG antibody, 30 μg/ml CT1 anti-HFE antibody, or 2 μg/ml anti-β_2-microglobulin antibody (Immunotech). Coverslips were then washed twice in PBS and incubated again for at least one hour at room temperature with 1:50 dilution of fluorescein isothiocyanate (FITC)-labeled swine anti-goat antibody (TAGO Immunologicals), FITC-labeled goat anti-mouse antibody (TAGO Immunologicals), or tetramethyl rhodamine isothiocyanate (TRITC) labeled anti-rabbit antibody (Cappel), respectively.

RESULTS

Identification of a Clone that Expresses Epitope-Tagged HFE under the Tetracycline Regulated Promoter- The tetracycline-responsive promoter system developed by Gossen and Bujard (Gossen and Bujard, 1992) was used to create a cell line in which fHFE expression could be tightly controlled. tTA HeLa cells stably express a tetracycline transactivatable (tTA) fusion protein with the *Escherichia coli* tetracycline-responsive element (tetR) binding domain and the VP16 activation domain from herpes simplex virus. In the absence of tetracycline, the fusion protein binds operator sequences upstream of the fWTHFE/pUHD 10-3 multiple cloning region, promoting expression of FLAG epitope-tagged HFE (fHFE). In the presence of tetracycline supplemented (tet+) medium, the tTA fusion protein binds tetracycline and releases from the tetracycline-responsive promoter, preventing fHFE transcription. Figure 1.1 shows fHFE, TfR, and Ft expression in fWTHFE/tTA HeLa cells that have been uninduced (tet +) or induced (tet-) at least 10 days. Western blot on lysates of $3x10^5$ cells detects no fHFE (~43 kDa) in tet+ cells with M2 anti-FLAG antibody. fHFE is easily detected in lysates of tet- cells. TfR (97 kDa) levels increase slightly (~50%) in cells expressing fHFE. A large decrease (over 10 fold) in the levels of Ft (19 and 21 kDa) is seen in cells expressing fHFE. These results imply that over-expression of HFE reduces intracellular iron load. The IRP modulates changes in TfR and Ft levels. At low intracellular iron concentrations the IRP binds to the IRE stem loop structure in the 3' untranslated portion of the TfR mRNA stabilizing the message and thus increasing TfR numbers. The same reduction in intracellular iron load results in the binding of the IRP to the 5' untranslated stem loop of Ft mRNA blocking translation and thus decreasing Ft levels.

fHFE lowers affinity of TfR for Tf in fWTHFE/tTA HeLa cells- Scatchard analysis of [^{125}I]-Tf surface binding to tet+ or tet- fWTHFE/tTA HeLa cells at 4°C reflects the ability of

HFE to modulate Tf binding to the TfR (Figure 1.2). The Kd the TfR for Tf increases from 1.2 to 11 nM Tf with expression of fHFE, indicating that the affinity of Tf for the TfR decreases when fHFE is over expressed. The linearity of the slope rather than a biphasic slope of the Scatchard plot shows that fHFE sufficiently saturates the TfR such that no high affinity binding sites are evident. This data also shows fHFE expression moderately increases the amount of Tf binding sites (52%) in keeping with the increased expression of TfR on Westerns (Figure 1.1).

Early association of fHFE and TfR- fHFE associates with the TfR at the cell surface in a manner that lowers the affinity of the TfR for Tf [(Feder et al., 1998) and this study]. To investigate where this association occurs in the biosynthetic pathway, we tracked the synthesis and association of fHFE and the TfR via endo H and pulse-chase analysis in fWTHFE/tTA HeLa cells (Figure 1.3). Endo H is an endoglycosidase that cleaves between the proximal GlcNAc residues of high mannose and hybrid, but not complex N-linked oligosaccharides. Loss of endo H sensitivity indicates that the protein has exited the endoplasmic reticulum (ER) and the cis-Golgi region of the biosynthetic pathway in pulse-chase experiments. The peptide sequence of HFE has three consensus sites for glycosylation at amino acids 110, 130, and 234. Immunoprecipitation of fHFE shortly after synthesis indicates little association between fHFE and the TfR (Figure 1.3, 0 min. chase). The TfR is capable of co-precipitating with fHFE within 30 minutes of chase when a significant portion of the TfR is completely sensitive to endo H [see 30 min. chase, lower bands of doublet at ~87 kDa (endo H-) and ~80 kDa (endo H+)]. Thus fHFE can associate with the TfR in the ER / cis-Golgi compartments. Between 30 minutes and 120 minutes of chase, the amount of TfR which co-precipitates with fHFE does not change indicating that HFE does not gain any more ability to bind the TfR over this period of time. In addition, within the first 120 minutes of chase most of the fHFE is rapidly degraded in that the levels of fHFE decrease 2.5 fold. PhosphorImager analysis of the amount of TfR which

immunoprecipitates with fHFE (taking into consideration the relative abundance of Met in each protein) indicates that after an overnight label the ratio of TfR to fHFE is 1.7 :1.

HFE associated with TfR is more stable- The relative stabilities of fHFE and TfR were compared over eight hours (Figure 1.4 panels A and B) to further investigate the turnover of the free and complexed fHFE. Cells were fully induced to express fHFE by withdrawal of tet from the medium for at least 3 days. Quantitation of the absolute amount of fHFE synthesized indicates that initially four times more fHFE is synthesized than TfR (Figure 1.4 panels A and C, 0 chase after 1 hr label). fHFE is rapidly degraded in the first four hours of pulse-chase (Figure 1.4 panels A and B). Degradation continues at a slower rate over the next four hours while the amount of TfR that co-immunoprecipitates with fHFE is unchanged (Figure 1.4 panel B). The ^{35}S quantitation shown here is specific to the experiment shown and representative of several experiments.

TfR immunoprecipitation shows constant levels of TfR for eight hours (Figure 1.4 panel C). Previous studies indicated the half life of the TfR in the absence of HFE is approximately 24 hours (Rutledge et al., 1991; Rutledge et al., 1994). Surprisingly, fHFE levels co-immunoprecipitating with the TfR are virtually unchanged over this time course (Figure 1.4 panel D). Quantitation of the relative amounts of fHFE and TfR demonstrate that one fHFE binds per TfR dimer (Figure 1.4 panel D), consistent with earlier reports (Lebron et al., 1998). These experiments indicate that when more fHFE is synthesized than TfR, fHFE is rapidly degraded whereas the complex between the TfR and fHFE is stable. Two possibilities could account for these results. Beta-2 microglobulin might be limiting in this system. In this case, if fHFE did not complex with β2 microglobulin it would be rapidly degraded. Alternatively, the TfR might stabilize the fHFE/β2 microglobulin complex.

fHFE recognizes a site on TfR other than that of Tf - The close association of fHFE and TfR over time and the lowered affinity of the TfR for Tf in fHFE expressing cells led us to investigate whether fHFE has the same TfR binding site as Tf. Earlier studies examining the association of Tf and the TfR indicated that the unglycosylated form of the TfR does not fold correctly and does not bind Tf (Reckhow and Enns, 1988; Williams and Enns, 1993; Yang et al., 1993). We investigated whether Asn-linked glycosylation was necessary for fHFE's association with the TfR. Tet+ and tet- fWTHFE/tTA HeLa cells were treated with tunicamycin to inhibit Asn-linked glycosylation. Western blot shows coimmunoprecipitation of unglycosylated TfR (~80 kDa) with fHFE (lane 8 in Figure 1.5, arrow). The discrepancy in the amount of unglycosylated TfR in tet+ and tet- cells immunoprecipitated with sheep anti-TfR is due to the total amount of TfR in the cell. Unglycosylated TfR from tet+ cells is detectable at a longer exposure (data not shown). Since TfR co-immunoprecipitation with fHFE is independent of glycosylation, the structural elements necessary for fHFE binding to the TfR are different than for Tf binding to the TfR.

To confirm that fHFE does not exclude Tf from the binding site of TfR, cell lysates of ~$1x10^6$ cells grown in tet+ or tet- medium were precipitated with Tf-agarose and analyzed by Western blot as described in Experimental Procedures. Simultaneous isolation of fHFE with TfR on Tf-agarose (Figure 1.6) confirmed that fHFE, recognizes a site on the TfR other than that of Tf.

fHFE co-localizes with TfR - Immunocytochemical staining of fHFE and the TfR was performed to determine their intracellular localization. fHFE synthesis is turned off in fWTHFE/tTA HeLa cells grown in the presence of tetracycline (tet+). Immunocytochemical staining of the TfR in cells grown for over 2 weeks in tet+ medium exhibits punctate cytoplasmic staining, and localization to a perinuclear compartment (Figure 1.7 panel A). The TfR is a marker for recycling endosomes, as 75-80% of the TfR

recycles through the endosomes while only 20-25% is found on the surface at any point in time (Williams and Enns, 1993). fHFE levels are undetectable in cells maintained in tet+ medium (Figure 1.7 panel B). The staining pattern of the TfR does not change in fWTHFE/tTA HeLa cells induced for fHFE expression (Figure 1.7 panel C). fHFE localizes to the same perinuclear compartment (Figure 1.7 panel D) and exhibits complete co-localization with the TfR. These results demonstrate that fHFE does not drastically alter the distribution of the TfR.

To confirm that the staining pattern we have seen is intrinsic to fHFE and is not a result of accumulation of fHFE in the biosynthetic pathway, fWTHFE/tTA HeLa cells were grown 48 hrs in tet- medium, then 18 hrs in tet+ medium to regulate the expression levels of fHFE by turning off the promoter. Addition of tetracycline shuts off transcription almost immediately (Gossen and Bujard, 1992). Staining for fHFE is essentially identical to cells maintained in tet- medium (Figure 1.7 panel F). At the time of immunostaining, fHFE levels were decreased but staining was still very distinct, in the same perinuclear compartment. Thus the perinuclear staining of the fHFE does not appear to be due to the transient passage through the biosynthetic pathway, but rather its intracellular localization at steady state.

fHFE co-localizes with Tf positive vesicles - To determine whether fHFE co-internalizes with the TfR, fWTHFE/tTA HeLa cells were allowed to take up Texas-Red labeled Tf for 30 minutes at 37˚C. The cells were washed with PBS, fixed, and stained as described in Experimental Procedures. Tet+ cells show co-localization of the TfR and Tf (Figure 1.8 panels A and B, respectively). TfR and Tf co-localization were not changed in cells expressing fHFE (Figure 1.8 panels C and D, respectively). Tet- cells also exhibited co-localization of fHFE and Tf (Figure 1.8 panels E and F, respectively). Combined with results from Figure 1.7, Tf, TfR and fHFE all appear to traffic through the same endocytic perinuclear compartment.

DISCUSSION

To continue to define the relationship between iron, Tf, TfR, and HFE, we have investigated the biosynthesis and trafficking of fHFE in relation to the TfR and have shown that HFE may regulate intracellular iron levels since its expression greatly decreases the levels of Ft and modestly increases the levels of TfR. Both Ft and TfR are encoded by mRNAs containing IREs in their UTRs [for review see (Klausner et al., 1993)]. The TfR mRNA is stabilized by IRP binding in a low cytoplasmic iron environment while the translation of Ft mRNA is blocked. Modulation of the TfR and Ft by fHFE in this manner implicates HFE in the regulation of intracellular iron load.

Scatchard analysis of ^{125}I- diferric Tf binding to tet+ and tet- cell lines shows Tf binding sites increase from $1.8x10^5$ TfR/ uninduced cell to $2.8x10^5$ TfR/ induced cell, confirming the elevated level of TfR seen on Western blot. The Scatchard data also shows that fHFE decreases the TfR affinity for Tf as seen in the increase in Kd from 1.2 nM to 11 nM. This is comparable to the 5 to 75 nM increase in Kd upon addition of 1 mM soluble β_2 microglobulin-HFE heterodimer to HeLa cells reported earlier (Feder et al., 1998). The previous report showed no significant increase in TfR number because HeLa cells were only exposed to soluble HFE for the length of the experiment, not for days in culture as in the experiments reported here. The tetracycline repressible system has the added benefit of comparing fHFE positive and negative states within the same clonal cell line, circumventing discrepancies that may arise due to clonal variation.

Our studies on the synthesis of fHFE and TfR and their susceptibility to endo H digestion demonstrate that these proteins are capable of associating with each other within 30 minutes of synthesis while the proteins are in the ER/cis-Golgi compartment. The pulse-chase analysis also indicates that a significant amount of fHFE is degraded between 90 and 120 minutes. The remainder of which is further processed by addition of complex

carbohydrates less sensitive to endo H digestion. This pool of higher molecular weight fHFE is protected from degradation by its association with TfR and/or β2-microglobulin.

The kinetics of degradation of the fHFE pool associated with TfR are much slower than the degradation rate of the non-TfR associated pool. Approximately 50% of the fHFE expressed in our system is degraded within 4 hours of synthesis while the amount of TfR remains steady. The pool of fHFE that co-precipitates on the TfR, however does not change significantly over the course of eight hours. These results suggest the complex is much more stable than fHFE alone. Quantitation of the steady state amount of fHFE co-immunoprecipitated with TfR yields a stoichiometry of two TfR for every fHFE, or one TfR dimer for every fHFE. The same stoichiometry has been reported by Lebron et al. using gel filtration (Lebron et al., 1998).

Not only does HFE have a different stoichiometry of binding to the TfR, but it binds to a different region of the TfR than Tf. Tunicamycin-treated fWTHFE/tTA HeLa tet- cells are capable of associating unglycosylated TfR and fHFE, as shown by the coimmunoprecipitation of the TfR with fHFE. Tf binding to the TfR is glycosylation-dependent because Tf does not show measurable binding to the unglycosylated TfR (Reckhow and Enns, 1988; Williams and Enns, 1991). Tf and HFE binding are not mutually exclusive since fHFE co-precipitates with TfR bound to Tf agarose. The original observation that HFE decreases the TfR binding affinity for Tf, but not in the number of binding sites is also in agreement with the present results (Feder et al., 1998).

Immunocytochemical staining of fHFE in tet- fWTHFE/tTA HeLa cells is punctate and perinuclear and co-localizes with the TfR. The pattern is similar to TfR immunostaining in tet+ fWTHFE/tTA HeLa cells that do not express fHFE. These results show that HFE does not drastically alter the trafficking of the TfR. The TfR is, traditionally, an excellent marker for recycling endosomes. Once a clathrin coated vesicle containing the TfR pinches off the plasma membrane, it loses its clathrin coat and lowers its internal pH. In these low pH endosomes, iron is released from Tf. TfR and apo-Tf remain

bound to each other and recycle to the cell surface. At neutral pH apo-Tf is released and the TfR is free to bind more diferric Tf [for further discussion see review (Enns et al., 1996)]. HFE has no identifiable internalization motif in its cytoplasmic domain, yet fHFE co-localizes with Tf and the TfR in perinuclear and cytoplasmic intracellular vesicles. HFE's intracellular location may therefore be dependent on its association and endocytosis with the TfR. The co-trafficking of fHFE and the TfR into endocytic compartments suggests that HFE may alter iron uptake inside the cell.

In this report, we conclude that TfR and HFE co-localize to endocytic compartments in a pattern typical of the TfR. This finding is important in that it indicates HFE's function may not be limited to its alteration of the Tf-TfR interaction on the cell surface, but that HFE may also play a role in the cellular uptake of iron from the endosome. The expression of fHFE in HeLa cells results in lower intracellular iron levels as reflected by the specific alteration of TfR and Ft levels in cells expressing fHFE. fHFE localization to Tf-positive vesicles suggests regulation of internal iron stores by HFE may occur within the endosome.

HH is a disease characterized by an increase in the set point for bodily iron load. HH patients have elevated levels of Tf iron saturation. The degree of Tf saturation is an indicator of bodily iron load. HFE's association with Tf may be critical for its role as a sensor of bodily iron load. With the expanding knowledge of novel functions of nonclassical MHC class I molecules, HFE has the potential to regulate iron homeostasis in a number of ways. HFE may act by itself, regulate other proteins involved in iron metabolism, regulate the level of iron transport through the endosomal membrane, or alter the kinetics of TfR cycling in a subtle manner. It will be important to characterize differences between regulation of Tf and iron uptake at the cellular level and in organs such as the liver, pancreas, pituitary, and heart which are damaged by iron overload in HH patients. It will also be important to compare such regulation with what occurs in the cells of the intestinal mucosa that are anomalously iron depleted in HH patients.

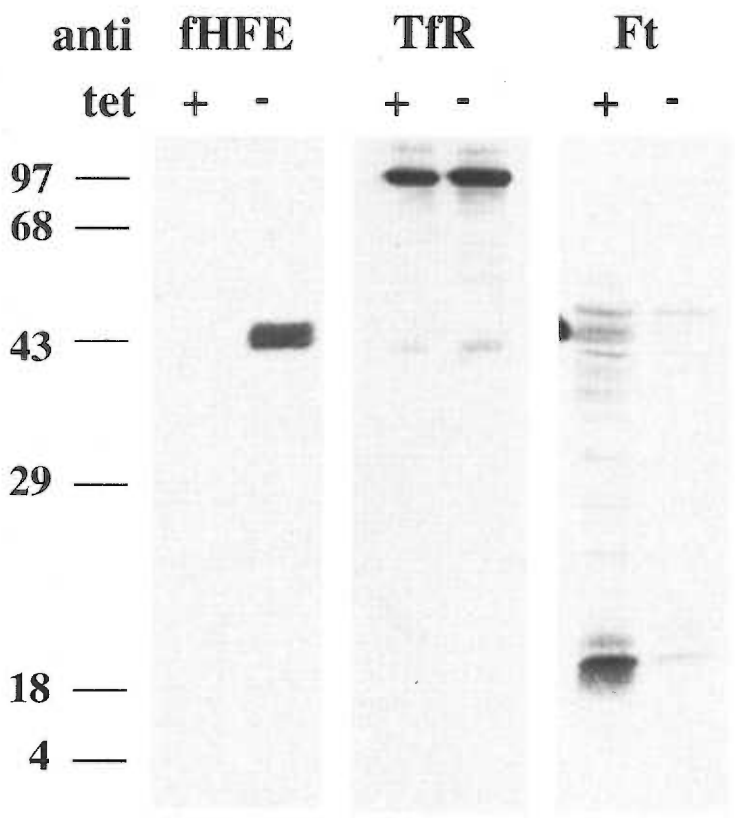

Figure 1.1. Inducible expression of fHFE in fWTHFE/tTA HeLa cells. Lysates of ~3 X 10^5 fWTHFE/tTA HeLa cells uninduced (tet+) or induced (tet-) for HFE expression were run on a 12% denaturing acrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-human TfR (1:10,000), mouse anti-FLAG (M2) (1:20,000) or sheep anti-Ft (1:100) antibodies and the appropriate HRP- conjugated secondary antibody (1:10,000). Chemiluminescent detection of ~97, ~43, and 21/19 kDa bands represent TfR, fHFE, and Ft, respectively. The increased TfR expression and decreased Ft expression in fHFE expressing cells is indicative of a decrease in intracellular iron load. These results are representative of three experiments without significant variation between experiments.

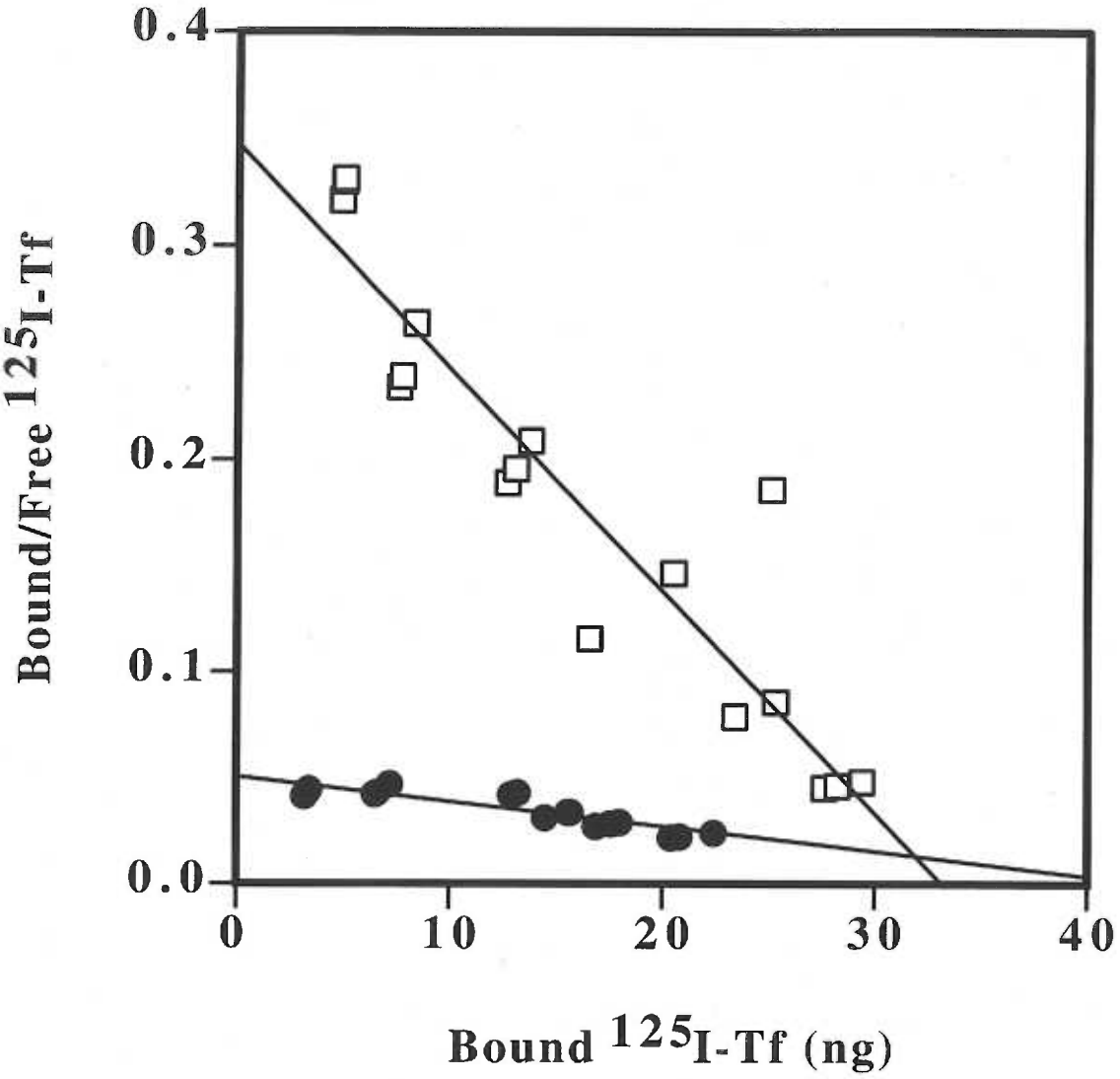

Figure 1.2. fHFE lowers TfR affinity for Tf in fWTHFE/tTA HeLa cells. Scatchard analysis of Tf binding to $1.38X10^6$ uninduced (tet+, open squares) or $1.17X10^6$ induced (tet-, closed squares) fWTHFE/tTA HeLa cells shows elevation of the dissociation constant from 1.2 to 11 nM Tf when fHFE is overexpressed. Change in x-intercept indicates surfaceTfR numbers increase from $1.82X10^5$ TfR/tet+ cell to $2.77X10^5$ TfR/tet- cell. Linear regression was determined in the Cricket Graph program using r^2 values. These results are representative of three experiments performed in triplicate data points without significant variation between experiments.

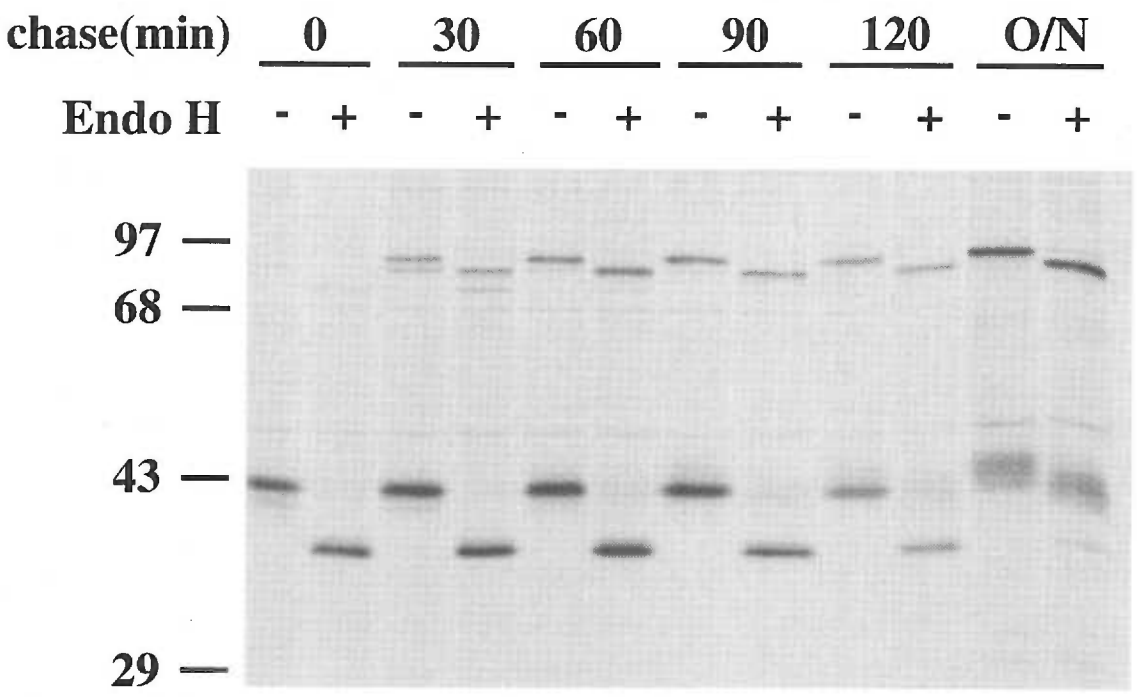

Figure 1.3. Early association of fHFE and the TfR. ~8.4X10^5 cells expressing fHFE were labeled for 10 minutes with 100 µCi [^{35}S]-methionine and chased with complete medium for 30 minute increments between 0 and 120. Alternately, cells were labeled ~18 hours (O/N) with 10 µCi [^{35}S]-methionine. fHFE immunoprecipitated with anti-FLAG was run on a 10% denaturing acrylamide gel under reducing conditions. TfR co-immuno-precipitates with fHFE within 30 minutes of synthesis and before it has become completely glycosylated. Endo β-N-acetylglucosaminidase H (endo H) cleaves oligosaccharides from high mannose and hybrid N-linked oligosaccharides. Loss of endo H sensitivity at any glycosylation site on the protein indicates it has exited the cis-Golgi. These results are representative of four experiments without significant variation between experiments.

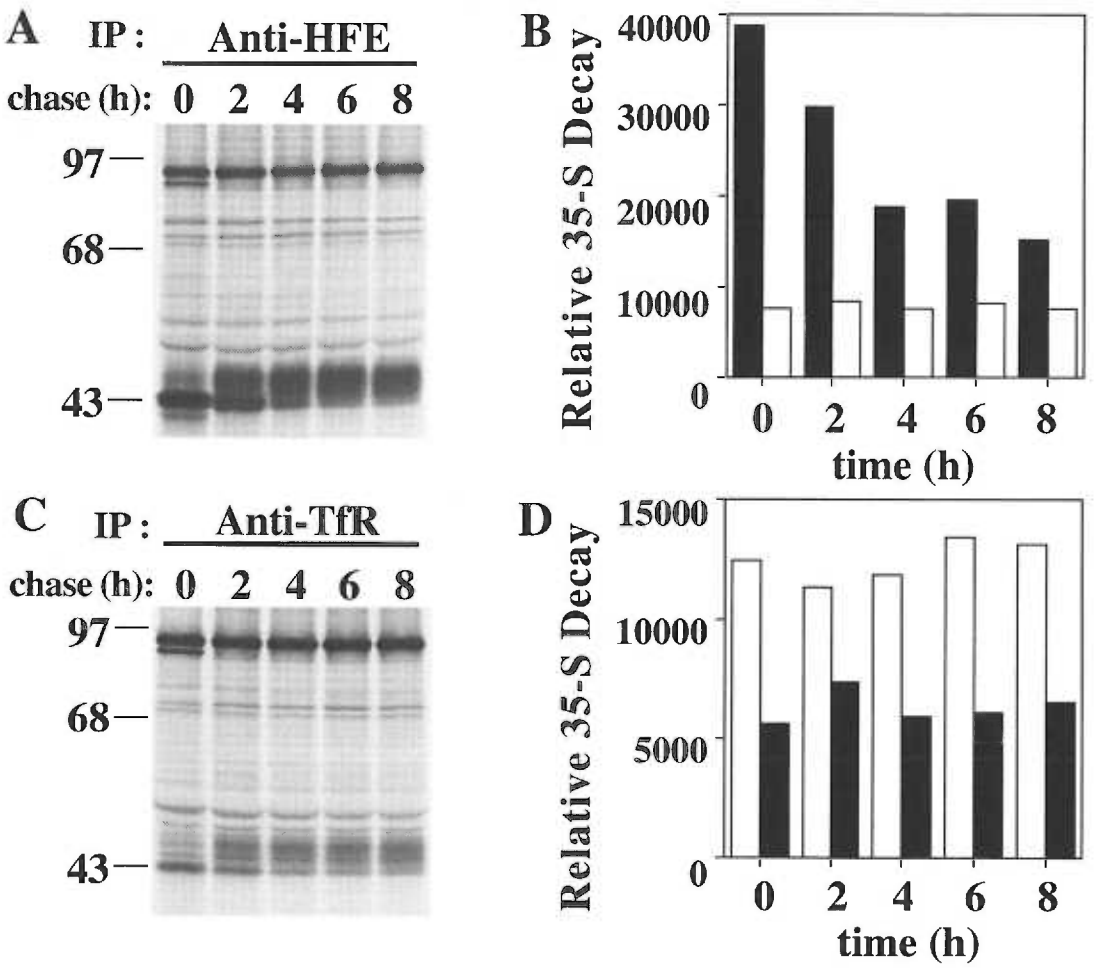

Figure 1.4. fHFE steady-state levels are reduced to TfR-associated pool. ~10^6 cells expressing fHFE were labeled one hour with 100 μCi [^{35}S]-methionine and chased with complete medium from two to eight hours. Immunoprecipitates were run on 10% denaturing acrylamide gels under reducing conditions. (A) Lysates immunoprecipitated with anti-FLAG antibodies. Despite degradation of fHFE after 4 hours of synthesis, the amount of co-immunoprecipitated TfR remains unchanged. (C) Lysates immunoprecipitated with anti-TfR antibody (4091). TfR and fHFE levels do not change significantly over the 8 hour timecourse. (B and D) Quantitation of fHFE (black bars) and TfR (white bars) co-immunoprecipitated with anti-FLAG antibody against fHFE (B) or 4091 antibody against TfR (D). These results are representative of four experiments without significant variation between experiments.

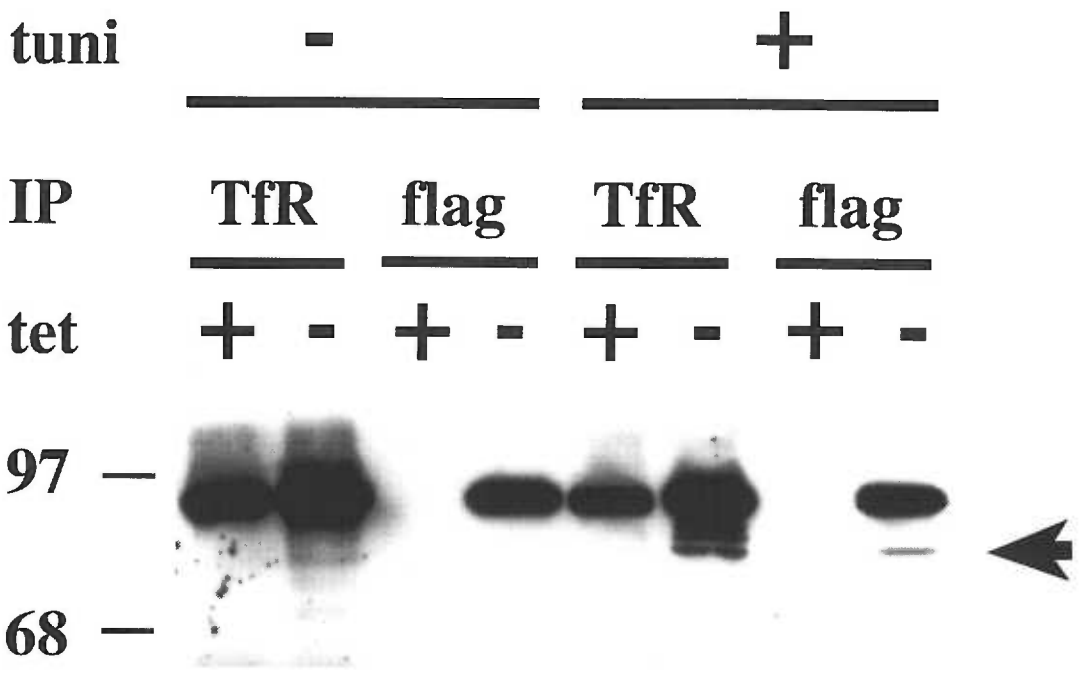

Figure 1.5. TfR co-immunoprecipitation with fHFE in tunicamycin treated cells. ~7X10^5 cells treated overnight with 5 μg/ml tunicamycin or mock treated with 0.05% DMSO were immunoprecipitated with sheep anti-human TfR or anti-FLAG antibodies. The immunoprecipitates were subjected to SDS-PAGE on a 10% denaturing gel under reducing conditions, transferred to nitrocellulose and probed with sheep anti-human TfR antibody (1:10,000) and swine anti-goat HRP-conjugated secondary (1:10,000). The arrow indicates unglycosylated TfR co-immunoprecipitated with fHFE. These results are representative of three experiments without significant variation between experiments.

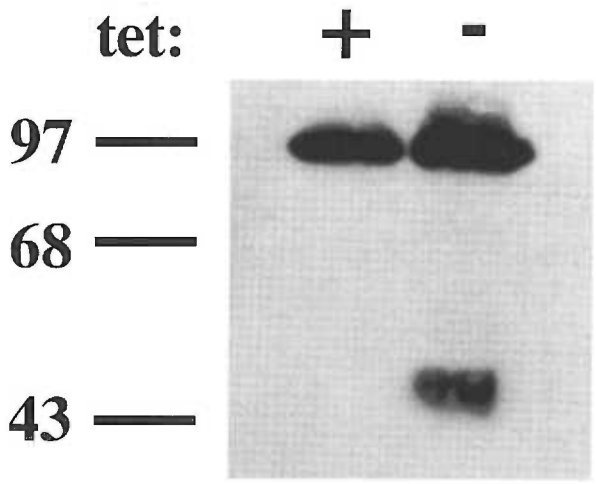

Figure 1.6. fHFE precipitates with the Tf-TfR complex. Lysates from ~5X10^5 tet+ or tet- cells were allowed to bind Tf-conjugated agarose for one hour at 4°C. Pellets were washed, eluted and subjected to SDS-PAGE on a 12% denaturing acrylamide gel under reducing conditions, transferred to nitrocellulose, and detected with sheep anti-human TfR and mouse anti-FLAG antibodies with their appropriate HRP-conjugated secondary antibody. Chemiluminescence detects ~97 and ~43 kDa species, providing further evidence for a binding site for HFE outside the Tf binding site on TfR.

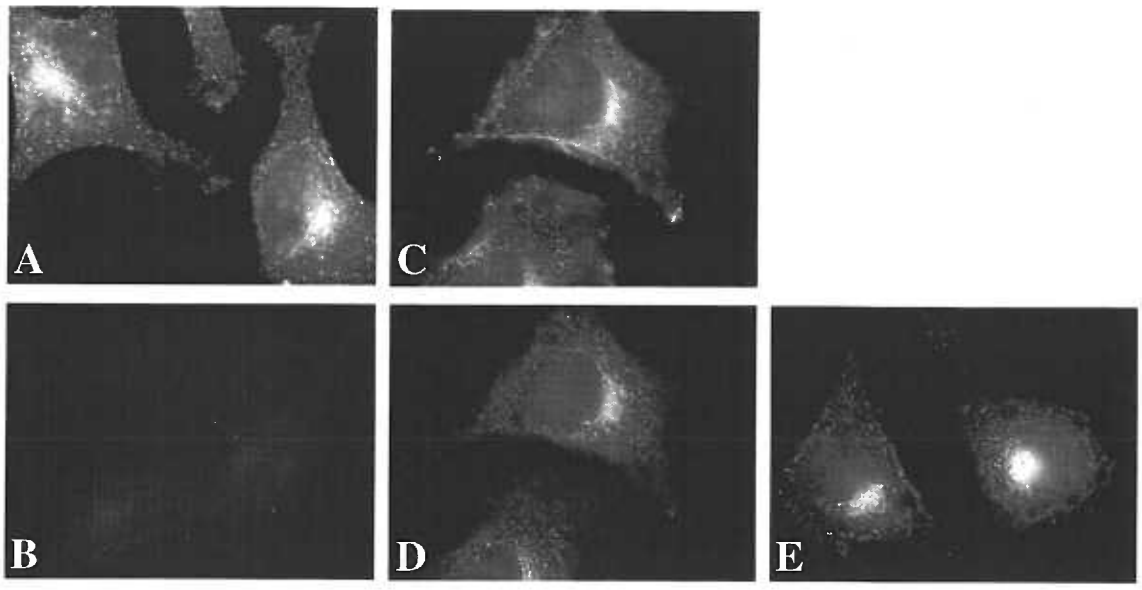

Figure 1.7. Co-localization of fHFE with the TfR in fWTHFE/tTA Hela cells. Permeabilized tet+ cells stained with mouse anti-human TfR (4093) and FITC anti-mouse antibodies show predominant TfR localization in a perinuclear compartment, with some at the cell surface. (B) Permeabilized tet+ cells stained with rabbit anti-HFE (CT1) and TRITC anti-rabbit antibodies show no specificity for HFE because tet turns off expression of fHFE and HeLa cells do not express detectable endogenous HFE. (C) Permeabilized tet- cells stained with mouse anti-human TfR (4093) and FITC anti-mouse antibodies show no change in TfR localization despite fHFE expression in these cells. (D) Permeabilized tet- cells stained with rabbit anti-HFE (CT1) and TRITC anti-rabbit antibodies show fHFE localizes to the same intracellular compartment as TfR with small amounts on the cell surface. (E) Permeabilized cells with decreased fHFE expression (i.e. passaged 18 hours in tet) stained with anti-HFE (CT1) and TRITC anti-rabbit antibody show a staining pattern identical to cells in (D). These results are representative of four experiments without significant variation between experiments.

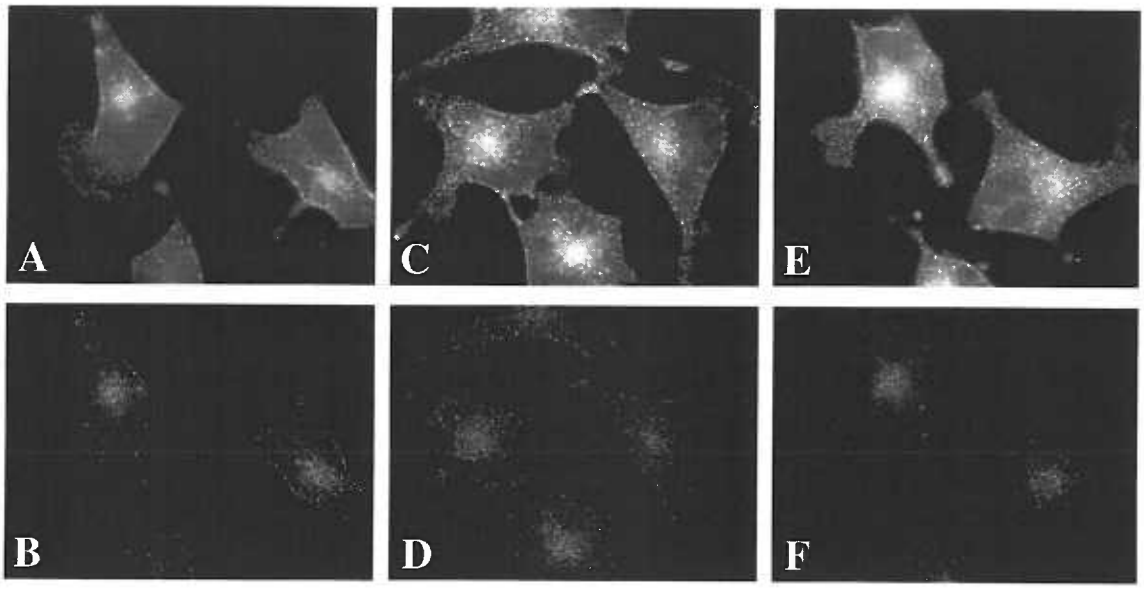

Figure 1.8. fHFE localizes to the same compartment as Tf. (A) Permeabilized tet+ cells stained with mouse anti-human TfR (4093) and FITC anti-mouse antibodies show TfR localization at the cell surface and in a perinuclear compartment. (B) Texas-Red Tf localizes to the same internal compartment as TfR in tet+ cells. (C) Permeabilized tet- cells stained with mouse anti-TfR (4093) and FITC anti-mouse antibodies show TfR localization does not change with induction of fHFE. (D) Texas-Red Tf continues to traffic through the same TfR labeled perinuclear compartment despite fHFE expresion. (E) Permeabilized tet- cells stained with mouse anti-FLAG (M2) and FITC anti-mouse antibodies show fHFE localization to the cell surface and in the same perinuclear compartment as TfR. (F) Texas-Red Tf localizes to the same perinuclear compartment as in (C), (D), and (E). TfR, Tf and fHFE occupy the same compartment in tet-fWTHFE/tTA HeLa cells. These results are representative of three experiments without significant variation between experiments.

CHAPTER 2

Interactions of the Ectodomain of HFE with the Transferrin Receptor are Critical for Iron Homeostasis in Cells

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SUMMARY

Expression of wild type HFE reduces the ferritin levels of cells in culture. In this report we demonstrate that the predominant hereditary hemochromatosis mutation, C282Y HFE, does not reduce ferritin expression. However, the second mutation, H63D HFE, reduces ferritin expression to a level indistinguishable from cells expressing wild type HFE. Further, two HFE cytoplasmic domain mutations engineered to disrupt potential signal transduction, S335M and Y342C, were functionally indistinguishable from wild type HFE in this assay, as was soluble HFE. These results implicate a role for the interaction of HFE with the transferrin receptor in lowering cellular ferritin levels.

INTRODUCTION

HH is a common, autosomal recessive disease of iron metabolism (Bothwell et al., 1995). Lack of functional HFE protein in the cells of the duodenal crypts leads to chronic absorption of iron (Feder et al., 1996; Levy et al., 1999; Parkkila et al., 1997; Santos et al., 1996; Zhou et al., 1998). The excess iron accumulates in the cells of parenchymal tissues leading to oxidative damage and multi-organ dysfunction (Bothwell et al., 1995). The pathology of HH indicates that HFE plays a significant role in regulated iron absorption and the maintenance of iron homeostasis, but the exact mechanism by which HFE facilitates iron homeostasis is not yet understood.

The predominant mutation in HFE that leads to HH is a missense mutation which leads to the conversion of a critical cysteine to tyrosine at amino acid 282 (Feder et al., 1996). HFE is a nonclassical major histocompatability complex (MHC) class I type molecule that requires beta 2-microglobulin binding for proper folding and efficient cell surface expression (Feder et al., 1998; Feder et al., 1997; Lebron et al., 1998; Parkkila et al., 1997; Waheed et al., 1997). The C282Y mutation prevents the formation of a disulfide bond that stabilizes the beta 2-microglobulin binding pocket in HFE (Lebron et al., 1998). The inability of this mutant to fold correctly and associate with beta 2-microglobulin (Feder et al., 1998) results in reduced amounts of HFE and inefficient trafficking to the cell surface (Feder et al., 1997). Lack of association with the TfR explains why it does not localize to the endosome as does wild type HFE (Gross et al., 1998; Ramalingam et al., 2000; Waheed et al., 1997).

A second HFE mutation has been identified in a significant number of individuals with HH. This missense mutation converts amino acid 63 from histidine to aspartic acid, disrupting the formation of a salt bridge with the aspartic acid at position 95 (Lebron et al., 1998). The frequency of this mutation is significantly increased in HH individuals that are heterozygous for the C282Y mutation. This mutation is often associated with an

intermediate iron loading phenotype (Brandhagen et al., 1999; Feder et al., 1996). The interaction of H63D HFE with the TfR is not significantly different from that of wild type HFE. It has approximately the same affinity for the TfR (Lebron and Bjorkman, 1999) and co-immunoprecipitates with the TfR (Feder et al., 1998). Because the association of HFE with the TfR is the main link between HFE and iron homeostasis, how this mutation contributes to iron overload is unclear.

Despite the efforts to understand the role of HFE in iron homeostasis, the precise mechanism behind its function remains unknown. Several groups have independently observed an iron deficient phenotype in cell lines transfected with HFE that is evidenced by increased TfR expression, decreased Ft expression, and an increase in IRP activity (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999). The labile iron pool is hypothesized to be reduced because HFE reduces iron uptake from Tf (Corsi et al., 1999; Ikuta et al., 2000; Lebron et al., 1999; Riedel et al., 1999; Roy et al., 1999).

In this report we have examined HFE disease alleles for an iron deficient phenotype in cultured cell lines based on their ability to reduce Ft levels when HFE is expressed. Ft levels were assayed to investigate the possibility that HFE uses its cytoplasmic domain to initiate a signal transduction cascade that might induce the low iron phenotype. Finally, Ft levels in cells treated with a soluble form of HFE were measured to determine whether the transmembrane or cytoplasmic domain of HFE might modify some other cytoplasmic component of iron metabolism that would produce the low iron phenotype. We conclude that HFE does not require the transmembrane or cytoplasmic domain to lower Ft levels in HEK 293 cells, but most likely does require cell surface expression and association with the TfR to induce the low iron phenotype.

MATERIALS AND METHODS

Site- directed mutagenesis of HFE

The isolation of the H63D cDNA and generation of the C282Y mutation have been previously described (Feder et al., 1997). A standard PCR mutagenesis approach was utilized to generate the S335M and Y342C mutations. Two overlapping fragments with the appropriate base changes were produced in a first round PCR reaction. The product of the first reaction was then combined with the primers previously described (Feder et al., 1997) to yield the HFE mutant with the FLAG epitope.

Cell lines

The fWTHFE/tTA HeLa cell line expressing FLAG epitope-tagged HFE (fHFE) under the tetracycline-responsive promoter has been previously described (Gross et al., 1998). Cells were grown in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE-) or without (HFE+) 2 μg/ml tetracycline. The HEK 293 cell lines were co-transfected with the plasmids for the individual HFE mutations described above, and a plasmid containing the coding sequence for human beta-2 microglobulin (Feder et al., 1997). Isolated HFE-expressing clones were grown in DMEM supplemented with 10% fetal bovine serum and 600 μg/ml G418.

Immunocytochemistry - Subconfluent HEK 293 cells (parental, wild type HFE, C282Y HFE, H63D HFE) grown on fibronectin treated coverslips were washed three times with 2 ml PBS and fixed 15 minutes in 3% paraformaldehyde at room temperature and washed an additional two times in PBS. Cells were blocked in 10% newborn calf serum in PBS with 0.5% Triton X-100 to permeabilize cells for at least 30 minutes at room temperature. The fixed cells were incubated for at least one hour at room temperature in 100 μg/ml 4093

mouse anti-human TfR antibody and 30 μg/ml EX1 rabbit anti-HFE antibody. Coverslips were then washed twice in PBS and incubated again for at least one hour at room temperature with 1:500 dilution of Alexa 594-labeled goat anti-mouse antibody or Alexa 488-labeled goat anti-rabbit antibody (Molecular Probes).

Cell treatments

Cells were plated into 35 mm dishes in DMEM with 10% fetal bovine serum and 50 nM human Tf. After three days in culture, the growth medium was changed and desferoxamine was added to a final concentration of 50 μM or $Fe(NTA)_4$ was added to a final concentration of 100 μM, or soluble HFE (a kind gift of Pamela Bjorkman, California Institute of Technology) was added to a final concentration of 0.5 or 1 μM. Cells were grown at least 16 hours in the treated medium before they were lysed .

Immunodetection of HFE and Ferritin

Cells were lysed with NET-Triton buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4). Samples (~300 μg) were diluted with Laemmli buffer (Laemmli, 1970) and subjected to electrophoresis on denaturing 12% SDS-polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis of HFE was performed as described previously using rabbit anti-HFE antibody EX1 (1:500 dilution) (Feder et al., 1997) and sheep anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000 dilution, Roche Molecular Biochemicals). Immunoblot analysis of Ft was performed as described previously (Gross et al., 1998) using rabbit anti-human Ft antibody (1:1000 dilution, Dako), and sheep anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000 dilution, Roche Molecular Biochemicals). Western blots were developed using chemiluminescence (Super Signal, Pierce) per the manufacturer's directions.

RESULTS

Co-localization with TfR is not Observed for the Primary HFE Disease Mutation- In collaboration with Dr. John Feder, Alivelu Basava, and Steven Stranes of Progenitor, Inc., wild type and mutant forms of HFE were stably expressed in HEK 293 cells. Immunoblot analysis identified stable expression of the ~40-45 kDa HFE product in all cell types except that of the parental HEK 293 cell line in which no detectable HFE was identified (Figure 2.1).

Immunohistochemical staining of HFE and the TfR was performed to confirm that the disease-causing mutated forms of HFE localized as previously described (Waheed et al., 1997). The TfR localized to a perinuclear compartment in HEK 293 cell lines, fitting with its role as an endosomal marker (Figure 2.2, panels A, C, and E and Figure 2.3, panels A, C, and E). While parental HEK 293 cells did not stain specifically for HFE (Figure 2.2 and Figure 2.3, panel B), wild type HFE was present in a perinuclear compartment that significantly co-localized with the TfR (Figure 2.2 and Figure 2.3, panel D). These results are consistent with the previously described localization of HFE in COS-7 cells(Waheed et al., 1997), and the co-trafficking of HFE and the TfR that we previously reported in HeLa cells (Gross et al., 1998).

The most severe HH disease mutation in HFE, C282Y HFE, did not co-localize with the TfR (Figure 2.2 panel F), exhibiting a reticular staining pattern that extended to the outer-most edges of the cell. This result is also in keeping with the inability of the C282Y HFE to reach the cell surface (Feder et al., 1997) and its localization to the endoplasmic reticulum in COS-7 cells (Waheed et al., 1997). Contrary to the C282Y HFE, the H63D HFE mutation, which has been linked to less severe HH pathology, does co-localize with the TfR in HEK 293 cells (Figure 2.3, panel F).

The Low Iron Phenotype in HFE-expressing Cells is not Observed for the Primary HFE Disease Mutation- Expression of HFE induces an iron deficient phenotype as indicated by reduced Ft levels in HeLa cells (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999; Roy et al., 1999). To confirm that HFE had the same effect in HEK 293 cells, analysis of Ft levels was performed for parental HEK 293 cells and those expressing wild type HFE (Figure 2.4). Immunoblot analysis revealed that HFE expression reduced Ft levels under standard tissue culture conditions. HFE did not prevent the cells from responding to changes in iron status, as Ft expression decreased in the presence of the iron chelator, Df, and increased with the addition of Fe-NTA. However, Ft levels in HFE-expressing cells remained below that of parental cells under iron depleted or iron loaded conditions because of their decreased basal Ft. Identical results were obtained in HEK 293 cells transfected with wild type HFE lacking the FLAG-epitope tag, indicating the FLAG epitope is not responsible for the low iron phenotype (data not shown). These results recapitulate the phenotype that was observed previously in fWTHFE/tTA HeLa cells (Gross et al., 1998; Roy et al., 1999).

While the HFE-specific reduction in cellular Ft levels has been independently observed by several investigators (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999; Roy et al., 1999), its correlation with iron homeostasis for the entire organism is not fully understood. To determine whether this low iron phenotype is reflected in the disease state, we compared the iron deficient phenotype for HEK 293 cells expressing wild type HFE with those expressing the two HH disease alleles (Figure 2.5). Immunoblot analysis revealed that under standard tissue culture conditions, C282Y HFE did not reduce Ft levels to that of wild type HFE-expressing cells. The Ft levels detected in C282Y HFE/HEK 293 cells were not significantly different from that of the HEK 293 parental cell line (data not shown). Conversely the H63D HFE disease allele, whose contribution to the disease state may not be as severe as the C282Y HFE, did recapitulate the wild type HFE phenotype. Ft

levels in the H63D HFE cells were reduced to a level similar to those of HEK 293 cells expressing wild type HFE.

The Low Iron Phenotype is not Dependent on Serine or Tyrosine Residues in the HFE Cytoplasmic Domain- Despite extensive characterization of the interaction between HFE and the TfR, the exact mechanism by which HFE lowers Ft levels has not been determined. One possible mechanism by which HFE could function would be through a signal transduction pathway initiated by the cytoplasmic domain of HFE. We assayed for the iron deficient phenotype in cell lines with either the Y342 or S335 in the cytoplasmic domain changed to C or M respectively (Figure 2.6). The original tyrosine and serine amino acids are potential candidates for phosphorylation. Immunoblot analysis indicated that the Y342C and S335M HFE proteins were able to reduce Ft levels below those of the HEK 293 parent. The Ft content of these cell lines was not significantly different from those of the HEK 293 cells expressing wild type HFE (data not shown), indicating these mutants were functional according to the limits of this assay.

The Low Iron phenotype is not Dependent on the HFE Transmembrane or Cytoplasmic Domains- To determine if other elements in the HFE cytoplasmic domain were involved, the ability of soluble HFE to induce the same low iron phenotype as the full length HFE protein in HEK 293 cells was assayed. A soluble form of HFE lacking the cytoplasmic and transmembrane domains has been used to investigate the interaction of HFE and the TfR (Lebron et al., 1998). This form of HFE has high affinity for the TfR (Feder et al., 1998; Lebron et al., 1998), and reduces the affinity of the TfR for Tf (Feder et al., 1998; Lebron et al., 1999)._ Incubation of cells with soluble HFE resulted in a reduction of Ft levels in parental HEK 293 cells in a concentration dependent manner (Figure 2.7). Like the results from the Y342C and the S335M mutants, these results also argue against the

possibility that the transmembrane or cytoplasmic domains of HFE modulate cytoplasmic components of iron metabolism.

DISCUSSION

A comparison of the wild type and disease-associated forms of HFE on Ft levels in cultured cells was assessed. We observed that wild type HFE expressed in HEK 293 cells produces the same low iron phenotype that was originally identified in HFE-expressing HeLa cells (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999; Roy et al., 1999).

C282Y HFE is severely reduced in function because it does not fold correctly (Lebron et al., 1998) as manifest by lack of association with beta-2 microglobulin (Feder et al., 1997), lack of efficient trafficking to the cell surface (Feder et al., 1997; Waheed et al., 1997), lack of association with the TfR (Feder et al., 1998), and the inability to maintain normal iron homeostasis in mice (Levy et al., 1999). In this study, we show that it does not localize to endosomes with the TfR, nor is it able to recapitulate the low iron phenotype induced by wild type HFE in HEK 293 cells. The observation that this mutation in HFE does not reduce Ft expression suggests that HFE must be efficiently expressed at the cell surface and/or tightly associate with the TfR to produce the low iron phenotype.

Unlike C282Y HFE, H63D HFE has been shown to traffic to the cell surface (Feder et al., 1997; Waheed et al., 1997) and to associate with the TfR (Feder et al., 1998). In this study, we show that the H63D HFE protein in HEK 293 cells does co-localize with the TfR in the endosome and does induce a low iron phenotype. These observations suggest that the H63D HFE protein would be functional *in vivo* and yet, the H63D allele occurs with increased frequency in individuals diagnosed with HH (Brandhagen et al., 1999; Feder et al., 1996). The difference between the disease phenotype observed in humans with the H63D mutation and the wild type phenotype observed in the H63D HFE-expressing cell culture system may be the level of expression of the protein. Since the H63D HFE protein lacks the ability to form a salt bridge that may be important to the stability of the protein (Lebron et al., 1998), the H63D HFE may be less stable than the wild type HFE. A small but significant decrease in functional HFE *in vivo* may lead to iron

overload over the course of a lifetime. The HH pathology resulting from the H63D mutation is milder than that of individuals homozygous for the C282Y HFE mutation.

The importance of the association between the extracellular domains of HFE and the TfR is underscored by the observation that mutations to the cytoplasmic domain of HFE do not affect the low iron phenotype. We observed that Y342C and S335M HFE proteins reduced Ft levels. These results indicate that the low iron phenotype is not the result of signal transduction through the tyrosine or serine amino acid residues in the cytoplasmic domain of HFE. Furthermore, the decrease in Ft levels observed in cells treated with soluble HFE supports the hypothesis that the phenotype is mediated solely through interaction of the extracellular domains of HFE and the TfR. However, these results do not rule out the possibility that HFE binding to the TfR ectodomain propagates conformational changes in the TfR cytoplasmic domain that might modulate signal transduction cascades or cytoplasmic components of cellular iron metabolism.

Our results support the hypothesis that HFE acts through the TfR-mediated pathway of iron uptake to regulate cellular iron homeostasis. Severe mutations in HFE, such as the C282Y mutation which prevents association with the TfR, do not manifest a low iron phenotype. These results predict that mutations in HFE that lower the affinity of HFE for the TfR, such as the W103A HFE protein (Lebron and Bjorkman, 1999), would not result in the low iron phenotype. Recent results indicate that this is indeed true (Ramalingam et al., 2000). Future experiments will be directed at determining the mechanism by which the HFE interaction with the TfR in the extracellular domains reduces iron uptake from Tf.

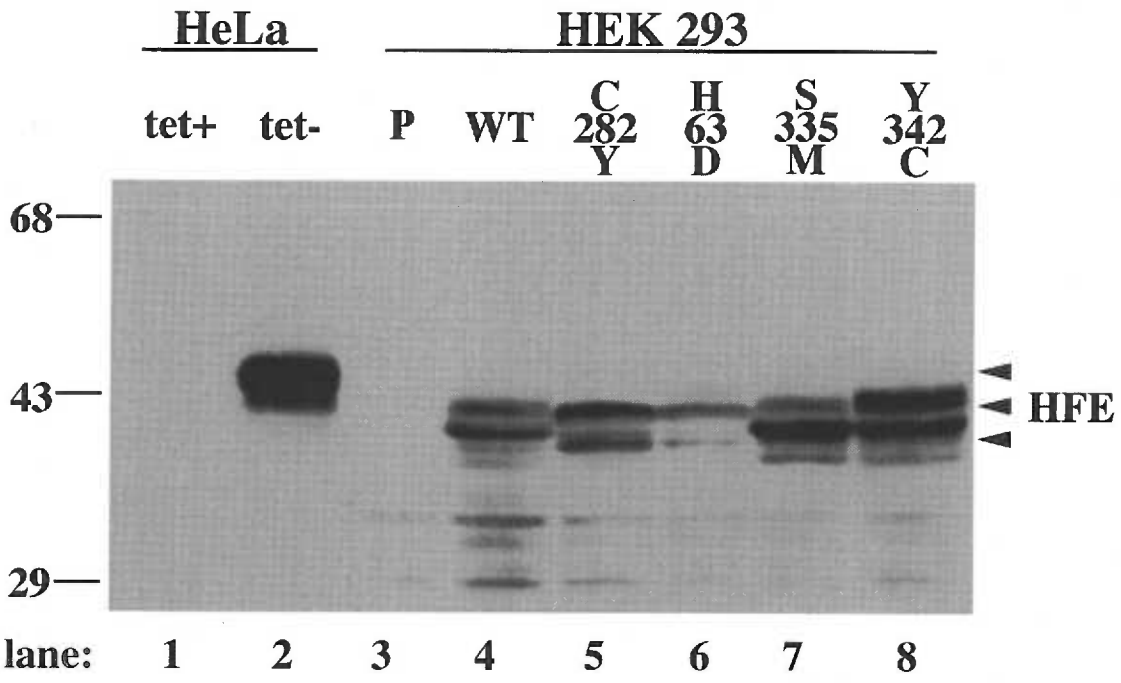

Figure 2.1. Characterization of stable HFE expression in WT-, C282Y-, H63D-, S335M-, and Y342C HFE/HEK 293 cell lines. Parental HEK 293 cells (lane 3), like fWTHFE/tTA HeLa cells with tetracycline added (tet+) to turn off HFE (lane 1), did not express detectable levels of HFE protein as assayed by immunoblot. Wild type (lane 4) and mutant HFE proteins (lanes 5-8) were stably expressed in HEK 293 cells. The molecular weight of HFE in these cells was slightly less than that of HFE in fWTHFE/tTA HeLa cells (lane 2), probably due to differences in the extent of glycosylation between the two cell lines. These results are representative of more than three independent experiments with similar results.

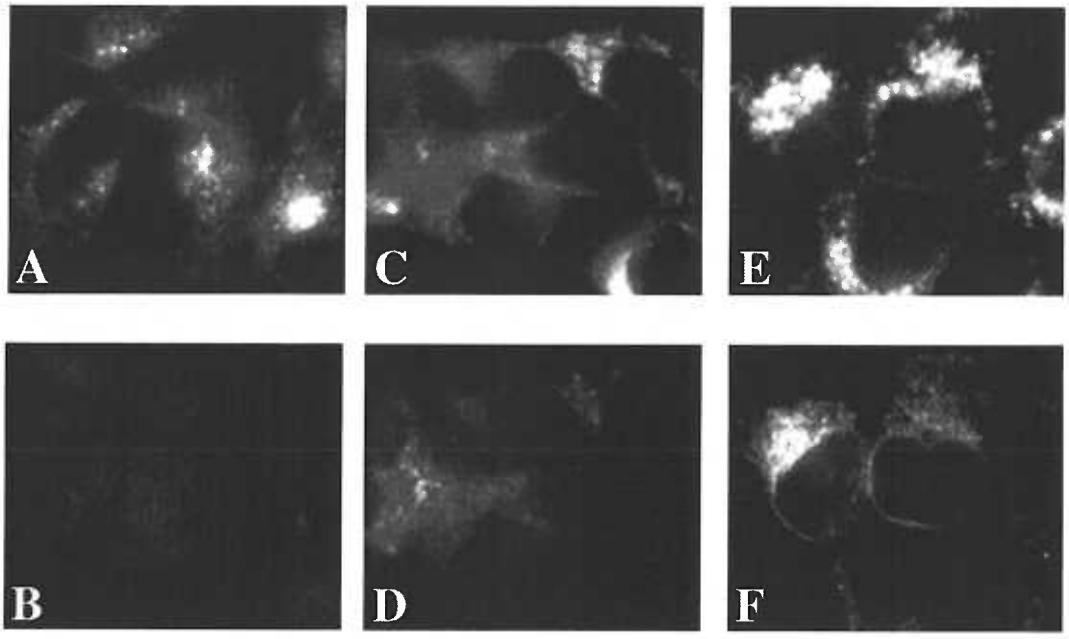

Figure 2.2 C282Y HFE does not co-localize with the TfR. (A) Permeabilized parental HEK 293 cells stained with mouse anti-human TfR (4093) and Alexa-594 anti-mouse antibodies show TfR localization at the cell surface and in a perinuclear compartment. (B) Rabbit anti-HFE (EX1) antibody and Alexa-488 anti-rabbit antibodies do not specifically stain parental HEK 293 cells. (C) Permeabilized HEK 293 cells transfected with wild type HFE stained as in (A) show similar TfR localization. (D) Wild type HFE, stained as in (B), colocalizes with TfR. (E) Permeabilized HEK 293 cells transfected with C282Y HFE stained as in (A) show similar TfR localization. (F) C282Y HFE, stained as in (B), does not colocalize with the TfR. These results are representative of three experiments without significant variation between experiments.

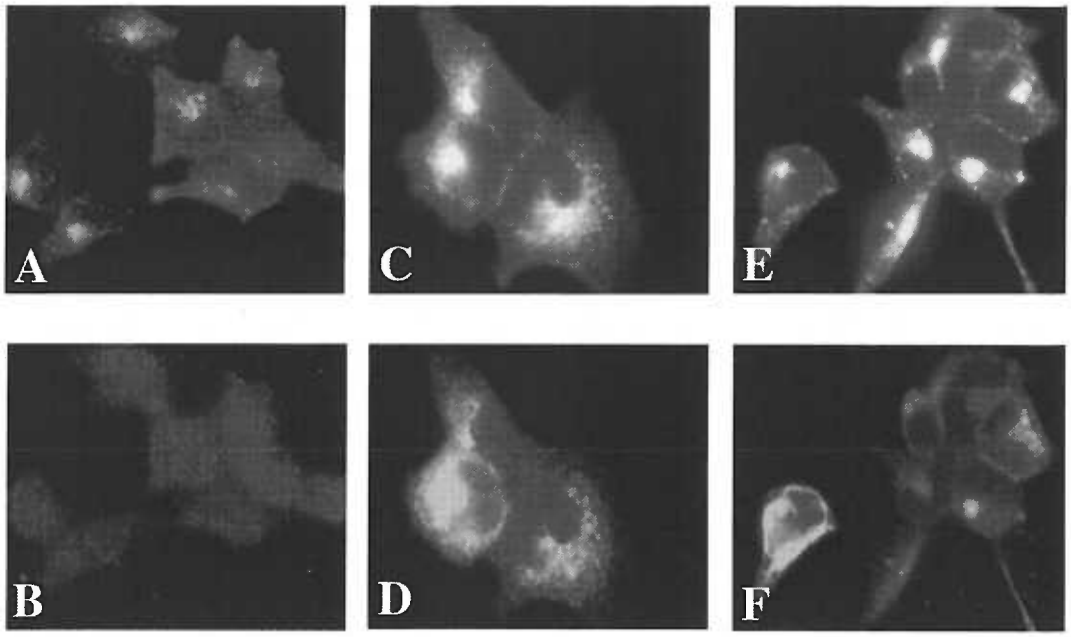

Figure 2.3 H63D HFE does co-localize with the TfR. (A) Permeabilized parental HEK 293 cells stained with mouse anti-human TfR (4093) and Alexa-594 anti-mouse antibodies show TfR localization and the cell surface and in a perinuclear compartment. (B) Rabbit anti-HFE (EX1) antibody and Alexa-488 anti-rabbit antibodies do not specifically stain parental HEK 293 cells. (C) Permeabilized HEK 293 cells transfected with wild type HFE stained as in (A) show similar TfR localization. (D) Wild type HFE, stained as in (B), colocalizes with TfR. (E) Permeabilized HEK 293 cells transfected with H63D HFE stained as in (A) show similar TfR localization. (F) H63D HFE, stained as in (B), does colocalize with TfR. These results are representative of three experiments without significant variation between experiments.

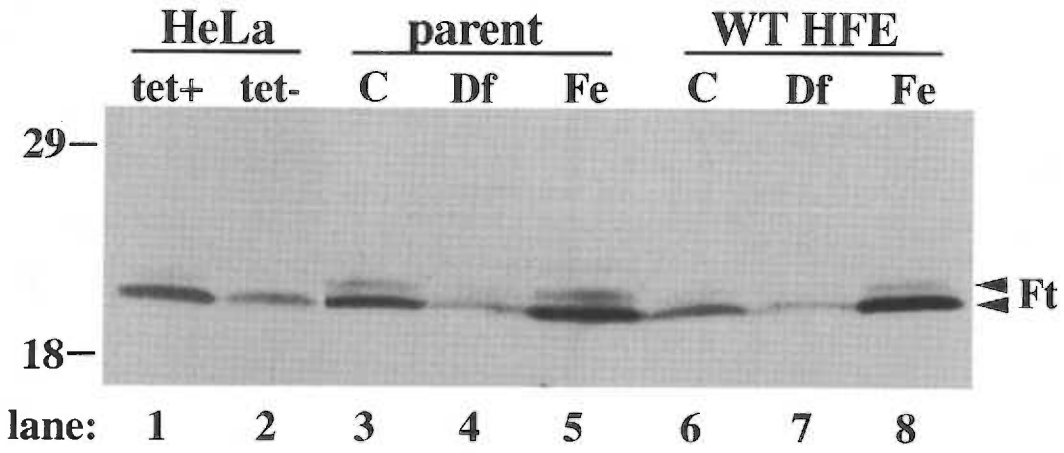

Figure 2.4. Wild type HFE-expression reduces Ft levels in HEK 293 cells. HFE expression reduced Ft levels (as assayed by immunoblot) in HEK 293 cells grown under standard tissue culture conditions (C, lane 6) when compared to the parental cell line (C, lane 3). Ft levels still responded to cellular iron status, however, as they decreased with the addition of desferoxamine, an iron chelator (Df, lane 7), and increased with the addition of $Fe(NTA)_4$, an iron salt (Fe, lane 8). These results are representative of more than three independent experiments with similar results.

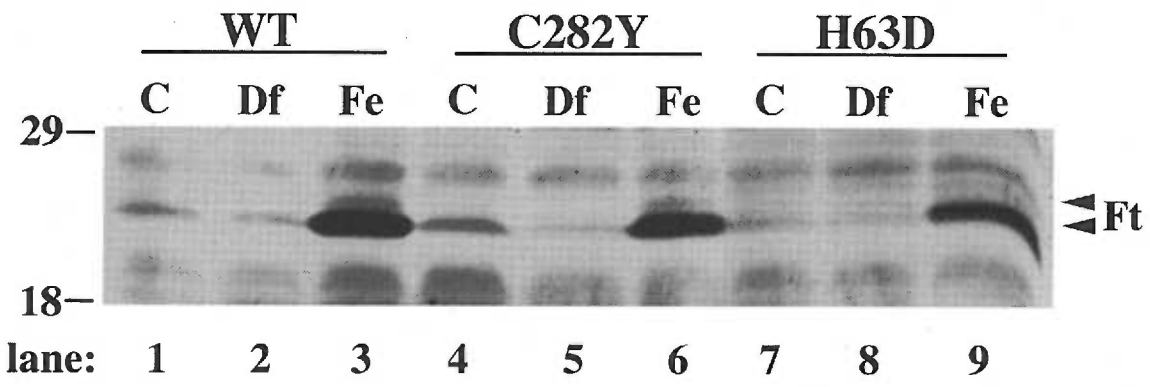

Figure 2.5. C282Y HFE-expression does not reduce Ft levels while H63D HFE-expression reduces Ft levels to that of wild type HFE in HEK 293 cells. Expression of the C282Y HFE disease allele protein product in HEK 293 cells grown under standard tissue culture conditions (C, lane 4) did not reduce Ft levels like that of wild type HFE (C, lane 1) as assayed by immunoblot. However, Ft levels still responded to cellular iron status as they decreased with the addition of desferoxamine (Df, lane 5) and increased with the addition of $Fe(NTA)_4$ (Fe, lane 6). Expression of the H63D HFE disease allele protein product in HEK 293 cells grown under standard tissue culture conditions (C, lane 7) reduced Ft expression to the same level found in wild type HFE-expressing cells (C, lane 1). As for wild type HFE, Ft levels responded to cellular iron status, decreasing in the presence of desferoxamine (Df, lanes 2 and 8) and increasing in the presence of $Fe(NTA)_4$ (Fe, lanes 3 and 9). These results are representative of more than three independent experiments with similar results.

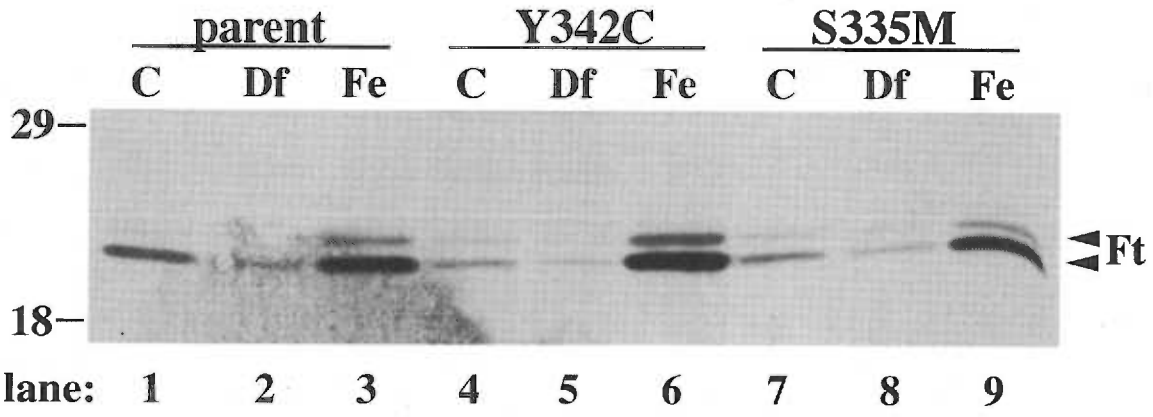

Figure 2.6. Y342C- and S335M HFE expression reduces Ft levels from that of parental HEK 293 cells. Expression of the Y342C (lanes 4-6) and S335M (lanes 7-9) HFE proteins in HEK 293 cells (as assayed by immunoblot) reduced Ft expression to levels below that of the parental cell line (lanes 1-3). Ft levels responded to cellular iron status. All cell types expressed less Ft in the presence of desferoxamine (Df, lanes 2, 5, and 8). Additionally, all cell types increased Ft expression in the presence of $Fe(NTA)_4$ (Fe, lanes 3, 6, and 9). These results are representative of more than three independent experiments with similar results.

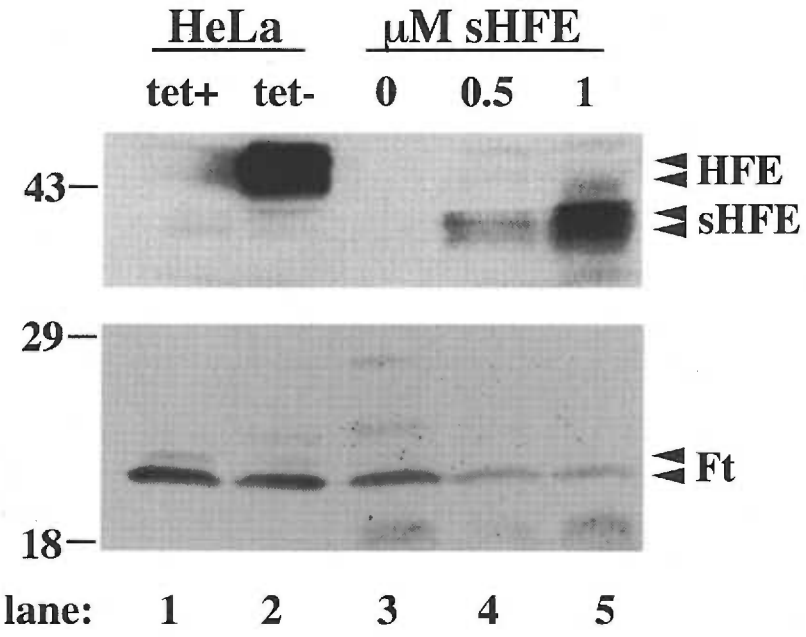

Figure 2.7. Soluble HFE reduces Ft expression in HEK 293 cells. (B) Addition of soluble HFE (sHFE) to the growth medium of parental HEK 293 cells (lanes 4-5) reduced Ft expression (panel B, lanes 4 and 5) as assayed by immunoblot. The reduction was not as extensive as that of full length HFE (compare lanes 1 and 2 versus 3 and 5). (A) Immunoblot analysis of sHFE confirmed the association of sHFE with these cells (lanes 4 and 5). These results are representative of more than three independent experiments with similar results.

CHAPTER 3

The Hereditary Hemochromatosis Protein, HFE, Specifically Regulates Tf-Mediated Iron Uptake in HeLa Cells

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SUMMARY

HFE is the protein product of the gene mutated in the autosomal recessive disease hereditary hemochromatosis. At the cell surface, HFE complexes with transferrin receptor, increasing the dissociation constant of transferrin for its receptor 10 fold. HFE does not remain at the cell surface, but traffics with the transferrin receptor to transferrin-positive internal compartments. Using a HeLa cell line in which the expression of HFE is controlled by tetracycline, we show that the expression of HFE reduces ^{55}Fe uptake from transferrin by 33%, but does not affect the endocytic or exocytic rates of transferrin receptor cycling. Therefore, HFE appears to reduce cellular acquisition of iron from transferrin within endocytic compartments. HFE specifically reduces iron uptake from transferrin, as non-transferrin-mediated iron uptake from Fe-NTA is not altered. These results explain the decreased ferritin levels seen in our HeLa cell system and demonstrate HFE's specific control over the transferrin-mediated pathway of iron uptake. These results also have implications for the understanding of cellular iron homeostasis in organs such as the liver, pancreas, heart, and spleen that are iron loaded in hereditary hemochromatotic individuals lacking functional HFE.

INTRODUCTION

HFE is the protein product of the gene mutated in the autosomal recessive disease HH which was first cloned in 1996 (Feder et al., 1996). It is therefore a relatively new member of the growing group of proteins involved in iron metabolism. HFE is remarkable in that it is a non-classical MHC class I type molecule, a characteristic that prevented its role in iron homeostasis from being recognized immediately. Over 80% of HH patients have the same mutation in the α_3 domain of HFE that prevents its heterodimerization with β_2 microglobulin (Feder et al., 1997). Consistent with HFE's classification as an MHC class I type molecule, this C282Y mutation has been shown to prevent cell surface expression and, presumably, function of the protein (Feder et al., 1997; Waheed et al., 1997). The generation of the HFE knockout mouse supports the finding that the C282Y mutation is, indeed, a loss of function mutation because the phenotype of the knockout mouse parallels the manifestation of HH in humans (Zhou et al., 1998).

Although the HFE gene was discovered by genetic mapping of individuals with a disease of iron overload, the protein's function in iron metabolism has not been immediately appreciated. The region of the HFE molecule normally associated with peptide binding and cell surface presentation in MHC class I molecules is too narrow to bind peptides. HFE does not appear to bind iron either (Lebron et al., 1998). HFE does, however, associate with the TfR, a well characterized member of the iron metabolic pathway. The TfR is a type II transmembrane protein that binds the serum protein, diferric Tf, at the cell surface. The Tf-TfR complex is constitutively taken into the cell through clathrin-mediated endocytosis. At the low pH of the endosome a conformational change in the TfR facilitates iron release from Tf (Bali and Aisen, 1992; Bali and Aisen, 1991; Bali et al., 1991; Sipe and Murphy, 1991). Apo-Tf remains bound to the TfR until the complex cycles back to the cell surface. At the neutral pH of the extracellular environment, apo-Tf is

quickly replaced by diferric Tf and is free to bind more iron [for further discussion, see reviews (Enns et al., 1996; Lok and Loh, 1998)].

HFE's association with the TfR has been demonstrated in tissues and in cell culture (Feder et al., 1998; Parkkila et al., 1997). Wild type HFE has been shown to associate with the TfR in the placenta (Parkkila et al., 1997) and to have the same immunohistochemical staining pattern as the TfR in the crypt cells of the intestine (Parkkila et al., 1997). In cultured cells, HFE traffics with the TfR between the cell surface and Tf-positive perinuclear compartments (Gross et al., 1998). The association between HFE and the TfR has been shown to lower the affinity of the TfR for its ligand 10 fold (Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998). Other non-classical MHC class I molecules have been shown to affect ligand affinity for other receptors, such as the HLA H-2 protein's ability to associate with the insulin receptor and reduce all insulin binding sites to low affinity sites (Due et al., 1986; Fehlmann et al., 1985; Ferm et al., 1996; Phillips et al., 1986; Verland et al., 1989) and the ability of anti-HLA antibodies to reduce EGF binding to its receptor (Schreiber et al., 1981; Schreiber et al., 1984).

The physiological importance of the interaction between HFE and the TfR is not immediately clear. Diferric-Tf is present at concentrations of approximately 5 μM in the blood (Ponka et al., 1998; Young et al., 1984). The dissociation constant of Tf for the TfR-HFE complex is approximately 11 nM. Thus, the binding of diferric-Tf to its receptor is saturated in the presence of HFE. These results emphasize the putative role of HFE as a regulator of iron homeostasis that acts through the TfR cycle rather than having a role as a transporter or iron binding protein.

To investigate its influence on cellular iron metabolism, the ability of HFE to specifically regulate the Tf-mediated pathway of iron uptake was tested. We find that HFE does not alter the cycling kinetics of the TfR. Rather, HFE reduces the amount of iron assimilated from Tf by ~33% without affecting non Tf-mediated uptake of Fe-NTA. This finding gives more insight into HFE's role as an iron regulator through the Tf-mediated

pathway. Additionally, this focuses attention on the role of Tf-iron stores as a critical part of the "sensing machinery" that regulates dietary iron uptake and the kinetics of metabolic iron recycling.

MATERIALS AND METHODS

Cell lines- The fWTHFE/tTA HeLa cell line expressing wild type fHFE under the tet responsive promoter has been previously described (Gross et al., 1998). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 400 µg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE-) or without (HFE+) 2 µg/ml tetracycline.

Iodination- Human diferric Tf (Intergen, Co.) was labeled with Na[^{125}I] (DuPont, NEN) using lactoperoxidase as previously described (Warren et al., 1997).

^{125}I-Tf Uptake Protocol- The rate of ^{125}I-diferric Tf uptake was determined as previously described (Warren et al., 1997) with the following modifications. Uptakes were performed on subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cultures (~1x10^6 cells) washed 2 times with 2 ml DMEM-20 mM HEPES, pH 7.4 and preincubated in the same medium for 15 minutes at 37°C with 5% CO_2. At the 0 minute mark, 1 ml of specific (DMEM-20 mM HEPES, 2 mg/ml ovalbumin, 50 nM ^{125}I-Tf) or non-specific (specific medium with 1 mg/ml cold Tf) medium was added to the appropriate cells. Cells were incubated at 37°C with 5% CO_2 for 2, 4, 6, or 8 minutes. Externally bound Tf was stripped with an acidic buffer (0.5 N acetic acid, 0.5 M NaCl) for 3 minutes at 4°C . Then the cells were washed 4 times with 2 ml 4°C final wash buffer (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4) before addition of solubilization detergent (0.1% Triton X-100, 0.1% NaOH) and counting in gamma counter (Packard, CobraII Auto-Gamma). Surface TfR numbers were determined by counting the amount of ^{125}I-Tf bound after incubation with 50 nM ^{125}I-Tf for 90 minutes on ice at 4°C. Following the 90 minute incubation, the media was removed and cells were washed 4 times with 2 ml 4°C final wash buffer before solubilization and counting.

^{125}I-Tf Efflux Protocol- The rate of Tf efflux was determined as previously described by McGraw and Maxfield (McGraw and Maxfield, 1990) with the following modifications. Monolayers of subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cells grown in 35 mm plates were washed three times with 2 ml DMEM-20 mM HEPES, then preincubated in the same medium for 15 minutes at 37°C and 5% CO_2. The media was removed and cells were incubated for 2 hours with specific (50 nM ^{125}I-Tf in DMEM-20 mM HEPES and 2 mg/ml ovalbumin) or nonspecific (specific medium with 1 mg/ml unlabelled Tf) medium at 37°C and 5% CO_2. Cells were washed with 2 ml 37°C mild acid buffer (500 mM NaCl, 50 mM MES, pH 5.0) for 2 minutes, then washed 2 times with 2 ml 37°C final wash buffer with 100 μM Df (deferoxamine mesylate; Ciba-Geigy Limited; Basel, Switzerland) and 3 μg/ml Tf to prevent loading and rebinding of apo ^{125}I-Tf. 1 ml 37°C medium (DMEM-20 mM HEPES, 2 mg/ml ovalbumin) with 3 μg/ml Tf and 100 μM desferoxamine was added at the 0 minute mark and then at each of the appropriate time points, plates were placed on ice and the appropriate *efflux*, *surface* and *internal* samples were collected:

Efflux- Media was removed by pipetting, cells were washed one time with 1 ml final wash which was pooled and counted in gamma counter (Packard, CobraII Auto-Gamma).

Surface- Cells were incubated in 1 ml acid wash (0.2 N acetic acid, 0.5 M NaCl) for 3 minutes at 4°C then washed 1 time with 1 ml final wash which was pooled and counted.

Internal- Cells were stripped and washed as per *Surface* samples, then solubilized in 2 ml solubilization detergent and counted.

TfR Distribution- The relative TfR distribution between cell surface (external) and internal compartments was determined as previously described (Garippa et al., 1996; McGraw and Maxfield, 1990) with the following modifications. Subconfluent HFE- and HFE+

fWTHFE/tTA HeLa cells grown in 35 mm plates were either incubated for 90 minutes with 100 nM ^{125}I-Tf at 37°C with 5% CO_2 for *total* and *internal* measurements or with 100 nM ^{125}I-Tf at 4°C for *external* measurements in 1 ml specific (100 nM ^{125}I-Tf in DMEM-20 mM HEPES) or nonspecific (specific medium with 1 mg/ml unlabelled Tf) medium at 4°C. At the end of the incubation time, cells were placed on ice. *Total* and *external* measurements were determined by washing 4 times with 2 ml final wash at 4°C, then solubilizing and counting. The amount of internalized ^{125}I-Tf was determined by stripping the surface for three minutes with acid wash, washing 4 times with 2 ml final wash, then solubilizing and counting in gamma counter (Packard, CobraII Auto-Gamma).

^{55}Fe Loading of Human Apo-Tf- $^{55}FeCl_3$ (NEN) was complexed to nitrilotriacetic acid in a 1:200 ratio (Fe:NTA). Then, the ^{55}Fe-NTA was incubated with Tf in a 2:1 ratio for 1 hr in carbonate buffer (10 mM $NaHCO_3$, 250 mM Tris-HCl). ^{55}Fe-Tf was separated from free ^{55}Fe on a 20 ml G-50 Sephadex (Sigma) column. The resulting Tf was 82% saturated with iron.

^{55}Fe-Tf Uptake Protocol- ^{55}Fe uptake from Tf was determined essentially as described above for ^{125}I-Tf uptake with the following modifications. Uptakes were performed on subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cells grown in 35 mm dishes. Specific medium contained 100 nM ^{55}Fe-Tf and 2 mg/ml ovalbumin in wash medium (McCoy's 5A with 20 mM HEPES). Nonspecific medium was the same as specific with the addition of 1 mg/ml unlabelled Tf. After 45, 90, 135, or 225 minutes of uptake, cells were placed on ice and externally bound Tf was stripped with an acidic buffer (0.2N acetic acid, 500 mM NaCl, 1 mM $FeCl_3$) for 3 minutes at 4°C. Cells were solubilized in 1 ml of the same solubilization detergent described above. Lysates were mixed with 6 ml UniverSol (ICN) and counted for 10 minutes in a scintillation counter (Beckman LS 6000SC) with a window

of 0-350 nm. TfR numbers were determined as described for the ^{125}I-Tf uptake protocol above.

^{55}Fe -NTA Uptake Protocol- ^{55}Fe -NTA uptake procedures were modified from a previously described protocol by Inman and Wessling-Resnick (Inman and Wessling, 1993). A ^{55}Fe-NTA solution was made by the addition of $^{55}FeCl_3$ to NTA buffer (80 μM NTA, 20 mM HEPES-TRIS) for a final concentration of 20 μM. Subconfluent fWTHFE/tTA HeLa cells grown in 35 mm dishes were washed 2 times with 2 ml of McCoy's 5A-20 mM HEPES at 37°C. Wash medium was replaced with 1 ml of specific (200 nM ^{55}Fe-NTA in wash medium) or nonspecific (specific medium with 1 mM Fe-NTA) medium. After 2, 5, 10, 15 or 30 minutes of uptake, cells were placed on ice and 3 ml 4°C quench buffer (1 mM Fe-NTA, 25 mM HEPES, 150 mM NaCl) was added for 20 minutes. Finally the cells were washed 3 times with 2 ml 150 mM NaCl at 4°C, solubilized and counted as for ^{55}Fe-Tf uptake protocol described above.

Iron Treatments- HFE- and HFE+ fWTHFE/tTA HeLa cells were seeded at ~$1.5x10^5$ cells per 35 mm dish. After 2 days, cells were treated with either 50 μM desferoxamine, 100 nM human diferric Tf (Intergen Co.), or 100 μM Fe-NTA overnight. Cells were lysed in NET-Triton (150 mM NaCl, 5 mM EDTA, and 10 mM Tris (pH 7.4) with 1% Triton X-100) after at least 12 hours of treatment.

Western Immunodetection- Cell extracts from $2x10^5$ cells were diluted with 4X Laemmli buffer (Laemmli, 1970) and subjected to electrophoresis on 12% polyacrylamide-SDS gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis was performed using sheep anti-human TfR serum [described previously (Warren et al., 1997) 1:10,000 dilution], and sheep anti-human Ft antibody (the Binding Site, 1:100 dilution) followed by swine anti-goat secondary antibody conjugated to horse-radish

peroxidase (Boehringer Mannheim). Chemiluminescence (SuperSignal, Pierce) was performed per manufacturer's directions.

RESULTS

fHFE Does Not Alter Tf Endocytosis- fHFE's influence on TfR cycling kinetics was measured to determine how HFE regulates iron levels in the cell. In previous studies, a cell line expressing an fHFE under the control of the tet repressible system was established (Gross et al., 1998). Induction of fHFE expression by the withdrawal of tetracycline from the medium resulted in decreased Ft levels. One mechanism by which HFE could lower the amount of iron taken up into cells would be by decreasing the rate of endocytosis of the TfR. fHFE co-traffics with TfR between the cell surface and Tf positive perinuclear compartments (Gross et al., 1998) and therefore might have some influence on TfR endocytic kinetics.

The rate of ^{125}I-Tf uptake was measured to test whether or not fHFE alters the endocytosis of the TfR and therefore the amount of Tf-mediated iron uptake in fWTHFE/tTA HeLa cells expressing fHFE (fWTHFE/tTA HeLa cells). Uptake experiments were performed in the presence of 50 nM ^{125}I-Tf. The association of HFE with the TfR decreases the affinity of the TfR for Tf resulting in an increase in the Kd from 1.2 nM to 11 nM (Gross et al., 1998). At 50 nM Tf, essentially all the TfRs are occupied with Tf, even in the presence of fHFE. Cells were incubated with ^{125}I-Tf for 2, 4, 6, or 8 minutes at 37°C with 5% CO_2. The surface was stripped of bound Tf and the amount of ^{125}I-Tf inside the cell was counted. Both HFE- and HFE+ cells take up approximately 0.20 Tf/ surface TfR/ minute (Figure 3.1). The same rates of internalization were measured in cells treated with 100 nM ^{125}I-Tf, confirming that the binding of Tf to its receptor was rapid and saturating (data not shown). fHFE does not significantly alter the amount of ^{125}I-Tf uptake per TfR in fWTHFE/tTA cells and therefore does not affect the kinetics of TfR endocytosis.

fHFE Does Not Alter TfR Exocytosis- The amount of iron taken up into cells is a function of the kinetics of TfR cycling which depends on both the endocytic and exocytic rates. Since fHFE had no effect on TfR endocytosis, the rate of ^{125}I-Tf release was measured to test whether fHFE alters the exocytic rate of the TfR in HFE- and HFE+ fWTHFE/tTA HeLa cells. Cells were loaded with ^{125}I-Tf for 2 hours at 37°C with 5% CO_2 to saturate internal and external TfR with Tf. Then, the cell surface was stripped of Tf, and fresh media was added to the cells. The Tf released from internal cellular compartments was collected and counted at 2, 4, 6, and 8 minute time points. HFE- and HFE+ fWTHFE/tTA HeLa cells do not differ significantly in their rate of TfR exocytosis (Figure 3.2).

TfR Distribution is Not Changed in fHFE-expressing Cells- Since the uptake and efflux kinetics of Tf do not change with the expression of fHFE, the steady state distribution of external and internal TfRs should also remain the same between cells expressing and not expressing fHFE. To test this prediction, steady state external and internal pools of ^{125}I-Tf were measured in control cells (HFE-) or in cells expressing fHFE (HFE+). fHFE expression does not significantly alter the relative surface and internal distributions of the TfR (Figure 3.3). Approximately 20% of cellular TfRs is found at the cell surface, while 80% is found in internal vesicles. The distribution of the TfR between the cell surface and internal compartments is similar that reported previously in other cell lines expressing similar amounts of the TfR (McGraw et al., 1991; Stoorvogel et al., 1989; Williams and Enns, 1993) Combined with the uptake and efflux data reported above, these findings support the conclusion that fHFE has no effect on TfR cycling kinetics even though HFE associates and co-traffics with the TfR (Gross et al., 1998).

fHFE Reduces Iron Uptake from Tf But Does Not Alter Non-Tf Mediated Iron Uptake- We wanted to determine whether HFE affected the amount of Tf-mediated or non-Tf -mediated iron uptake since no effect on the cycling kinetics of the TfR was detected. HFE-

or HFE+ fWTHFE/tTA HeLa cells were incubated in the presence of 100 nM ^{55}Fe-loaded Tf for up to 240 minutes and the rate of ^{55}Fe uptake from Tf for HFE- and HFE+ cells was determined. Control cells take up ^{55}Fe from Tf at a rate of 0.291 pmoles Fe/10^6 cells/minute, whereas cells expressing fHFE take up ^{55}Fe from Tf at a rate of 0.193 pmoles Fe/10^6 cells/minute (Figure 3.4). These data show that fHFE expressing cells take up approximately 33% less iron from Tf than control cells that do not express fHFE.

To determine whether fHFE's influences were restricted to the Tf-mediated pathway of iron uptake or if fHFE additionally affected the non Tf-mediated iron uptake pathway, HFE- and HFE+ fWTHFE/tTA HeLa cells were incubated in the presence of 200 nM ^{55}Fe-NTA for up to 30 minutes. No significant difference was seen in iron uptake between HFE- and HFE+ cells (Figure 3.5). Combined, these results support the conclusion that HFE's regulation of iron uptake is specific to the Tf-mediated iron uptake pathway. The effect of HFE on Tf-mediated iron uptake is therefore a direct effect on the Tf-derived iron pathway rather than an alteration of all cellular iron metabolism.

Ft Levels are Responsive to Iron Treatment in fWTHFE/tTA HeLa Cells- The response of HFE- or HFE+ fWTHFE/tTA HeLa cells to iron loading or iron chelation was observed by detection of TfR and Ft levels on western blots. These experiments were performed to test whether HFE affects the ability of the cells to compensate for changing iron levels in the media as predicted by the known influences of IRPs on Ft and TfR expression. IRPs are the most extensively characterized mode of regulating intracellular iron concentrations. In the presence of low intracellular iron, IRPs bind to IREs in the mRNA of iron-regulated proteins. This interaction either stabilizes the mRNA by binding to the 3' end of the transcript (as is the case for TfR mRNA), or blocks translation by binding to the 5' end of the transcript, close to the site of translation initiation (as is the case for Ft mRNA). Thus, at low intracellular iron levels, the cell increases iron uptake by increasing TfR levels. It decreases iron storage by lowering Ft levels. When intracellular iron concentrations are

high enough to keep iron bound in the IRP, the IRP no longer binds the IRE. This acts to reduce iron uptake from Tf by reducing TfR levels and increasing Ft levels.

The response of cells to iron depletion and iron loading was measured in HFE- and HFE+ fWTHFE/tTA HeLa cells grown for 2 days in untreated growth medium and treated for at least 12 hours with growth medium supplemented with 50 μM desferoxamine to chelate iron, human Tf, or Fe-NTA. Under control conditions, cells expressing fHFE have no detectable Ft over background while those that do not express HFE show significant amounts of Ft (Figure 3.6). HFE- and HFE+ cells show increased Ft expression following treatment with 100 nM human Tf. HFE- cells increase Ft levels only about 2 fold upon inspection of the western blot. HFE+ cells have slightly less Ft than HFE- cells for the same treatment condition but this is a significant increase over the undetectable Ft levels in HFE+ cells without Tf treatment. In the same way, both HFE- and HFE+ cells showed increased Ft expression following treatment with 100 μM Fe-NTA.

TfR levels also changed in Figure 3.6 as predicted by the known influence of IRPs. TfR levels are slightly elevated in fHFE expressing cells versus HFE- cells in keeping with their iron depleted status. Both HFE- and HFE+ cells treated with desferoxamine show increased TfR levels above that in untreated cells. For both HFE- and HFE+ cells, TfR levels slightly decrease upon treatment with human Tf or Fe-NTA as compared to untreated cells. These results demonstrate that the outcome of the iron-responsive mechanisms used by the cell are not perturbed by fHFE expression. Rather, fHFE imposes an additional but separate homeostatic mechanism.

The sensitivity of the cellular IRP response to iron was also tested in the presence and absence of fHFE. Even at Tf concentrations below saturation of the TfR and fHFE-TfR complex, such as at 5 nM, Ft levels are greater for both HFE- and HFE+ cells than without Tf treatment (Figure 3.7). However, due to the difference in the Tf binding affinity and the reduced iron uptake from Tf, HFE+ cells express consistently smaller amounts of Ft than HFE- cells under the same Tf treatment conditions. Ft levels are also

increased in HFE- and HFE+ cells when treated with Fe-NTA at concentrations as low as 50 nM. Ft levels for HFE+ cells remain lower than HFE- cells due to their initially depressed intracellular iron levels. The observation that the cells respond to Tf-derived iron at much lower concentrations than NTA derived iron is consistent with the association constants and the kinetics of the protein components of those systems. Detection limits of the enhanced chemiluminescence and X-ray film prevent quantitative conclusions from these data, however the qualitative results provide strong evidence that HFE does not prevent the IRP-dependent regulation of Ft and TfR expression. Rather HFE alters the "set point" of intracellular iron levels in a Tf-mediated manner.

DISCUSSION

The mechanism by which HFE controls iron homeostasis is not yet known. We have previously reported that fHFE specifically reduces Ft levels in fHFE-expressing HeLa cells, showing a direct relationship between HFE expression and cellular iron homeostasis (Gross et al., 1998). fHFE traffics with the TfR to Tf-positive perinuclear vesicles, which are one site of cellular iron absorption (Gross et al., 1998). Regulated Tf and non-Tf mediated iron uptake pathways have both been described for the HeLa tissue culture system (Kaplan et al., 1991; Penhallow et al., 1986; Planas-Bohne and Duffield, 1988) [for further discussion, see review (de Silva et al., 1996)]. This study shows that fHFE specifically decreases the Tf-mediated pathway of iron uptake.

fHFE has no effect on the kinetics of the TfR cycling in cells. If HFE were to slightly decrease the endocytic rate or increase the exocytic rate of the TfR, less Tf and therefore less iron would be taken up per unit time. At concentrations of Tf that saturate Tf binding to the HFE/TfR complex (50-100 nM) no difference in the uptake or release of ^{125}I-Tf from HFE- or HFE+ fWTHFE/tTA HeLa cells was detected. These results were confirmed by measuring the steady state distribution of TfRs. No redistribution of TfRs was detected between the cell surface and internal compartments when fHFE is expressed at steady state.

Although fHFE does not affect the TfR cycling kinetics, it does regulate cellular iron homeostasis through the Tf-mediated iron uptake pathway. At saturating concentrations of ^{55}Fe-loaded Tf (100 nM), 33% less iron was taken up by fWTHFE/tTA HeLa cells expressing fHFE. Since saturating amounts of Tf were used in these experiments, the ability of fHFE to lower the TfR affinity for Tf is not the mechanism responsible for the observed decrease in cellular iron. This experiment does not differentiate between release of iron from Tf and transport across the endosomal membrane to the cytoplasm. fHFE might effect either of these steps in Tf-mediated iron transport.

Since Ft levels are also reduced for HFE+ fWTHFE/tTA HeLa cells in culture, these results suggest that acquisition of iron from fetal bovine Tf in tissue culture media takes place through the TfR. Despite the larger dissociation constant of fetal bovine Tf for the human TfR, iron is still removed from the bovine Tf but the uptake is reduced in HFE+ cells.

fWTHFE/tTA HeLa cells were incubated in the presence of 200 nM ^{55}Fe-NTA to confirm that fHFE specifically regulates Tf-mediated iron uptake. Iron presented in this form bypasses the Tf-TfR pathway and enters the cell directly through a transporter (Inman and Wessling, 1993; Sturrock et al., 1990). Candidate transporters are SFT and DCT1/Nramp2 although other, currently unidentified, cell surface iron transporters may exist as well (Kaplan et al., 1991; Trinder et al., 1996). No significant difference was seen in the amount of non-Tf bound iron taken up by HFE+ cells as compared to HFE- cells, suggesting that HFE acts specifically through the Tf/TfR-mediated pathway of iron uptake.

Western blots were used to determine whether cells expressing fHFE could respond to bioavailable iron by increasing their Ft levels. Under control tissue culture conditions, HFE- cells have more Ft than HFE+ cells. Cells that do not express fHFE are capable of depleting Ft levels under low iron conditions, such as in the presence of the iron chelator desferoxamine. Both HFE- and HFE+ fWTHFE/tTA HeLa cells respond to iron loading via the Tf-mediated and non-Tf mediated pathways by increasing intracellular Ft levels and decreasing TfR levels. These results emphasize that cells expressing fHFE still regulate Ft levels under high and low iron conditions, presumably through the translational regulation of Ft by the IRPs. fHFE has simply changed the total amount of iron that is stored within the cell.

These studies demonstrate that in a nonpolarized cell line, HFE reduces the amount of iron taken up from the Tf-mediated iron uptake pathway. Several factors are involved in iron uptake from the endosome, any one of which could be affected by HFE. One characteristic of the endosome is its relatively low pH [between 5.5 and 6.5, depending on the cell type (Sipe and Murphy, 1991)]. This low pH is responsible for a conformational

change in both Tf and the TfR that facilitates the release of iron (Bali and Aisen, 1992; Bali and Aisen, 1991; Bali et al., 1991; Sipe and Murphy, 1991). HFE might regulate the lumenal pH of the endosome, preventing efficient removal of iron from Tf. In conflict with this hypothesis is evidence that suggests increases in endosomal pH slows recycling of the TfR (Johnson et al., 1993). We do not see any perturbations of TfR recycling, suggesting the endosomal pH is close to normal.

We favor the hypothesis that HFE prevents the pH-induced conformation of the TfR which potentiates the release of iron from Tf. Aisen and colleagues and Sipe and Murphy showed the importance of the TfR-induced conformational change in Tf for the release of iron in the endosome (Bali and Aisen, 1992; Bali and Aisen, 1991; Bali et al., 1991; Sipe and Murphy, 1991). At pH 6.0 the association of Tf with the TfR approximately doubles the rate of loss of iron from Tf (Egan et al., 1993). If this change is prevented then less iron would be taken up into cells. One caveat of this hypothesis is the observation that binding of soluble TfR to soluble HFE is not detectable at pH 6.0 (Lebron et al., 1998). For this mechanism to be responsible for the lower uptake of iron into cells, either HFE does interact with the TfR at the slightly higher pH of many endosomes or the membrane bound forms of the TfR and HFE interact with each other at endosomal pHs.

Alternatively, HFE might regulate the function of one of the known endosomal iron transporters such as Nramp2/DCT1 or SFT (Gutierrez et al., 1997; Su et al., 1998), thereby reducing iron transport across the endosomal membrane. HFE might simply use its association with the TfR for endocytosis. Once in the endosome, HFE might complex with transmembrane proteins responsible for transporting iron into the cytoplasm. HFE could modulate the transporters affinity for iron or its kinetics of iron transport across the endosomal membrane. Thus, HFE may be involved in maintaining homeostatic equilibrium of iron through its regulation of such a transporter. By modulating transporter activity in Tf-positive endosomes, HFE could facilitate appropriate flux of Tf-derived iron across the intestinal enterocyte. These three hypothesis need to be tested.

With the addition of the present findings from the fWTHFE/tTA HeLa system, we propose a new model for HFE's role in iron regulation (Figure 3.8). HFE imposes an additional regulatory step for iron uptake in HFE-expressing cells. IRE-mediated iron regulation does not appear to be sufficient to regulate iron homeostasis since organisms lacking HFE or Tf, but having functional IRPs eventually succumb to iron overload. We propose that HFE limits iron uptake from Tf by reducing the amount of iron released from Tf. In this way, HFE expression keeps Tf saturation higher in the blood. Without HFE (as is the case in HH patients), cells clear more iron from circulating Tf. The increased clearance of iron from the blood results in the increased uptake of iron from the intestine.

We have shown previously that HFE co-traffics with the TfR from the time of its initial synthesis to its cycling through Tf-positive endosomes (Gross et al., 1998). HFE may traffic with the TfR from the basolateral plasma membrane to endocytic vesicles of the enterocytes. This would be consistent with the perinuclear immunohistochemical staining of HFE (Parkkila et al., 1997) and TfR localization (Shah and Shen, 1994) in the cells of the intestinal crypts. HFE in the enterocyte endosome, where iron derived from apical dietary uptake and iron derived from bodily Tf stores meet, may play a key role in the regulation of iron transport out of the enterocyte into the blood. The mechanisms for such events remain unresolved.

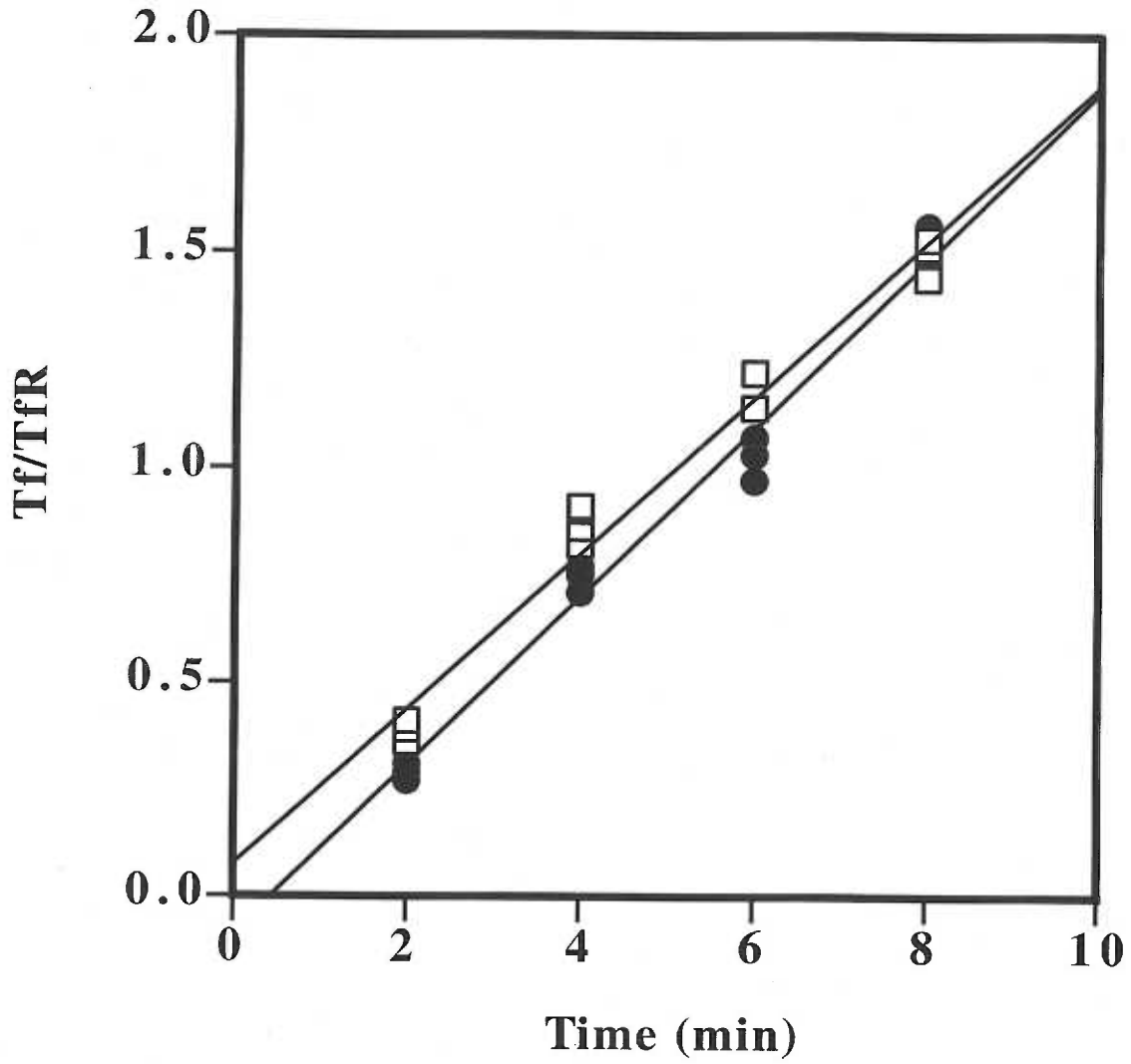

Figure 3.1. fHFE does not significantly alter Tf endocytosis. ^{125}I-Tf uptake was measured in 7.2X10^5 HFE- (open squares) or 7.0X10^5 HFE+ (closed circles) fWTHFE/ tTA HeLa cells. The rate of ^{125}I-Tf uptake per surface receptor per minute for HFE- and HFE+ cells was 0.198 (r^2= 0.901) and 0.213 (r^2=0.925), respectively. Linear regression was determined in the Cricket Graph program. These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

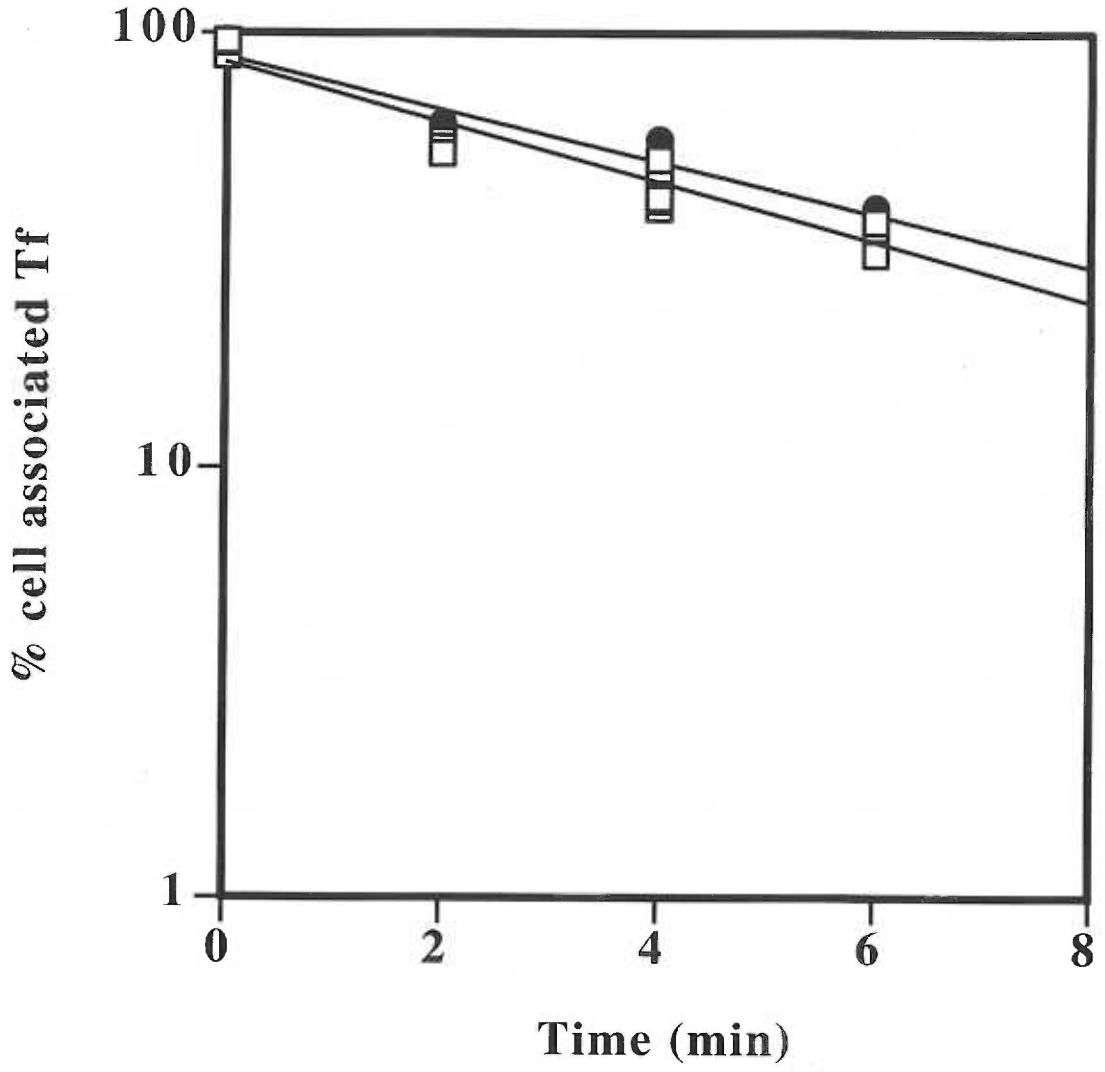

Figure 3.2. fHFE does not significantly alter Tf exocytosis. ^{125}I-Tf release was measured in 9.3X10^5 HFE- (open squares) or 7.7X10^5 HFE+ (closed circles) fWTHFE/tTA HeLa cells. The rate of ^{125}I-Tf release as a percentage of the total cell associated Tf per minute for HFE- and HFE+ cells was 0.052 (r^2=0.916) and 0.047 (r^2= 0.929), respectively. Linear regression was determined in the Cricket Graph program. These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

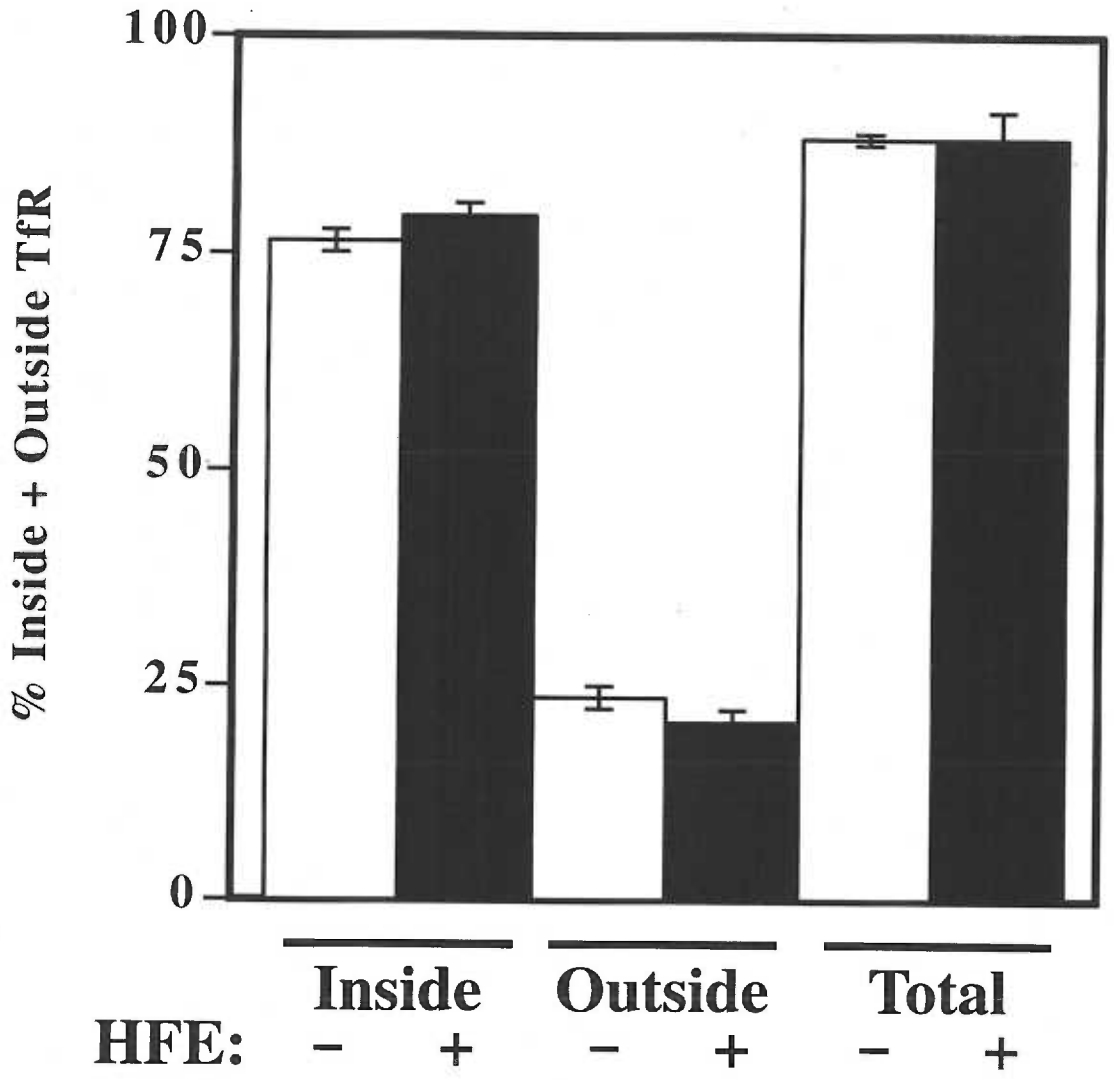

Figure 3.3. fHFE does not significantly alter TfR distribution. TfR distribution was measured in $7.6X10^5$ HFE- (open bars) or $6.1X10^5$ HFE+ (closed bars) fWTHFE/ tTA HeLa cells. The percentage of receptors on the cell surface or in internal compartments did not differ significantly between the two cell types. Both express approximately 20% of TfR on the cell surface (outside) and 80% in intracellular vesicles (inside). These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

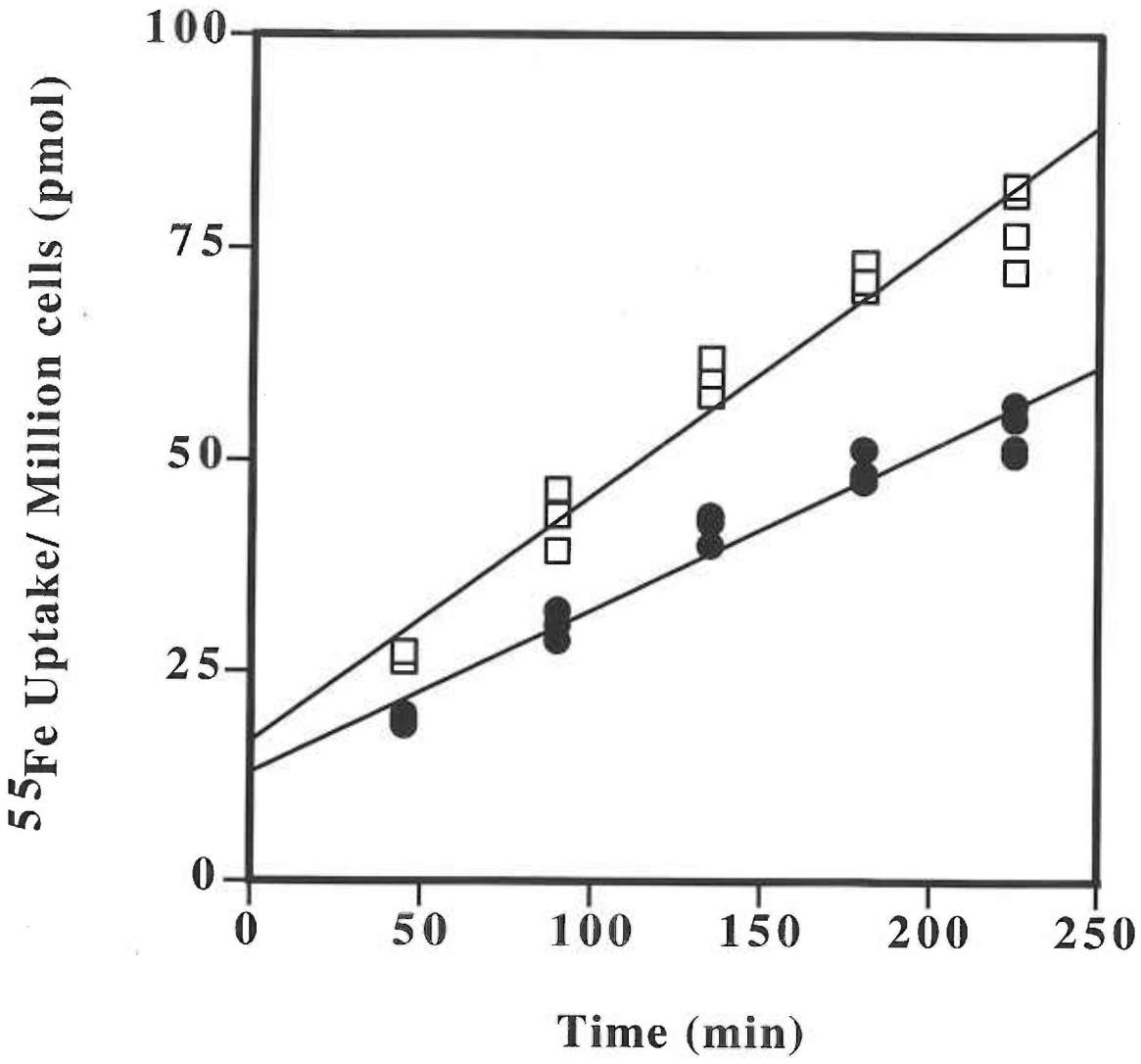

Figure 3.4. fHFE significantly reduces iron uptake from Tf. ^{55}Fe uptake from Tf was measured in 6.8X10^5 HFE- (open squares) or 5.6X10^5 HFE+ (closed squares) fWTHFE/tTA HeLa cells. The rate of ^{55}Fe uptake per minute per 10^6 HFE- and HFE+ cells from Tf was 0.291 (r^2=0.939) and 0.193 (r^2=0.923), respectively -- a 33% reduction specific to HFE expression. Linear regression was determined in the Cricket Graph program. These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

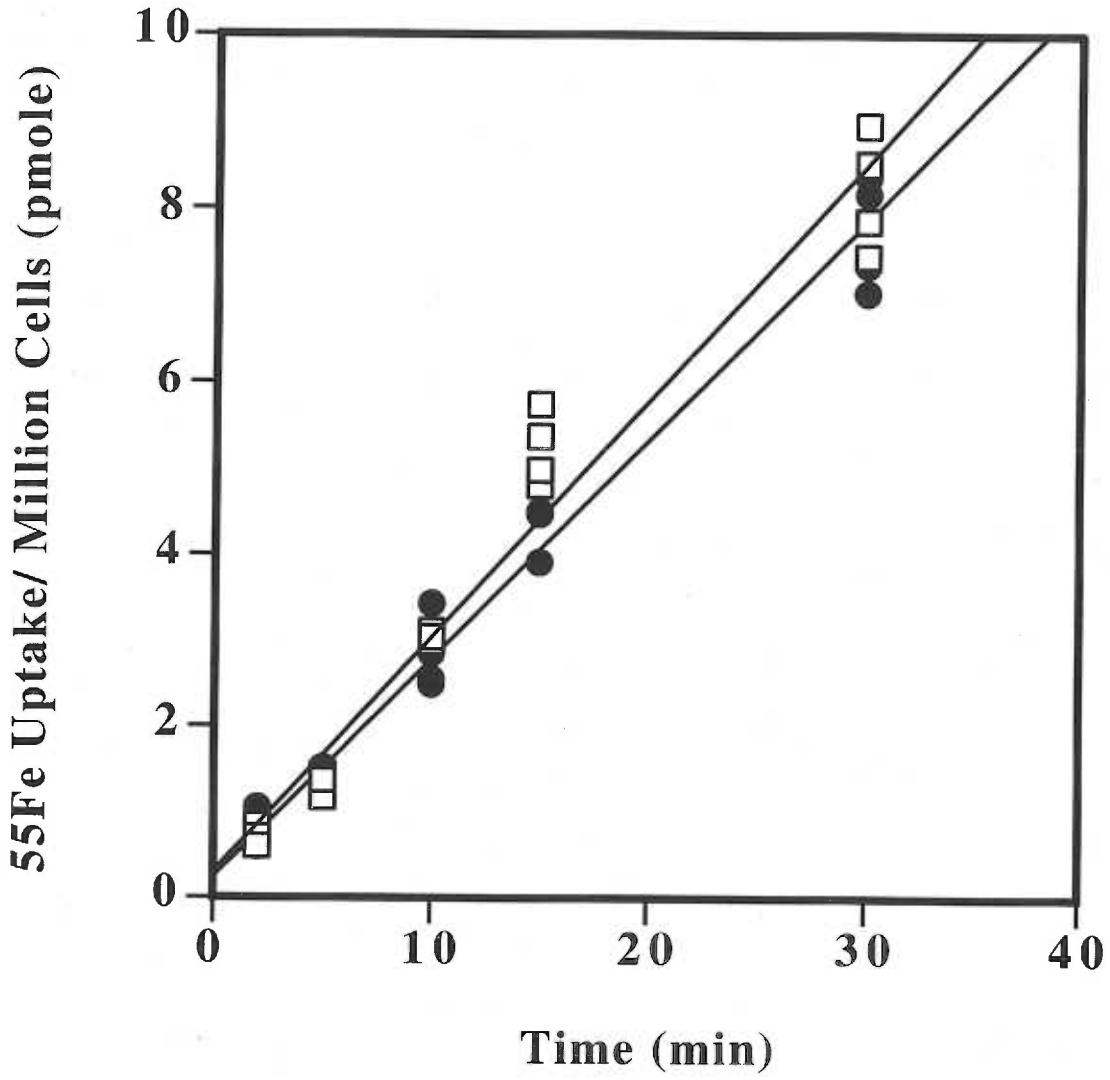

Figure 3.5. fHFE does not significantly alter non-Tf mediated iron uptake. ^{55}Fe uptake from ^{55}Fe-NTA was measured in $1.5X10^6$ HFE- (open squares) or $1.8X10^6$ HFE+ (closed circles) fWTHFE/tTA HeLa cells. The rate of ^{55}Fe uptake in pmoles per 10^6 cells per minute for HFE- and HFE+ was 0.275 (r^2=0.964) and 0.255 (r^2=0.975), respectively. Linear regression was determined in the Cricket Graph program. These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

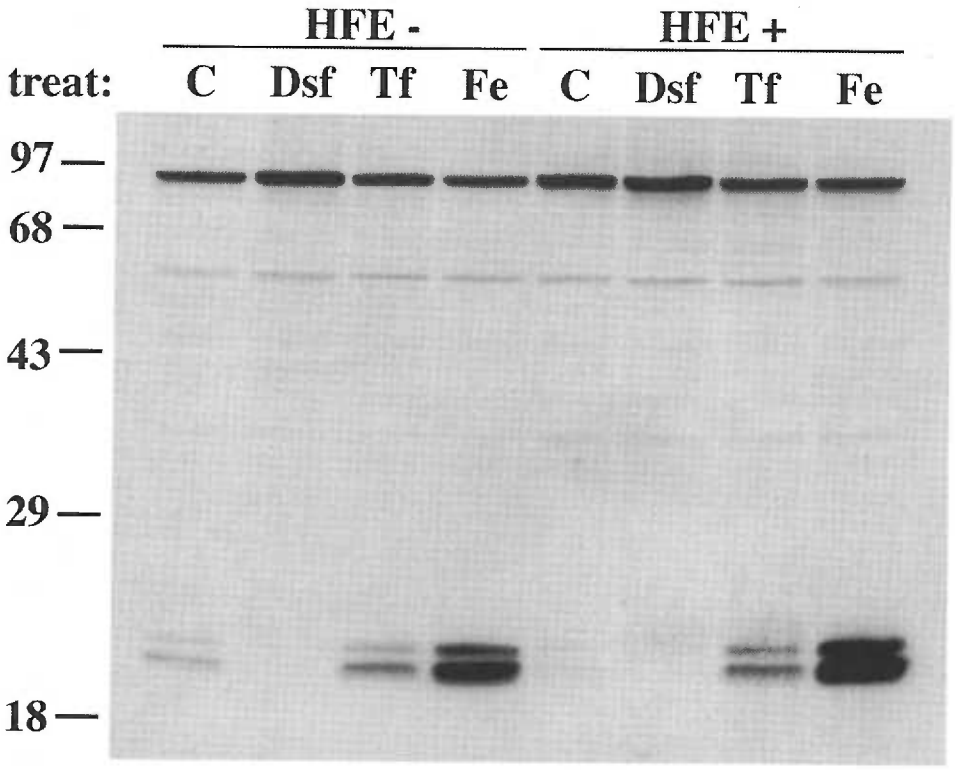

Figure 3.6. Ft levels are responsive to iron treatment in fWTHFE/tTA HeLa cells. Lysates of $2X10^5$ HFE- or HFE+ fWTHFE/tTA HeLa cells were run on a 12% denaturing polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-human TfR (1:10,000) and sheet anti-Ft (1:100) antibodies and swine anti-goat HRP-conjugated secondary antibody (1:10,000).Chemiluminescent detection of ~94, and 21/19 kDa bands represent TfR and Ft, respectively. The increased TfR expression and decreased Ft expression in fHFE expressing cells is consistent with previously reported results. Both cells types increase Ft levels in the presence of 100 μM $Fe(NTA)_4$, an iron source, and increase TfR levels in the presence of 50 μM Dsf, an iron chelator. These results are representative of four experiments without significant variation between experiments.

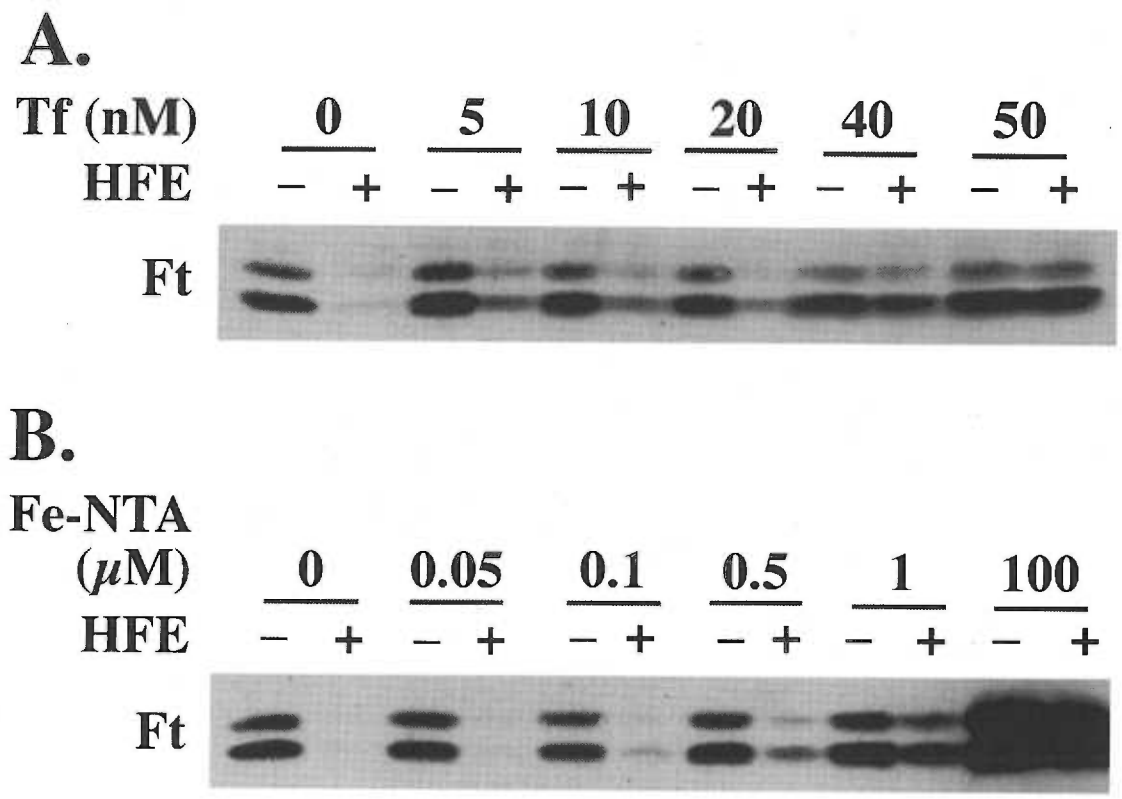

Figure 3.7. Sensitivity of fWTHFE/tTA HeLa cells to iron treatment. Lysates of $4X10^5$ HFE- or HFE+ fWTHFE/tTA HeLa cells treated 12 hours with indicated amounts of (A) Tf or (B) Fe-NTA were run on a 12% denaturing polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-Ft (1:100) antibodies and swine anti-goat HRP-conjugated secondary antibody (1:10,000). Sensitivity to Tf and NTA derived iron sources is within the concentration range consistent with the association constants of the TfR and iron transport proteins.

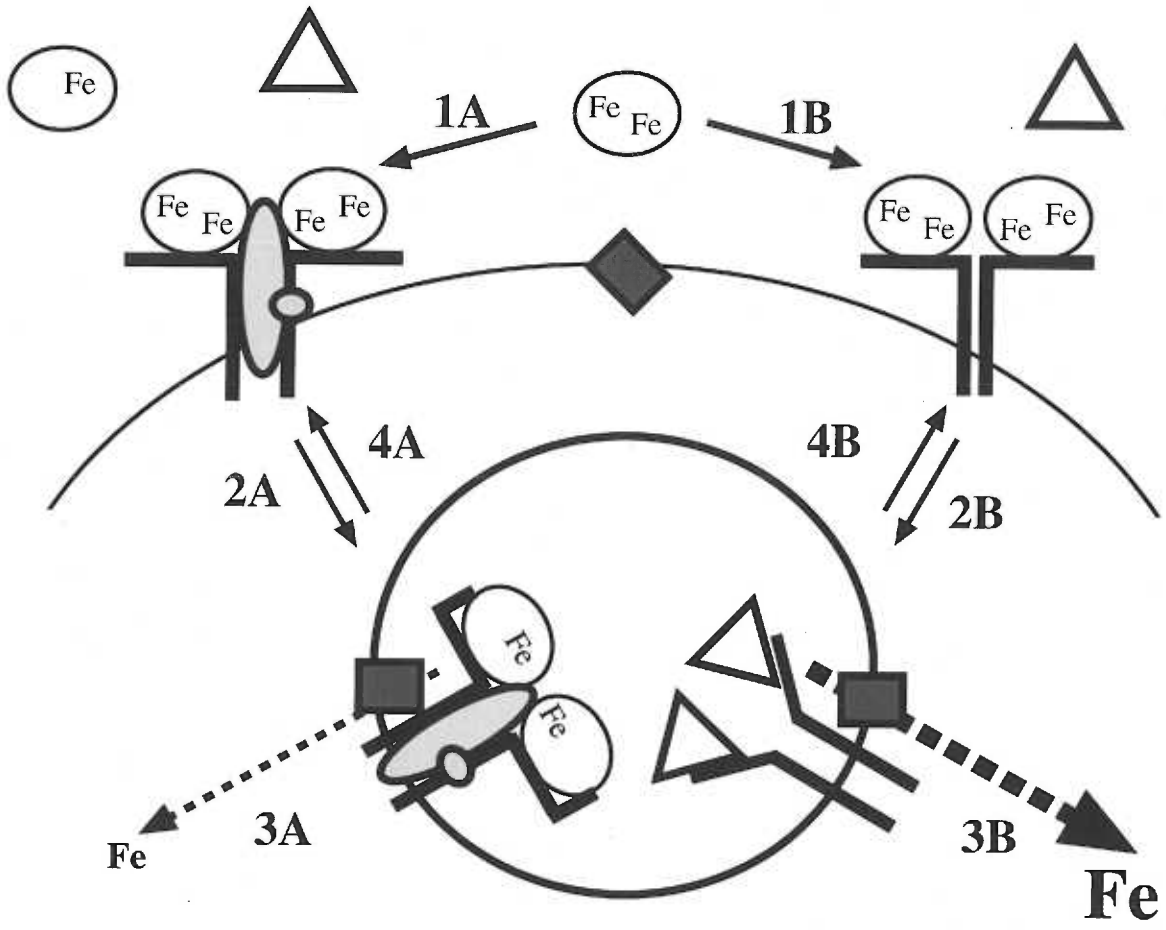

Figure 3.8. Model of HFE action on iron homeostasis in nonpolarized cells. Diferric Tf is present at a concentration of approximately 5 μM in the blood and is therefore capable of saturating all TfRs, even in the presence of HFE (arrow 1A). HFE traffics with Tf and TfR to the endosome (arrow 2A). Once the endosome has acidified, iron is released from Tf and transported through an endosomal iron transporter such as DMT1 or SFT (arrow 3A). In the presence of HFE, TfR does not potentiate the full release of iron from Tf, leaving some iron bound to Tf. Tf then cycles out of the endosome and back to the cell surface, resulting in release of ferric- rather than apo-Tf (arrow 4A). TfR that is not associated with HFE (as is the case in HH patients) also binds diferric-Tf preferentially at the cell surface (arrow 1B). TfR internalization is identical to the TfR/HFE complex. Tf then cycles out of the endosome and back to the cell surface where apo-Tf is released (arrow 4B). By facilitating the cellular uptake of less iron and keeping plasma Tf saturation higher, HFE effectively reduces the dietary iron demand.

CHAPTER 4

Investigation of HFE and Transferrin Competition for the Transferrin Receptor: A Cellular Study

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Manuscript in Progress

SUMMARY

The hereditary hemochromatosis gene product, HFE, is closely associated with the transferrin-mediated pathway of iron uptake. HFE cycles with the transferrin receptor to endosomal compartments where it specifically reduces the amount of iron assimilated from transferrin. Recent reports have indicated that soluble HFE and transferrin compete for the same or overlapping binding sites on the transferrin receptor. We have assayed the competitive nature of soluble HFE and transferrin on the transferrin receptor of cells in culture. Additionally, we have tested for competition between transferrin and membrane bound HFE for internalization with the transferrin receptor. We report that while the soluble form of HFE can compete with transferrin, it is not a very potent competitor. Additionally, we find that membrane bound HFE and the transferrin receptor maintain a strong association in the presence of saturating transferrin. This result indicates that the association of the membrane bound form of HFE with the transferrin receptor may be stabilized by domains outside of that which associates with the transferrin binding site on the transferrin receptor.

INTRODUCTION

HH is the most common inherited disease of people of Northern European Caucasian descent (Barton and Bertoli, 1996). Individuals with the disease inherit a founder mutation in the HFE gene (Feder et al., 1996). The most severe mutation prevents proper folding and cell-surface presentation of this protein required for the maintenance of iron homeostasis (Feder et al., 1997; Lebron et al., 1998; Waheed et al., 1997). HFE deficiency leads to a chronic increase in iron absorption from the duodenum and iron overload of parenchymal tissues (Feder et al., 1996; Levy et al., 1999; McLaren et al., 1991; Rothenberg and Voland, 1996; Santos et al., 1996; Zhou et al., 1998).

HFE's exact role in organismal iron homeostasis remains unclear, but is likely to be closely related to its role in the regulation of cellular iron homeostasis (Andrews and Levy, 1998; Cox and Kelly, 1998; Kuhn, 1999). The observation that HFE associates with the TfR (Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998; Parkkila et al., 1997) has focused much attention on HFE's modulation of iron uptake through the Tf-mediated iron uptake pathway. HFE is capable of associating with the TfR early in the biosynthetic pathway (Gross et al., 1998; Salter-Cid et al., 1999) and cycles with the TfR to endocytic compartments (Gross et al., 1998). The TfR, expressed on the cell surface, binds the serum protein diferric Tf with nanomolar affinity. The receptor-ligand complex is constitutively endocytosed at which point the pH inside the vesicle drops, potentiating iron release from Tf (Bali and Aisen, 1991; Sipe and Murphy, 1991). Iron is transported across the endosomal membrane and incorporated into iron containing enzymes or stored in Ft [for review, see (Enns et al., 1996; Lok and Loh, 1998)].

Several investigators have observed that HFE reduces iron uptake through the Tf-mediated iron uptake pathway (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). The decreased iron influx establishes a lower set point for the cellular labile iron pool despite up-regulation of TfR and down-regulation of Ft (Corsi et al., 1999;

Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). This observation supports the hypothesis that HFE is a regulator of cellular iron homeostasis and may apply directly to the iron-dependent regulation of proteins responsible for organismal iron absorption and utilization (Eisenstein, 1998; Kuhn, 1999). Despite these observations concerning the effect of HFE on iron uptake from Tf, the nature of the interaction between HFE and the TfR in cells is not well understood. Several observations have been made concerning the HFE-TfR complex that are of primary importance when interpreting the mechanism of HFE action on Tf-derived iron uptake.

When HFE and the TfR are expressed in cells in culture, they form a complex of relatively high stability and are easily coimmunoprecipitated (Feder et al., 1998; Parkkila et al., 1997). This HFE-TfR complex has a steady state stoichiometry of one HFE per TfR dimer (Gross et al., 1998) and ~10 fold reduced affinity for diferric Tf (Feder et al., 1998; Gross et al., 1998). Since the concentration of diferric Tf in the plasma is close to 5 μM (Lane, 1975; Ponka et al., 1998), the relative effect of this affinity change on Tf uptake is minimal. If the concentration of diferric Tf at the cell surface is sufficient to saturate the HFE-TfR complex (~50 nM), there is no reduction in Tf uptake (Ikuta et al., 2000; Roy et al., 1999).

Experiments performed in solution with soluble HFE and soluble TfR, which lack the transmembrane and cytoplasmic domains, produced some results that were significantly different from those obtained from the full length forms. Soluble HFE and soluble TfR associate with nanomolar affinity at pH 7.4 (Lebron et al., 1998). While the stoichiometry of soluble HFE and soluble TfR in solution is one HFE per TfR dimer (Lebron et al., 1998), in the HFE-TfR co-crystals, the observed stoichiometry is two HFE per TfR dimer (Bennett et al., 2000). The difference in stoichiometry may result from the high concentration of soluble HFE in the crystal (approximately 8 mM). How closely the HFE-TfR complex in the cell membrane resembles this crystal structure remains under investigation.

Another caveat to the interpretation of the HFE-TfR complex is the observation that soluble HFE binds the TfR weakly in the presence of high concentrations of diferric Tf (Lebron et al., 1999). One interpretation of these results is that soluble HFE competes directly with Tf for binding to the TfR. Such an interpretation is not inconsistent with the observation that HFE associates with the TfR over a very large surface and overlaps with the hypothesized Tf binding site on the TfR (Bennett et al., 2000; Dubljevic et al., 1999). Overlapping binding sites for HFE and Tf on the TfR would suggest that HFE and Tf cannot simultaneously bind the same TfR. The application of this result is critical when interpreting the results of experiments that assay Tf uptake in HFE-expressing cells (Ikuta et al., 2000; Roy et al., 1999). If membrane bound HFE association with the TfR is identical to that of soluble HFE, saturating concentrations of Tf would be expected to displace membrane bound HFE from the TfR dimer, leaving it at the cell surface when the TfR internalizes.

In this report, we set out to further characterize the nature of the HFE-TfR interaction with Tf. We have used the soluble HFE molecule in competition experiments with Tf to characterize its qualities as a competitor of Tf binding. Additionally, we have assayed for the ability of Tf to compete full length fHFE from the TfR in fWTHFE/tTA HeLa cells. We conclude that while HFE is a competitor of Tf binding, it has low potency and the physiologic implications of such an interaction are unclear. Additionally, we conclude that concentrations of Tf that saturate the HFE-TfR complex do not disrupt the HFE-TfR interaction as measured by the ability of fHFE to internalize with the TfR.

MATERIALS AND METHODS

Cell lines- The fWTHFE/tTA HeLa cell line expressing wild type fHFE under the tet responsive promoter has been previously described (Gross et al., 1998). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE-) or without (HFE+) 2 μg/ml tet. The parental tTA HeLa cell line was grown in DMEM supplemented with 10% fetal bovine serum and 400 μg/ml G418 (Geneticin, Calbiochem), only. All cells were grown at 37°C with 5% CO_2 and propagated not longer than ninety days.

Iodination- Human diferric Tf (Intergen, Co.) was labeled with Na[^{125}I] (DuPont, NEN) using lactoperoxidase and used for Scatchard analysis as previously described (Warren et al., 1997).

Soluble HFE- Human recombinant soluble HFE has been described elsewhere (Lebron et al., 1998) and was a kind gift of Dr. Pamela J. Bjorkman, Howard Hughes Medical Institute, California Institute of Technology.

Competition Assays- Competition assays were performed in 35 mm dishes containing ~1X10^6 parental tTA HeLa cells. Cells were placed on ice and washed twice with 2 ml DMEM with 20 mM HEPES, pH 7.4 at 4°C. Competition medium containing 2 nM ^{125}I diferric Tf and the appropriate concentration of competitor in DMEM with 20 mM HEPES and 2 g/L ovalbumin, pH 7.4 at 4°C was added. The reaction was allowed to equilibrate on ice for 90 minutes at 4°C. Following equilibration, the cells were washed on ice four times with 2 ml final wash buffer (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4) at 4°C, harvested in solubilization detergent (0.1% Triton X-100, 0.1% NaOH) and counted in a gamma counter (Packard, CobraII Auto-Gamma).

Calculation of Ki and IC_{50}- The Ki and IC_{50} of diferric Tf or soluble HFE for the TfR were calculated from a representative competition experiment. A Pseudo-Hill plot (or logit-log plot) was generated by calculating log[percent of ^{125}I-Tf bound/ (100 - the percent of ^{125}I-Tf bound)] and plotting it against the logarithm of the concentration of the competitor. Linear regression was determined in the Cricket graph program using r^2 values and was used to generate a line of which the intercept on the abscissa is the IC_{50}. The Ki was calculated from the IC_{50} using the equation developed by Cheng and Prusoff (Cheng and Prusoff, 1973), Ki = IC_{50} / (1+ [^{125}I-Tf]/Kd). In all cases, the [^{125}I-Tf] was 2 nM. The Kd used for ^{125}I-Tf for the TfR alone was 1 nM, but the Kd used for ^{125}I-Tf for the HFE-TfR complex was 10 nM in accordance with our previously published results (Gross et al., 1998).

Assay for endocytosis of HFE in the presence of saturating Tf- This assay was performed essentially as described by Schmid and Carter (Schmid and Carter, 1990) with the following modifications. Approximately $1X10^6$ subconfluent HFE+ fWTHFE/tTA HeLa cells in 35 mm dishes were placed on ice and washed three times with PBS, pH 8.0 at 4°C. Cells were biotinylated on ice for 30 minutes with 0.5 g/L NHS-SS-Biotin (Pierce Chemical Co.) in PBS, pH 8.0 at 4°C according to the manufacturers instructions. The biotinylation was quenched when cells were then washed on ice three times with 2 ml PBS with 10 mM glycine, pH 8.0 at 4°C and incubated on ice for 10 minutes at 4°C. Cells were washed twice with 2 ml PBS with 10 mM glycine, pH 8.0. The quenching step was repeated two more times. After the last wash, cells were incubated in the presence of DMEM with 10% fetal bovine serum and 100 nM human diferric Tf (Sigma) for 20 minutes at 37°C, and 5% CO_2. Following the incubation at 37°C, cells were placed back on ice and washed three times with 2 ml PBS, pH 8.0. Then cells were washed twice with 2 ml MTN buffer (10 mM MESNA, 50 mM Tris, 100 mM NaCl, pH 8.6) and incubated

on ice at 4°C in MTN buffer to reduce the NHS-SS-biotin and remove it from the cell surface. The reducing step was repeated two more times. Then, the cells were incubated on ice in 200 mM iodoacetamide for 10 minutes at 4°C. Finally, the cells were washed twice with 2 ml PBS, pH 7.4 and cells were solubilized with NET-Triton (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4 with 1% Triton X-100). The amount of internalized, biotinylated TfR and fHFE was determined as described below.

Assay for redistribution of fHFE in the presence of saturating Tf- Approximately $1X10^6$ subconfluent HFE+ fWTHFE/tTA HeLa cells in 35 mm dishes were incubated 16 hours in the presence of 100 nM human diferric Tf. After the incubation, the cells were placed on ice and washed three times with PBS, pH 8.0 at 4°C. Cells were biotinylated on ice for 30 minutes with 0.5 g/L NHS-SS-Biotin (Pierce Chemical Co.) in PBS, pH 8.0 at 4°C according to the manufacturer's instructions. Cells were washed on ice four times with 2 ml PBS, pH 8.0 at 4°C, then solubilized with NET-Triton.

Strepavidin precipitation- Biotinylated cell extracts were incubated with 100 µl strepavidin agarose (Pierce) with rotation for one hour at 4°C. The strepavidin agarose was pelleted by centrifugation at 14000 Xg for one minute. The supernatant was removed to a clean tube by pipetting and prepared for Western immunodetection as described below. The pellet was also prepared for immunodetection as described below.

Western Immunodetection- Cell extracts or strepavidin bead precipitates were diluted with Laemmli buffer (Laemmli, 1970) and subjected to electrophoresis on 8 or 10% SDS-polyacrylamide gels under reducing conditions. The proteins were transferred to nitro-cellulose. Immunoblot analysis was performed using sheep anti-human TfR serum [described previously (Warren et al., 1997) at 1:10,000 dilution] or mouse anti-FLAG antibody, M2, (Kodak, 1:20,00 dilution) followed by the appropriate secondary antibody

conjugated to HRP (Roche Molecular Biochemicals, 1:10,000 dilution) and chemiluminescence (SuperSignal, Pierce) per manufacturer's directions.

RESULTS

Soluble HFE reduces the affinity of the TfR for diferric Tf on parental tTA HeLa cells without reducing the number of Tf binding sites- Scatchard plots on HFE+ fWTHFE/tTA HeLa cells showed that HFE reduces the affinity of the TfR for diferric Tf (Gross et al., 1998). They also indicated that HFE increased the number of total Tf binding sites. The most likely explanation for this increase is the activation of IRP RNA binding activity that stabilizes the TfR message and increases TfR number (Corsi et al., 1999; Riedel et al., 1999; Roy et al., 1999). The 52% up-regulation of surface TfR number we reported in HFE+ fWTHFE/tTA HeLa cells seemed consistent with the increase in TfR that was detected on Western blot (Gross et al., 1998).

In light of the crystal structure of the HFE-TfR complex which proposed overlap between the HFE and Tf binding sites on the TfR (Bennett et al., 2000; Dubljevic et al., 1999) and the recent data concerning the competitive nature of soluble HFE with Tf (Lebron et al., 1999), the interpretation of the Scatchard analysis on HFE+ fWTHFE/tTA HeLa cells has become more complicated. It is possible that each full length HFE bound to a TfR dimer reduced the number of Tf binding sites to half the number of TfRs in our fWTHFE/tTA HeLa cells. However, this decrease in Tf binding sites could have been compensated by an IRP-induced up-regulation of the TfR greater than two fold. The IRP-mediated increase would equalize the number of Tf binding sites when compared to HFE-fWTHFE/tTA HeLa cells. To assay whether HFE reduces the number of surface Tf binding sites, we tested the interaction of soluble HFE with the surface TfR on the parental tTA HeLa cells by Scatchard analysis (Figure 4.1). Because the experiment is performed at 4°C, soluble HFE cannot induce changes in the labile iron pool that would activate IRPs and affect surface TfR number.

^{125}I-diferric Tf binding to the surface of parental tTA HeLa cells in the presence of 16 nM or 32 nM soluble HFE showed that soluble HFE binding does not change the total

number of Tf binding sites. Addition of 16 nM soluble HFE increased the dissociation constant of Tf binding from a Kd of 2.0 nM (Figure 4.1 panel A, open squares) to 6.0 nM (Figure 4.1 panel A, closed circles). Analysis with the KinetAsyst program revealed that soluble HFE acts most like a competitive inhibitor at 16 nM, calculating a Ki for soluble HFE of approximately 16.1±1.8 nM. Addition of 32 nM soluble HFE increased the dissociation constant of Tf binding from a Kd of 2.7 nM (Figure 4.1 panel B, open squares) to a Kd of 7.8 nM (Figure 4.1 panel B, closed circles). Analysis with the KinetAsyst program revealed that soluble HFE also acts like a competitive inhibitor at 32 nM, calculating a Ki for soluble HFE of approximately 16.8±1.0 nM. The linearity of the slopes rather than a biphasic slope of the Scatchard plots shows that soluble HFE sufficiently saturates the TfRs at 16 and 32 nM such that no high affinity binding sites (i.e. free TfR) are evident.

Soluble HFE displaces Tf from binding sites on parental tTA HeLa cells but is not a potent competitor- To determine whether soluble HFE could act directly as a competitor of diferric Tf for binding to the TfR, higher concentrations of soluble HFE were used to displace ^{125}I-diferric Tf from the TfR on parental tTA HeLa cells (Figure 4.2). The IC_{50} of Tf for the TfR was estimated through the use of a Pseudo-Hill plot (described above) to be 4.0 nM. When this value was used for the determination of the Ki according to the method by Cheng and Prusoff (Cheng and Prusoff, 1973), the estimated value was 1.3 nM. This value is similar to our previous estimate of the Kd of Tf for the TfR (1 nM) (Gross et al., 1998).

The IC_{50} of soluble HFE for the TfR was also estimated through the use of a Pseudo-Hill plot to be 69.2 nM. The resulting Ki was estimated to be 57.7 nM. When calculating the Ki for soluble HFE, the Kd of Tf was set equal to 10 nM to account for the change in affinity for Tf when HFE is bound (Gross et al., 1998). The Ki determined for soluble HFE in this experiment is very different than the Ki of 16.5 nM that was estimated

by Scatchard analysis and the KinetAsyst program. The slope of the competition by diferric Tf generated by the Pseudo-Hill plot was –0.946 (r^2=0.928). The slope of the competition by soluble HFE was only -0.507 (r^2=0.844). The flattening of the slope indicates negative cooperativity for soluble HFE binding.

Diferric Tf does not prevent endocytosis of full length fHFE - We have shown previously that the amount of Tf uptake at 100 nM Tf does not change with expression of fHFE, indicating that the same amount of Tf is internalized per surface TfR in HFE- and HFE+ fWTHFE/tTA HeLa cells (Roy et al., 1999). The same result could have been achieved if Tf displaced fHFE from the TfR and was then internalized by the TfR. To determine whether saturating amounts of Tf could compete full length fHFE off the TfR, the amount of endocytosed fHFE was determined in the presence or absence of 100 nM Tf (Figure 4.3). The amount of surface labeled TfR internalized in 20 minutes did not change with incubation in the presence of 100 nM diferric Tf, as expected (compare Figure 4.3, lane 1 versus lane 2). Likewise, the amount of surface labeled fHFE internalized in 20 minutes did not change significantly with incubation in the presence of 100 nM diferric Tf (compare Figure 4.3, lane 5 versus lane 6), indicating that both fHFE and Tf can internalize when Tf is saturating.

Diferric Tf does not redistribute full length fHFE- To assay for a steady state redistribution of HFE in the presence of diferric Tf, we assayed the amount of fHFE on the cell surface and in internal compartments after treatment with diferric Tf for 16 hours (Figure 4.4). The distribution of the TfR did not change in the presence of 100 nM Tf (compare Figure 4.4 lanes 1 versus 2), consistent with previous findings that the TfR constitutively endocytoses (Ajioka and Kaplan, 1986) and consistent with the distribution of the TfR in our fWTHFE/tTA HeLa cells (Roy et al., 1999). Likewise, the distribution of fHFE did not

change significantly in the presence of 100 nM Tf (compare Figure 4.4 lanes 5 versus 6) indicating that Tf does not displace fHFE from the TfR or interfere with its cycling.

DISCUSSION

We have compared the interaction of soluble HFE and the TfR with the membrane bound fHFE and the TfR in an attempt to further understand the nature of the association between these two proteins of iron metabolism. At low concentrations, soluble HFE has a relatively high Ki, or dissociation constant (16.5 nM) for the TfR, even in the presence of Tf. The interaction of soluble HFE with the TfR is consistent with that of a competitive inhibitor of diferric Tf based on the interpretation of the Scatchard plot. Soluble HFE does not decrease the total number of Tf binding sites on the cell surface. With an increase in the concentration of soluble HFE from 16 nM to 32 nM, the Kd of Tf for the soluble HFE-TfR complex was increased from 6 nM to 7.8 nM. Despite the fact that the Kd of the TfR for Tf increased between 16 nM soluble HFE and 32 nM soluble HFE, the Ki of approximately 16.5 nM for soluble HFE was not significantly different between the two conditions.

To further investigate the interaction between soluble HFE and the TfR, direct competition experiments were performed against diferric Tf. Soluble HFE competed Tf from the TfR above 1 nM soluble HFE, but it was not a very potent competitor, having an IC_{50} (69.2 nM) more than a log higher than that of Tf (4.0 nM). This is reflected in the calculation of the Ki of soluble HFE (57.7 nM) being more than 40 times higher than that of Tf (1.3 nM). The competition of Tf by soluble HFE also shows negative cooperativity, indicating that the binding of soluble HFE, itself, to the TfR may reduce the probability of more soluble HFE binding.

To determine whether the competition of HFE and Tf for the TfR could be demonstrated in cells, the ability of diferric Tf to compete membrane bound fHFE from the TfR in fWTHFE/tTA HeLa cells was assayed indirectly. We reasoned that if Tf competed with membrane bound fHFE for the TfR as it competes with soluble HFE, the membrane bound fHFE associated with the TfR would be replaced with Tf. Since HFE requires

association with the TfR to internalize (Ramalingam et al., 2000), the membrane bound HFE would be left at the cell surface while the Tf-TfR complex internalized. However, the same amount of fHFE internalizes in the presence of 100 nM diferric Tf as in the absence of Tf, and a saturating amount of Tf did not significantly alter fHFE distribution. It is important to note that the biotin-based experimental protocol used for these experiments lacks precision. Over several experiments (CNR-351-352, CNR-354-356, CNR-360-364, CNR-366, CNR-368-370), some variation was observed for the distribution of fHFE and the TfR with Tf treatments. However, fHFE routinely internalized in the presence of 100 nM diferric Tf.

From these data, we conclude that the membrane bound form of HFE remains associated with the TfR even in the presence of diferric Tf. The disparity between these results and those generated with the soluble HFE protein (Lebron et al., 1999) may indicate that interactions exist between the TfR and HFE ectodomains that are sufficient to maintain the HFE-TfR complex. Such interactions must be independent of the Tf binding site, but are not implausible since HFE and the TfR interact over a relatively large surface area (Bennett et al., 2000). Additionally, the transmembrane and cytoplasmic domains might stabilize this complex in a manner that the soluble HFE lacks.

Another explanation for the disparity between the results generated with the soluble HFE protein versus fHFE expressed in cells in culture may be the sequence of association with the TfR. The evidence for competition of HFE and Tf for the TfR is the observation that soluble HFE can only bind the TfR efficiently when the TfR is not occupied by Tf (Lebron et al., 1999). While Tf binding may preclude access to the HFE binding site on the TfR, the reverse is not necessarily true. Although HFE reduces the affinity of the TfR for Tf, it may not preclude Tf binding. In cells, HFE and the TfR can associate very early in the biosynthetic pathway and may emerge from the secretory pathway as a preformed complex (Gross et al., 1998). *In vivo*, the sequence of binding to the TfR is HFE first and Tf second. In this way, HFE does not have to compete with Tf for binding to the TfR.

While HFE binding may change the affinity of the TfR for Tf, and even compete for the same binding site on the TfR, these results suggest that the HFE-TfR complex is stable for purposes of internalization. HFE may still have a role in the endosome as a part of the Tf-mediated cellular iron uptake pathway.

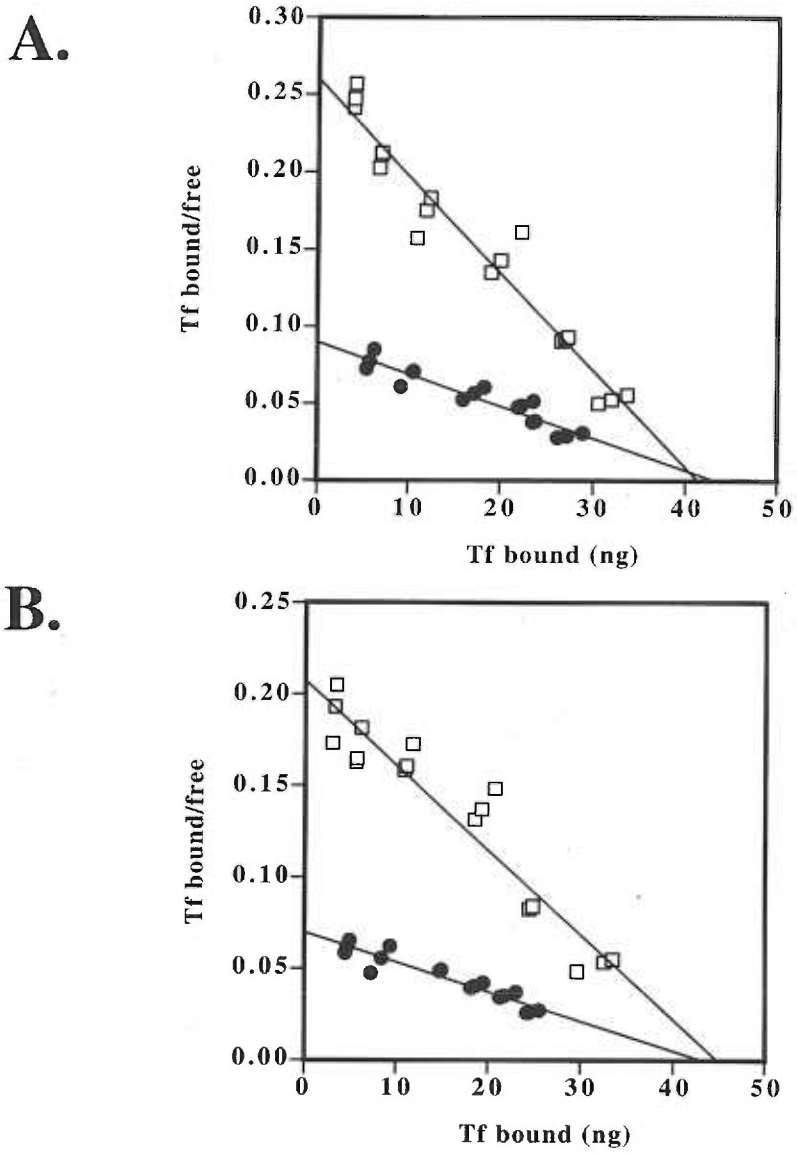

Figure 4.1 Soluble HFE reduces the affinity of the TfR for diferric Tf. Scatchard plot of diferric Tf binding to parental tTA HELa cells shows elevation of the dissociation constant of Tf in the presence of soluble HFE. (A) 16 nM soluble HFE increases the dissociation constant from 2.0 nM (open squares, no HFE) to 6.0 nM (closed circles, 16 nM HFE). (B) 32 nM soluble HFE increases the dissociation constant from 2.7 nM (open squares, no HFE) to 7.8 nM (closed circles, 32 nM HFE). No significant change in the x-intercept indicates the number of surface Tf binding sites remained the same. Linear regression was determined in the Cricket Graph program using r^2 values. These experiments were performed in triplicate but were not repeated.

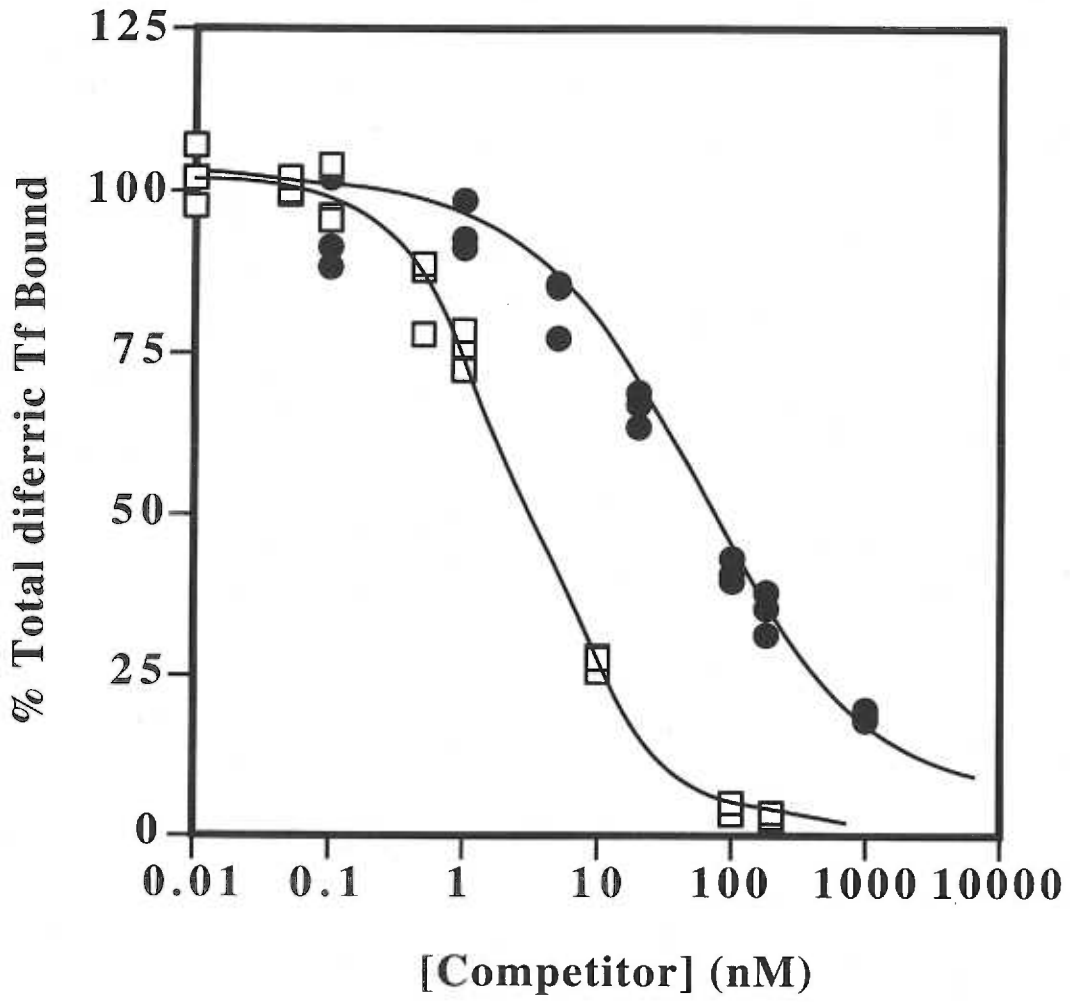

Figure 4.2. Soluble HFE displaces diferric Tf from the TfR. Competition analysis was performed for soluble HFE against diferric Tf on the TfR of parental tTA HeLa cells. The IC_{50} calculated for diferric Tf (open squares) was 4.0 nM, giving a Ki of 1.3 nM. The IC_{50} calculated for soluble HFE (closed circles) was 69.2 nM, giving a Ki of 57.7 nM. Replotting the data as a logit-log curve (Pseudo-Hill) plot revealed negative cooperativity for soluble HFE competition of diferric Tf (slope = -0.507, r^2= 0.844) but not for diferric Tf competition of diferric Tf (slope = -0.946, r^2= 0.928. Linear regression was performed in the Prism and Cricket graph programs using r^2 values. These results are representative of more than three experiments performed in triplicate with no significant variation between experiments.

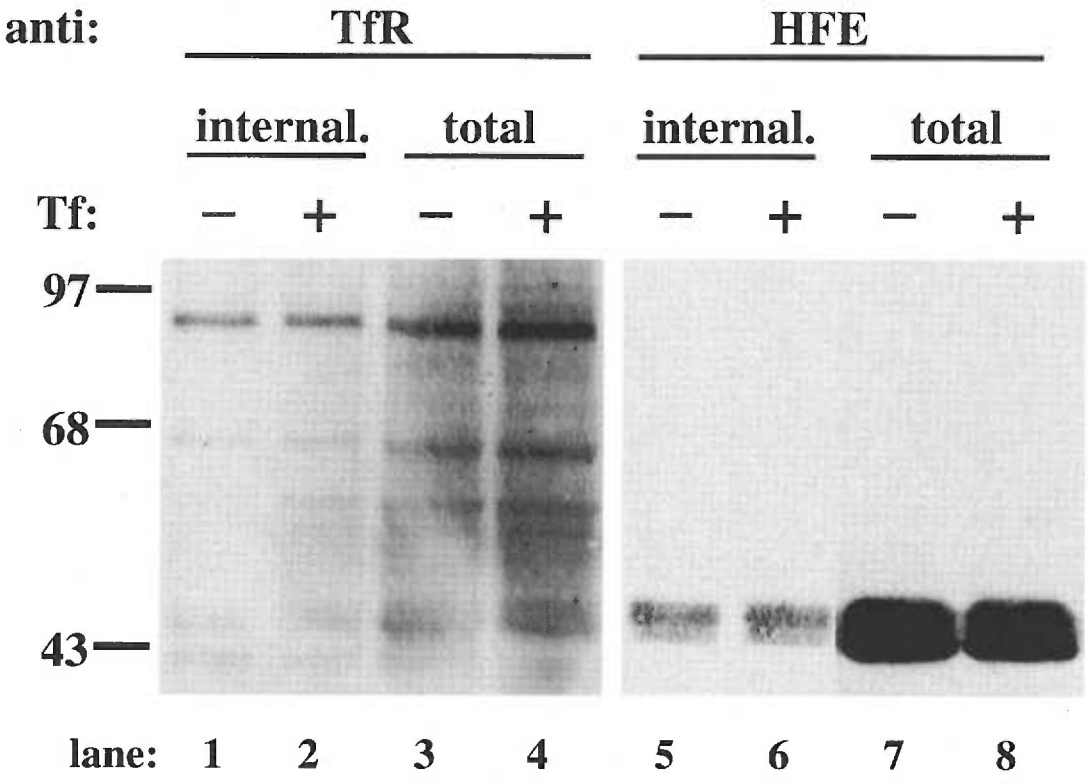

Figure 4.3. Diferric Tf does not prevent endocytosis of full length fHFE. Surface proteins of HFE+ fWTHFE/tTA HeLa cells were biotinylated. Then, cells were incubated in the presence (+) or absence (-) of 100 nM diferric Tf for 20 minutes at 37°C, during which time the TfR internalized with Tf and/or HFE bound. The surface was stripped of biotin, and the protected biotinylated proteins (internal.) were precipitated on strepavidin agarose. Biotinylated proteins were run on an 8% denaturing acrylamide gel for the TfR or a 10% denaturing acrylamide gel for HFE under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-human TfR (1:10,000) or mouse anti-FLAG (M2, 1:10,000 for HFE) antibodies and the appropriate HRP-conjugated antibody. The amount of internalized TfR or internalized HFE is much less than the total amount TfR of HFE. No significant difference in TfR internalization or HFE internalization was detected in the presence of 100 nM diferric Tf. These results are representative of two experiments without significant variation between results.

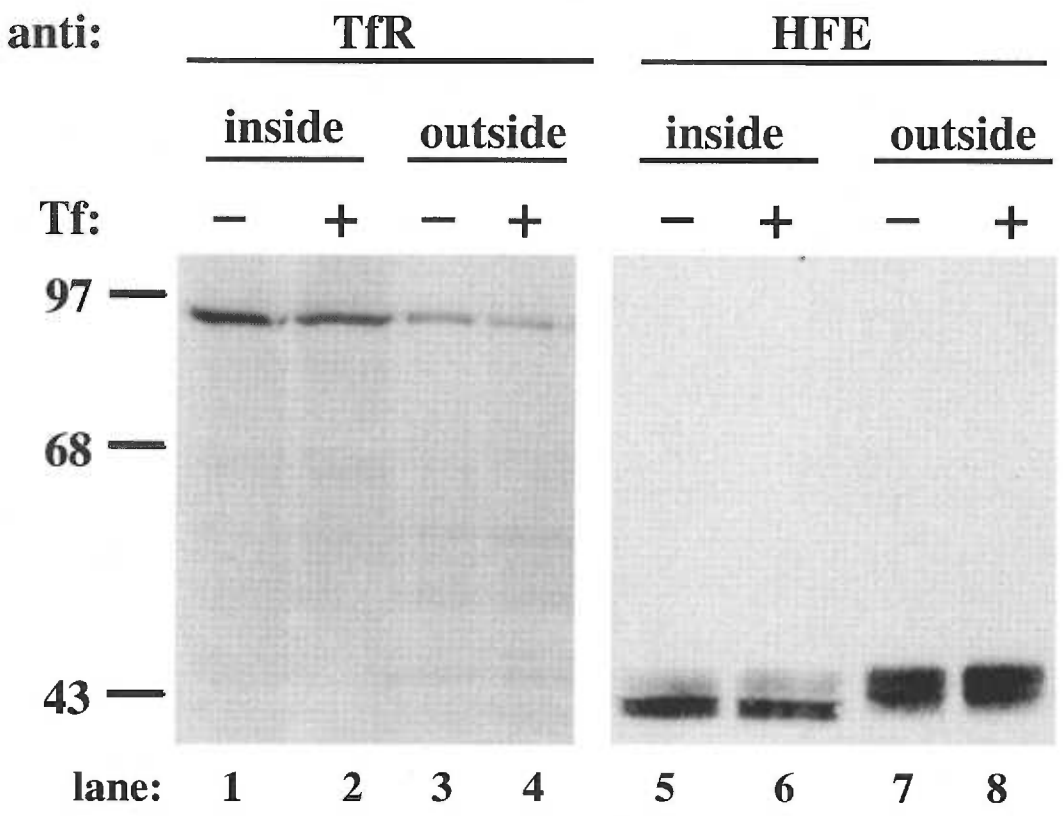

Figure 4.4. Saturating diferric Tf does not redistribute fHFE. HFE+ fWTHFE/tTA HeLa cells were incubated at least 16 hours in standard growth medium (Tf-), or growth medium with 100 nM diferric Tf (Tf+). Surface proteins were biotinylated and isolated with strepavidin agarose beads. Unlabeled (inside) proteins and biotinylated (outside) proteins were run on an 8% denaturing acrylamide gel for TfR or a 10% denaturing acrylamide gel for HFE under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-human TfR (1:10,000) or mouse anti-FLAG (M2 1:10,000 for HFE) and the appropriate HRP-conjugated secondary antibody. No significant difference was noted in the distribution of the TfR or HFE between internal endocytic compartments and the cell surface, indicating that HFE cycled with the TfR in the presence of saturating diferric Tf. These results are representative of two experiments without significant variation between experiments.

CHAPTER 5

The Effect of HFE Expression on Molecular Components of the Tf-Mediated Iron Uptake Pathway in HeLa Cells

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SUMMARY

The hereditary hemochromatosis gene product, HFE, is a proposed regulator of iron metabolism. In cultured cell lines, HFE traffics with the transferrin receptor to endosomal compartments where it reduces transferrin-mediated iron uptake. In this report, we tested several hypotheses by which HFE could reduce iron uptake from transferrin. We conclude that HFE does not significantly alter the pH dependence of the interaction of the transferrin receptor with transferrin, nor does it significantly alter endosomal pH. We also provide some preliminary evidence indicating that HFE does not alter iron transport through iron transporters active at low pH. The most intriguing result from this study was that while HFE increased the dissociation constant of the transferrin receptor for Fe_CTf, it did not reduce Fe_CTf binding to the same magnitude that it reduces diferric transferrin binding. We propose that such differences could result in more efficient uptake of unsaturated transferrin species, resulting in reduced transferrin-mediated iron uptake.

INTRODUCTION

HFE is the protein product of the gene mutated in HH (Feder et al., 1996). Loss of HFE function prevents efficient maintenance of iron homeostasis and leads to the accumulation of iron in parenchymal tissues (Levy et al., 1999; Rothenberg and Voland, 1996; Santos et al., 1996; Zhou et al., 1998). HFE's association with the Tf-mediated pathway of iron uptake is well established. HFE binds the TfR with high affinity (Lebron et al., 1998; Lebron and Bjorkman, 1999) and may compete with diferric Tf for binding to the TfR (Lebron et al., 1999), lowering the apparent affinity of the TfR for diferric Tf (Feder et al., 1998; Gross et al., 1998; Lebron and Bjorkman, 1999; Salter-Cid et al., 1999). The physiological relevance of this competition is not understood because diferric Tf is present at a concentration in the blood plasma which is sufficient to compete HFE from the TfR (Lane, 1975; Lebron et al., 1999). Despite this uncertainty, we and others have shown that HFE-expressing cells have reduced iron uptake from the same amount of diferric Tf when compared to cells that do not express HFE (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). Additionally, HFE establishes a lower set point for the labile iron pool (Corsi et al., 1999; Riedel et al., 1999). The mechanism by which HFE might reduce the accumulation of iron from Tf may occur at one of three stages in the Tf-mediated iron uptake pathway: 1) the selectivity of the HFE-TfR complex for Tf species; 2) the pH dependent release of ferric iron from Tf; or 3) the reduction and transport of ferric iron across the endosomal membrane.

Tf circulates through the blood plasma and binds a maximum of two ferric iron atoms with a dissociation constant less than 1×10^{-22} M. Tf exists as four species with approximately 35% of its iron binding sites saturated. Apo-Tf contains no iron, while monoferric forms bind iron in either the amino terminal iron binding site (Fe_NTf) or the carboxy terminal iron-binding site (Fe_CTf). Diferric-Tf accounts for the remaining species (Leibman and Aisen, 1979). Cell surface TfRs bind diferric Tf with high affinity (Kd = ~1

nM) (Enns and Sussman, 1981; Mason et al., 1998; Young et al., 1984), followed by Fe_CTf and Fe_NTf (Mason et al., 1998; Young et al., 1984) with Kd in the tens of nanomolar (Mason et al., 1998; Young et al., 1984), and apo-Tf with very low affinity (Kd = >200 nM) (Mason et al., 1998; Tsunoo and Sussman, 1983; Young et al., 1984).

Apo-Tf and the monoferric forms of Tf are present at three to five times the concentration of diferric Tf in the plasma (Lane, 1975; Ponka et al., 1998). However, the increased affinity of the TfR for diferric Tf over other Tf species results in the majority of TfRs bound by diferric Tf. While HFE increases the dissociation constant of the TfR for Tf 10 fold (Feder et al., 1998; Lebron and Bjorkman, 1999; Roy et al., 1999) and may compete with diferric Tf for the TfR (Lebron et al., 1999), HFE is not a very potent competitor when compared to diferric Tf, making the physiological significance of this competition unclear (chapter 4). However, if HFE were to equalize the affinity of the TfR for the different Tf species, it might alter the sensitivity of the HFE-TfR complex and the cellular labile iron pool to the iron saturation of plasma Tf.

The pH dependent interaction between the TfR and Tf also plays a critical role in the Tf-mediated iron uptake pathway. The Tf-TfR complex and Tf-HFE-TfR complex cycle between the cell surface and endosomal compartments. Once a vesicle endocytoses, it is acidified by a membrane bound H+/ATPase (Forgac, 1999). In this acidic compartment, pH-dependent conformational changes take place in Tf and the TfR. The acidic endosomal pH decreases the affinity of Tf for ferric iron, but additional changes in the TfR potentiate more efficient iron loss from Tf (Bali and Aisen, 1991; Sipe and Murphy, 1991). A comparison of the co-crystal structure of HFE and the TfR with the crystal structure of the TfR alone may indicate that some conformational changes occur in the TfR when HFE is bound (Bennett et al., 2000). Whether HFE-induced conformational changes in the TfR prevent it from assuming the conformation necessary to potentiate efficient loss of iron from Tf is not known.

Iron must cross the endosomal membrane if it is to be used by the cell. Iron that is not transported across the endosomal membrane cycles back out to the cell surface where it is bound by apo-Tf. At least two types of transport mechanisms are hypothesized for the endosomal compartment. The first, DMT1, is selective for ferrous iron (Fleming et al., 1997; Gunshin et al., 1997). Since iron is released from Tf in the ferric form, it must be reduced by an unidentified ferrireductase before it can be transported by DMT1. A second protein, SFT, has been identified that is thought to stimulate absorption of iron (Gutierrez et al., 1997). Whether SFT is truly an iron transporter or a modulator of another iron transport pathway is not yet known. Whether HFE has a direct affect on absorption of iron through either of these transport pathways is also unknown.

To understand the mechanism behind HFE's ability to specifically reduce iron uptake from Tf, we have characterized the interaction of HFE with these components of the endosomal iron uptake pathway in HeLa cells.

MATERIALS AND METHODS

Cell lines- The fWTHFE/tTA HeLa cell line expressing wild type fHFE under the tet responsive promoter has been previously described (Gross et al., 1998). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE-) or without (HFE+) 2 μg/ml tet. The parental tTA HeLa cell line was grown in DMEM supplemented with 10% fetal bovine serum and 400 μg/ml G418 (Geneticin, Calbiochem), only. All cells were grown at 37°C with 5% CO_2 and propagated not longer than ninety days.

Iodination- Human diferric Tf (Intergen, Co.) was labeled with Na [^{125}I] (DuPont, NEN) using lactoperoxidase as previously described (Warren et al., 1997).

Apo- and Monoferric Tf- Human recombinant Tf that contains the mutation D63S binds iron weakly in the amino terminus (Fe_CTf). Human recombinant Tf that contains the mutation D392S binds iron weakly in the carboxy terminus (Fe_NTf). Both human recombinant proteins have been described elsewhere (Mason et al., 1998) and were a kind gift of Dr. Anne Brown Mason, University of Vermont College of Medicine, Burlington, Vermont.

Competition of ^{125}I -diferric Tf with Tf mutants- The competition of ^{125}I -diferric Tf with several Tf mutants was used to assay their relative affinities for the TfR or the fHFE-TfR complex. Approximately 1X10^6 subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cells in 35 mm dishes were placed on ice and washed twice with 2 ml DMEM-20 mM HEPES, pH 7.4 at 4°C. 1 ml of DMEM-20 mM HEPES, pH 7.4 at 4°C with 2 mg/ml ovalbumin was added to the cells with 2 nM ^{125}I -diferric Tf and increasing concentrations of cold competitor as indicated in the figure legends. After a 90 minute incubation at 4°C, when

binding had reached equilibrium, cells were washed 4 times with 2 ml 4°C final wash buffer (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4) before addition of solubilization detergent (0.1% Triton X-100, 0.1% NaOH) and counting in gamma counter (Packard, CobraII Auto-Gamma).

pH specificity of iron unloading from Tf- The pH dependence of iron unloading from the Tf-TfR or Tf-fHFE-TfR complexes was determined as described previously (Dautry-Varsat et al., 1983) with the following modifications. Approximately $1X10^6$ subconfluent HFE- or HFE+ fWTHFE/tTA HeLa cells in 35 mm dishes were placed on ice and washed twice with 2 ml DMEM-20 mM HEPES, pH 7.4 at 4°C. 1 ml of DMEM-20 mM HEPES, pH 7.4 at 4°C with 2 mg/ml ovalbumin and 35 nM ^{125}I-diferric Tf was added to the cells. After a 90 minute incubation at 4°C, when binding had reached equilibrium, cells were washed twice with 2 ml 4°C final wash buffer. 1 ml binding buffer at 4°C [25 mM NaOAc, 150 mM NaCl, 2 mM $CaCl_2$, 50 μM desferoxamine (deferoxamine mesylate; Ciba-Geigy Limited; Basel, Switzerland) adjusted to pH 4.0, 4.5, 5.0, or 5.5; or 25 mM PIPES, 150 mM NaCl, 2 mM $CaCl_2$, 50 μM desferoxamine adjusted to pH 6.0, 6.5, 7.0; or 24 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 50 μM desferoxamine adjusted to pH 7.5] was added for 10 minutes on ice in which time the Tf-TfR or Tf-fHFE-TfR complex is hypothesized to unload iron. Any released iron was bound by the desferoxamine present in the buffer, leaving apo-Tf species bound to the surfaces of the cells. The binding buffer was removed and the cells were washed three times with 2 ml final wash buffer (pH 7.4). Apo-Tf species that were generated at the lower pHs remained bound to the TfR at low pH, but were released from the cell surface and removed with the increased pH of the final wash. Samples were solubilized in solubilization detergent and counted in a gamma counter.

Measurement of endosomal pH- The methods for measuring pH of endosomes have been described previously (Presley et al., 1993). Briefly, subconfluent HFE- or HFE+ fWTHFE/tTA HeLa cells and tet+ or tet- (mock induced) tTA HeLa cells were seeded on coverslips and grown for 2 days. Cells were labeled with medium containing 10 μg/ml Tf labeled with both rhodamine and fluorescein (F-R-Tf). Fluorescence images were collected between 5 and 15 min. of labeling with a Zeiss 63X planapochromat NA 1.4 oil immersion objective. Images were collected with a Zeiss Axiovert microscope using a Bio-Rad MRC-600 confocal attachment, with a 515-545 nm fluorescein and 575 nm rhodamine emission filter sets, and 488 nm excitation. A pH calibration curve was generated by plotting the average value of the rhodamine to fluorescein fluorescence ratio from cells fixed and maintained in a range of pH calibration buffers with 10 μM nigericin. The rhodamine to fluorescein ratio increases as the pH becomes more acidic because rhodamine fluorescence is pH independent, while fluorescein fluorescence is quenched as the pH decreases. Image processing was performed as described previously (Presley et al., 1993). Ratios of rhodamine to fluorescein fluorescence were calculated for individual endosomes (represented as pixels), and histograms were plotted of the pixel frequency for various R/F ratio values.

^{55}Fe-NTA Uptake Protocol- ^{55}Fe-NTA uptake procedures were modified from a previously described protocol by Inman and Wessling-Resnick (Inman and Wessling, 1993). A ^{55}Fe-NTA solution was made by the addition of $^{55}FeCl_3$ to NTA buffer (80 μM NTA, 20 mM HEPES-TRIS) for a final concentration of 20 μM. Approximately $1X10^6$ subconfluent fWTHFE/tTA HeLa cells grown in 35 mm dishes were washed 2 times with 2 ml of McCoy's 5A-20 mM HEPES, pH 6.0 at 37°C. Wash medium was replaced with 1 ml of specific (200 nM ^{55}Fe-NTA in wash medium), pH 6.0 or nonspecific (specific medium with 1 mM Fe-NTA) medium, pH 6.0. After 2, 5, 10, 15 or 30 minutes of uptake, cells were placed on ice and 3 ml 4°C quench buffer (1 mM Fe-NTA, 25 mM

HEPES, 150 mM NaCl) was added for 20 minutes. Finally the cells were washed 3 times with 2 ml 150 mM NaCl at 4°C and solubilized in 1 ml of solubilization detergent (0.1% Triton X-100, 0.1% NaOH). Lysates were mixed with 6 ml UniverSol (ICN) and counted for 10 minutes in a scintillation counter (Beckman LS 6000SC) with a window of 0-350 nm.

RESULTS

Apo-Tf does not compete with diferric Tf for binding to the fHFE-TfR complex- Several groups have determined that HFE increases the dissociation constant of the TfR for diferric Tf at pH 7.4 (Feder et al., 1998; Gross et al., 1998; Lebron and Bjorkman, 1999; Salter-Cid et al., 1999), but this is only one of four Tf species that are present in the blood plasma. We reasoned that if HFE did not increase the dissociation constant of the TfR for mono- or apo-Tf species 10 fold, as it does diferric Tf, the result would be more efficient uptake of unsaturated Tf species by the HFE-TfR complex, resulting in reduced Tf-mediated iron uptake. To determine whether HFE affects the dissociation constant of the TfR for apo-Tf, competition of apo-Tf with ^{125}I-diferric Tf for surface TfRs was performed. Because HFE reduces the affinity of the TfR for diferric Tf, less ^{125}I-diferric Tf was associated with HFE+ cells at each data point. Competition is expressed, therefore, as a percentage of total ^{125}I-diferric Tf bound for HFE- or HFE+ cells. Unlabeled diferric Tf competes readily for ^{125}I-diferric Tf on the TfR alone (data not shown) and on the fHFE-TfR complex (Figure 5.1, closed triangles). Unlabeled apo-Tf did not compete for the diferric Tf binding site on either the TfR (Figure 5.1, open squares) or fHFE-TfR complexes (Figure 5.1, closed circles) at concentrations as high as 320 nM. This result is consistent with the reported dissociation constant greater than 200 nM (Tsunoo and Sussman, 1983; Young et al., 1984) of apo-Tf for the TfR at pH 7.4. However, because the affinity of the TfR and the fHFE-TfR complex is so low for apo-Tf, the relative differences between them could not be determined.

The dissociation constant of human recombinant diferric Tf from the TfR is not significantly different from the dissociation constant of glycosylated diferric Tf - To determine the dissociation constants of the TfR and the fHFE-TfR complex for monoferric forms of Tf, we employed human recombinant Tf mutants that cannot bind iron in either

the amino or carboxyl terminal lobes. These proteins are not glycosylated. To confirm that the glycosylation state of Tf does not affect its dissociation from the TfR, we compared the competition of human diferric Tf (Figure 5.2, open squares) and recombinant diferric Tf (Figure 5.2, closed circles) for human ^{125}I-diferric Tf on the TfR. No significant difference was noted between the dissociation constant of human or recombinant diferric Tf for the TfR. The Ki calculated for either human or recombinant diferric Tf for the TfR was ~2.5 nM as determined from the method described by Cheng and Prusoff (Cheng and Prusoff, 1973) (Ki = IC_{50}/[1+([ligand]/Kd)]). The Ki calculated from these equations is the equivalent of the Kd for dissociation from the TfR. These values are comparable to those reported previously for the dissociation constant of the TfR for human diferric Tf (Gross et al., 1998).

fHFE decreases the affinity of the TfR for Fe_CTf- To determine whether HFE affects the dissociation of monoferric Tf from the TfR, competition of recombinant Fe_CTf with human ^{125}I-diferric Tf for surface TfRs was performed with the D63S recombinant Tf mutant which does not bind iron with high affinity in the amino terminal lobe. Again, because HFE reduces the affinity of the TfR for diferric Tf, less ^{125}I-diferric Tf was associated with HFE+ cells at each data point. Competition is expressed, therefore, as a percentage of total ^{125}I-diferric Tf bound for HFE- or HFE+ cells. Fe_CTf competes with diferric Tf better than apo-Tf on the TfR alone (Figure 5.3, open squares) and on the fHFE-TfR complex (Figure 5.3, closed circles). This result is consistent with the increased affinity of Fe_CTf for the TfR when compared to apo-Tf (Mason et al., 1998; Young et al., 1984). fHFE increases the dissociation constant of the TfR for Fe_CTf by almost four fold. The IC_{50} for Fe_CTf was estimated through the use of a pseudo-Hill plot to be 72.2 nM for the TfR dimer and 163.7 nM for the fHFE-TfR complex. When these values were used for the determination of the Ki using the equation described by Cheng and Prusoff (Cheng and Prusoff, 1973) (Ki = IC_{50}/[1+([ligand]/Kd)]), the estimated Ki was 36 nM for the TfR dimer and 136 nM on the

fHFE-TfR complex. The Ki calculated for Fe_CTf is the equivalent of the Kd for its dissociation from the TfR. For the TfR alone, the Kd of Fe_CTf is close to the 40 nM Kd reported by Young and Bomford for Fe_CTf (Young et al., 1984).

Lack of Fe_NTf binding to the TfR or fHFE-TfR complex- Competition of Fe_NTf with ^{125}I-diferric Tf for surface TfRs was performed with the D392S Tf mutant which does not bind iron with high affinity in the carboxy terminal lobe. Again, because HFE reduces the affinity of the TfR for Tf, less ^{125}I-diferric Tf was associated with HFE+ cells at each data point. Competition is expressed as a percentage of total ^{125}I-diferric Tf bound for HFE- or HFE+ cells. We found that Fe_NTf does not significantly compete against diferric Tf for binding to the TfR alone (Figure 5.4, open squares) or to the fHFE-TfR complex (Figure 5.4, closed circles). The previously reported dissociation constant of Fe_NTf from the TfR is approximately 36 nM (Young et al., 1984). Because we did not observe any significant binding of Fe_NTf to the TfR or the fHFE-TfR complex, we cannot determine whether fHFE alters the dissociation constant of this Tf species from the TfR.

fHFE does not alter the pH dependence of the Tf-TfR interaction- The unloading of iron from the Tf-TfR complex is highly dependent upon pH-induced conformational changes in both the Tf and TfR proteins. We reasoned that if HFE prevented conformational changes in Tf and/or the TfR that are responsible for potentiating iron loss from Tf at low pH, this would result in less iron uptake from Tf and more ferric Tf species exiting the cell after cycling through the endosome. To assay for such changes, we examined the pH profile of ^{125}I-diferric Tf conversion to ^{125}I apo-Tf on the TfR alone or on the fHFE-TfR complex. We quantitated the amount of ^{125}I-diferric Tf that remained bound to the TfR after dropping the pH to potentiate iron loss, and returning the pH to 7.4 to remove the apo ^{125}I-Tf generated at the low pH. Returning the system to pH 7.4 is necessary for the removal of apo-Tf, as it has high affinity for the TfR at low pH, and the difference between ^{125}I-

diferric Tf and ^{125}I apo-Tf cannot be distinguished because they have the same amount of associated radioactivity. Again, because HFE reduces the affinity of the TfR for Tf, less ^{125}I-diferric Tf was associated with HFE+ cells at each data point. Binding is therefore expressed as a percentage of total ^{125}I-diferric Tf bound for HFE- or HFE+ cells. The amount of ^{125}I-diferric Tf that remained associated with the TfR dropped significantly between pH 5.5 and 5.0 (Figure 5.5, open squares). Likewise, the amount of ^{125}I-diferric Tf that remained associated with the fHFE-TfR complex dropped significantly between pH 5.5 and 5.0 (Figure 5.5, closed circles). While the pH dependence of iron unloading is the same for the TfR versus the fHFE-TfR complex in this experiment, the unloading profile is 0.5 pH units less than that previously reported (Dautry-Varsat et al., 1983; Klausner et al., 1983). Klausner and colleagues and Dautry-Varsat and colleagues both reported that ^{125}I-diferric Tf was removed between pH 6.0 and 5.5

fHFE does not increase the pH of endosomal compartments- Since there was no change in the pH dependence of iron loss from Tf for the fHFE-TfR complex when compared to the TfR alone, we tested the hypothesis that HFE alters endosomal pH directly. Endosomal acidification is an ATP-dependent process that occurs as the newly formed endosome traffics from its origin at the plasma membrane to the perinuclear sorting compartment. We reasoned that if HFE reduced the activity of the endosomal proton pump, this would result in an increase in endosomal pH and a reduction in pH dependent conformational changes in the Tf-TfR complex that are necessary for iron release from Tf.

Fluorescence microscopy was used to quantitate the pH of Tf positive endosomes in collaboration with Dr. Tim McGraw and Michael Lampson of Cornell University. Rhodamine (R) and fluorescein (F) were coupled individually to Tf. The labeled Tf was then internalized by the cells for 5 to 15 minutes. Rhodamine fluorescence is not pH dependent and is therefore a measure of the amount of Tf internalized. Fluorescein fluorescence is quenched with a decrease in pH. Thus, the ratio of the emission of

fluorescent energy from rhodamine and fluorescein labeled Tf (R/F) is increased as the vesicles become more acidic. Imaging and quantitation of the fluorescence from individual vesicles allowed us to calculate the distribution of vesicles in the pH range below 6.2, between 6.2 and 6.8, and greater than 6.8. The peak value (R/F ratio X 50) of the pH distribution of the vesicles in HFE- fWTHFE/tTA HeLa cells was measured at approximately 45 (Figure 5.6, panel A). The peak value (R/F ratio X 50) of the pH distribution of vesicles in HFE+ fWTHFE/tTA HeLa cells was also 45 (Figure 5.6, panel B). No significant difference was noted between the two. As a positive control, cells were treated with methylamine to increase the endosomal pH. The shift in distribution toward more alkaline pH was easily detected by the shift in the peak R/F ratio to approximately 30, indicating that this protocol detects such differences in endosomal pH (Figure 5.6, panel C).

fHFE does not reduce ferric iron uptake at pH 6.0- The results reported above indicate that HFE does not affect the unloading of iron from the Tf-TfR complex. We reasoned that HFE may modulate another component of the endosomal iron uptake pathway, such as an endosomal iron transporter, either through direct protein-protein interaction, or by regulation of a modifier of this pathway. We have already reported that HFE has no effect on non-Tf mediated iron transport at pH 7.4. However DMT1, a hypothesized endosomal iron transporter, is also a proton symporter and requires a low pH environment such as the acidic endosome for activity. We assayed the efficiency of $Fe(NTA)_4$ uptake from the cell surface at pH 6.0 in an attempt to activate this transporter. No significant difference in ^{55}Fe iron uptake was observed between HFE- (Figure 5.7, open squares) and HFE+ (Figure 5.7, closed circles) cells.

DISCUSSION

HFE specifically reduces iron uptake from Tf (Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). HFE has also been shown to compete with diferric Tf for the TfR (Lebron et al., 1999) and to increase the dissociation constant of diferric Tf from the TfR approximately 10 fold (Feder et al., 1998; Gross et al., 1998; Lebron and Bjorkman, 1999; Salter-Cid et al., 1999). While this change in Tf and TfR association would significantly affect diferric Tf binding and uptake at concentrations of diferric Tf below ~50 nM, the concentration of diferric Tf in the blood plasma is nearly 5 μM (Lane, 1975; Ponka et al., 1998). When the HFE-TfR complex cycles in the presence of at least 50 nM diferric Tf, no change in the rate of Tf uptake per surface TfR occurs (Ikuta et al., 2000; Roy et al., 1999). Though one report indicates that the cycling of the TfR back to the cell surface may be slowed when HFE is expressed (Ikuta et al., 2000), we did not observe a reduction in Tf efflux from HFE-expressing cells, nor did we observe a redistribution in the TfR that should be accompanied by a change in the TfR exocytic kinetic constant (Roy et al., 1999). Assuming that HFE does not alter the cycling kinetics of the TfR, the observed reduction in iron uptake from Tf must be the result of an increase in the uptake of unsaturated Tf species, a decrease in the release of iron from Tf or reduced transport of iron across the endosomal membrane.

We reasoned that HFE might reduce diferric Tf-mediated iron uptake by altering the affinity of the TfR for unsaturated Tf species. Competition experiments showed that apo-Tf did not significantly compete with diferric Tf for binding to the TfR or to the fHFE-TfR complex. Of interest, however, is the observation that fHFE increased the dissociation constant of Fe_cTf from the TfR 3.7 fold. This difference is not as great as the 8-10 fold increase in the dissociation constant of diferric Tf from the TfR that is induced by HFE (Feder et al., 1998; Gross et al., 1998). We have previously reported that HFE reduces iron uptake from Tf (Roy et al., 1999). These experiments were performed with 85%

saturated Tf, providing, at most, 30% Fe_CTf. Bringing the affinity of diferric Tf and Fe_CTf for the fHFE-TfR complex closer together than the relative affinities of diferric Tf and Fe_CTf for the TfR alone could provide a mechanism by which HFE increases the uptake of unsaturated Tf species when compared to the TfR alone. Such competition between unsaturated and saturated Tf species might explain the decrease in iron uptake we originally observed from Tf (Roy et al., 1999).

While others have reported a similar affinity of Fe_CTf and Fe_NTf for the TfR (Young et al., 1984), we found the dissociation constant of Fe_NTf from both the TfR and the fHFE-TfR complex to be significantly more than that of Fe_CTf. The point mutations in the human recombinant Tf proteins used in our assay are independent and in opposite lobes of the Tf molecule. However, some evidence suggests there may be some cooperativity involved with iron binding in the amino terminal lobe of Tf (Bali and Aisen, 1992). It is possible that the Fe_NTf used in these experiments is not fully saturated with iron in the amino terminal lobe, but a mixture of apo- and Fe_NTf instead. This might explain why the apparent dissociation constant of Fe_NTf is so much greater than that of Fe_CTf. The ability to saturate the Fe_NTf species with iron in the amino terminal lobe should be assessed for the purpose of repeating these experiments. The dissociation constants of the TfR and the HFE-TfR complex for monoferric Tf species would be more accurate when determined by Scatchard plot if enough Fe_CTf and Fe_NTf could be generated that bound iron in a single site during experimentation. The affinity of the TfR and the HFE-TfR complex for Fe_NTf is also of interest because two times more Fe_NTf is present in the blood plasma than Fe_CTf, making it a more likely candidate for competition with diferric Tf (Lane, 1975).

The TfR facilitates unloading of iron from Tf in the endosome. Because HFE binding might alter the conformation of the TfR (Bennett et al., 2000; Lawrence et al., 1999), we tested the hypothesis that HFE impairs the TfR-induced iron unloading from Tf. fHFE does not significantly inhibit the conversion to apo- or monoferric Tf species in our assay since we observed unloading of iron from Tf between pH 5.5 and pH 5.0, whether

or not fHFE was expressed. The sensitivity of this assay may not fully reflect the events of iron unloading that occur in the endosome, however. It is possible that the fHFE-TfR complex does prevent efficient unloading of iron, but only from one lobe of Tf. This would result in monoferric Tf forms that would cycle back out of the endosome. In our assay, however, the ^{125}I-monoferric Tf forms have reduced affinity for the fHFE-TfR complex and may therefore be removed with ^{125}I-apo-Tf in the final wash. It is also possible that a similar experiment performed with pH points closer together would provide more information. For example, if the Tf-TfR complex releases iron from Tf at pH 5.4, but the HFE-TfR complex does not release iron from Tf until the pH drops to 5.2, the assay used here could not differentiate between these two pH values.

Endosomal pH is regulated by the activity of the H+/ATPase. We tested the hypothesis that HFE reduces the activity of the H+/ATPase by determining the endosomal pH in HFE- and HFE+ fWTHFE/tTA HeLa cells. If HFE were to reduce the activity of the H+/ATPase, the pH of HFE+ endosomes would increase. We found no significant difference between the mean pH of HFE- and HFE+ endosomes. Because fHFE does not seem to have an effect on endosomal pH, we conclude that the reduction in iron uptake from Tf is not due to impaired release of iron from Tf in a significantly more alkaline environment. However, this assay does not distinguish among vesicle pH below 6.2. We observed unloading of iron from the TfR and the fHFE-TfR complex below pH 5.5. If fHFE prevented further acidification below 6.2, it would not be perceived due to the sensitivity of this assay.

While endosomal iron uptake is dependent on the Tf-TfR complex, other components of the endosomal membrane are also responsible for iron transport into the cell. We assayed the efficiency of non-Tf mediated iron uptake at pH 6.0 in HFE- and HFE+ fWTHFE/tTA HeLa cells to determine whether HFE might reduce the activity of the endosomal iron transport system. We found no significant difference in non-Tf mediated iron uptake at pH 6.0 when fHFE was expressed. While this assay is not specific for

endosomal iron uptake, the two identified endosomal iron transport proteins DMT1 and SFT are hypothesized to cycle between the cell surface and endosomal compartments. The activity of these transporters should contribute to the observed iron absorption, as well as other unidentified iron transport systems.

DMT1 and SFT are recognized components of endosomal iron transport, but they have not been fully characterized in a wide variety of cell types. We have observed that DMT1 is expressed to a significantly lower degree in HeLa cells than in HEK 293 cells (CNR-372 unpublished results). This might indicate that DMT1 is not the primary transporter for endosomal iron uptake in HeLa cells. Neither has robust expression of SFT been observed in the HeLa cell line. While the inability to detect these proteins on immunoblot certainly does not exclude their presence and activity in these cells, it may implicate the existence of another transporter of the endosomal iron uptake pathway in HeLa cells.

Finally, because Tf binds and delivers ferric iron to the endosome, a ferrireductase has been hypothesized to exist within the endosomal membrane. Conversion of iron from the ferric to ferrous iron state and back again is a well-documented component of many systems of iron uptake and transport (Askwith and Kaplan, 1998). We have not tested directly for the ability of HFE to modulate the activity of such a component of endosomal iron uptake. However, if HFE were to reduce the activity of the ferrireductase, the efficiency of Tf derived ferric iron transport from the endosome would likely be reduced and might explain the mechanism by which HFE reduces iron uptake from Tf. Identification of the protein components of the endosomal iron uptake pathway in HeLa cells including the actual iron transporter(s) and the ferrireductase would significantly impact the ability to design and test for HFE-dependent modulation of this pathway.

We have tested several hypotheses that might uncover the mechanism by which HFE reduces iron uptake from Tf in the endosome. These data are preliminary and would benefit from future experiments that test the same mechanisms. The most intriguing result

from this series of experiments is the difference in the Ki of Fe_CTf for diferric Tf on the TfR versus the fHFE-TfR complex. Future experiments that compare the delivery of iron to HFE- versus HFE+ fWTHFE/tTA HeLa cells under different conditions of Tf saturation may reveal a "sensing" mechanism for HFE, or an ability of HFE to distinguish between states of iron saturation. An ability such as this would provide the cells in which HFE is expressed with a mode of communication between themselves and the status of body iron stores.

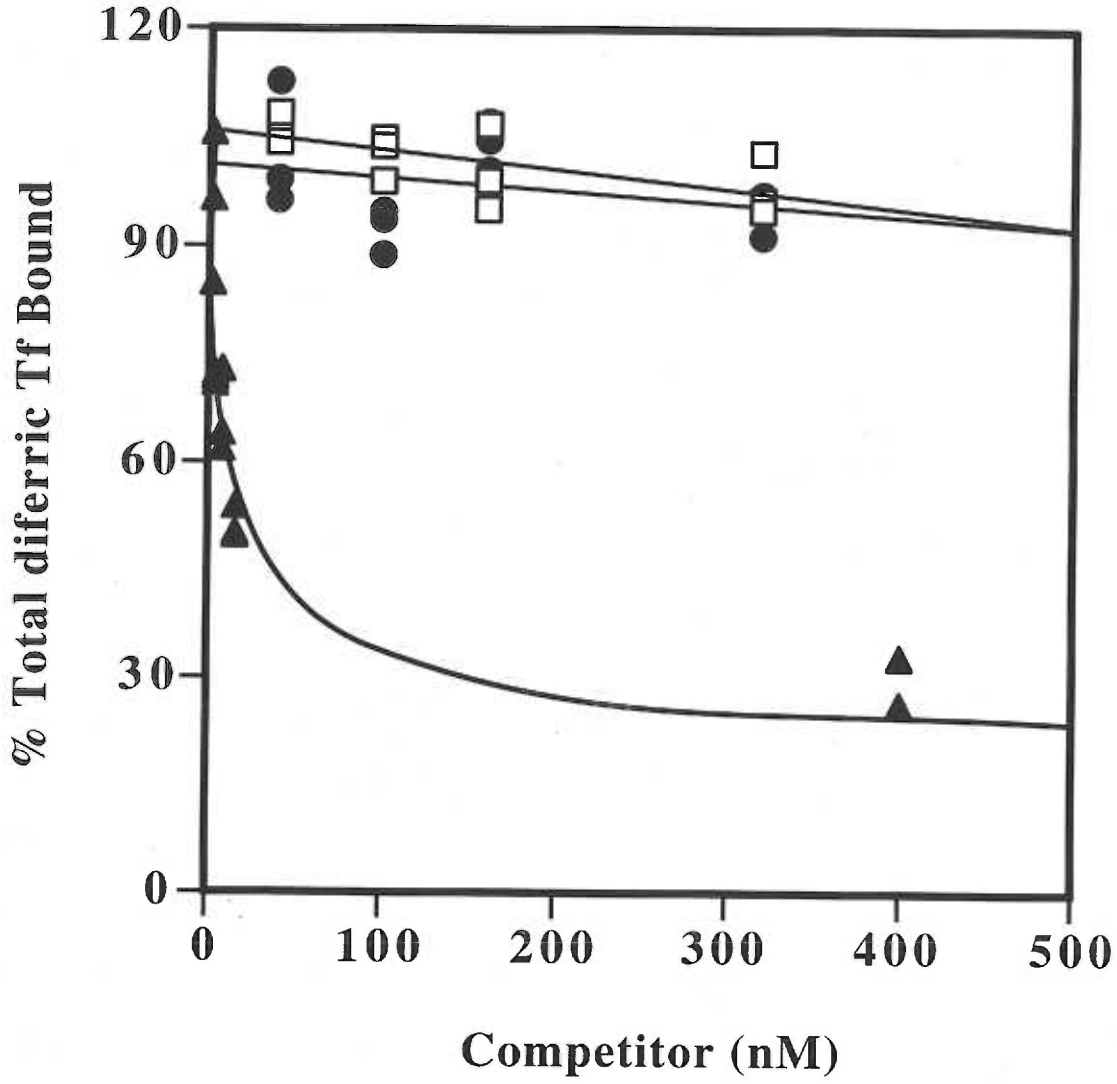

Figure 5.1. Apo-Tf does not compete with diferric Tf for binding to the TfR or the fHFE-TfR complex. Competition of 40 to 320 nM apo-Tf for ^{125}I-diferric Tf on HFE- (open squares, r^2= 0.386) or HFE+ (closed circles, r^2= 0.078) fWTHFE/tTA HeLa cells indicates that apo-Tf does not obviously compete with diferric Tf for the TfR or the HFE-TfR complex. Competition of diferric Tf for ^{125}I-diferric Tf on HFE+ fWTHFE/tTA HeLa cells (closed triangles, r^2= 0.838), shows that ^{125}I-diferric Tf can be displaced. Curves were fit using the Cricket Graph program. This experiment was performed in triplicate, but was not repeated.

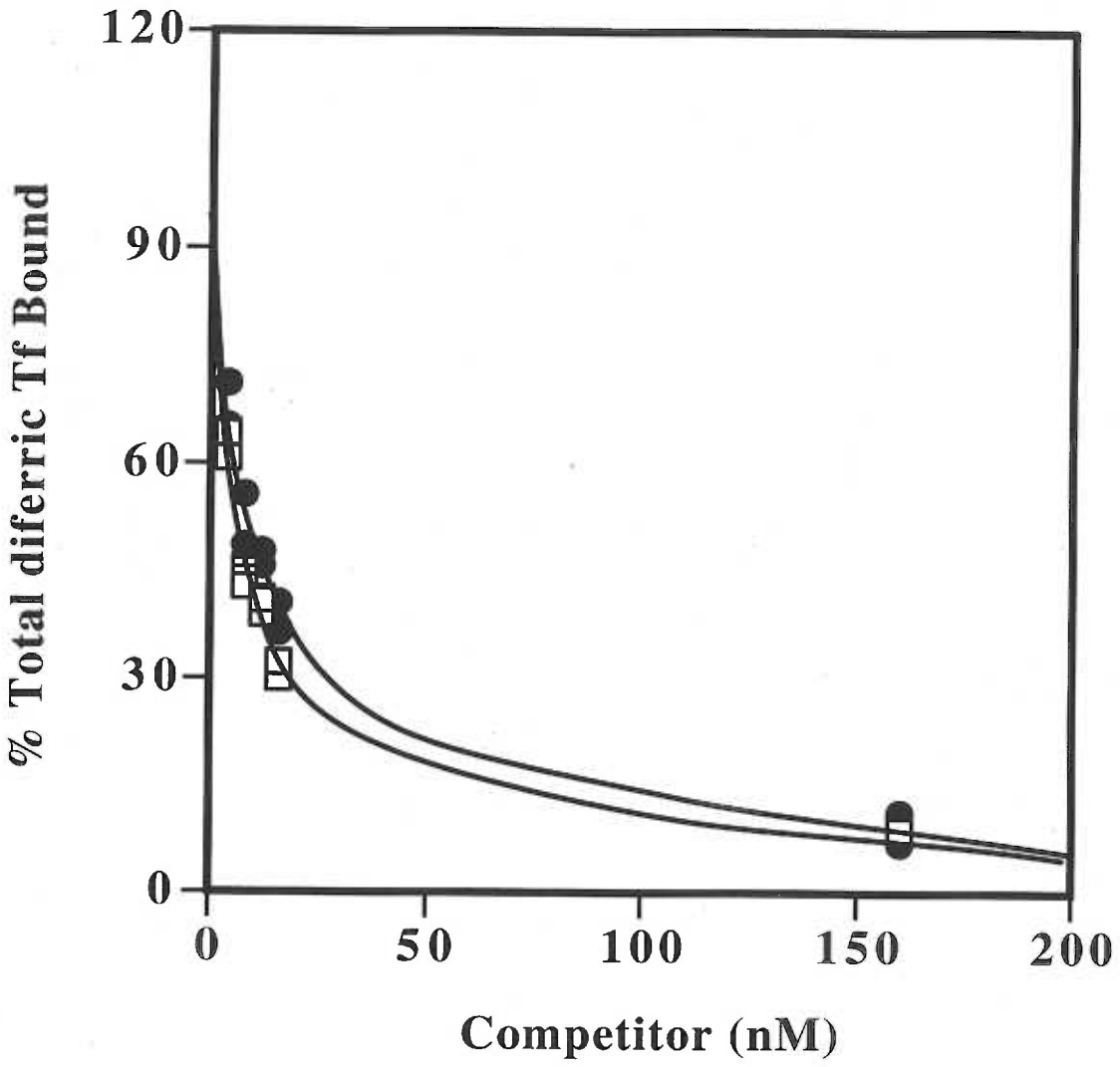

Figure 5.2. Human unglycosylated diferric Tf is indistinguishable from glycosylated diferric Tf for competition of ^{125}I-diferric Tf. Competition of 4 to 160 nM diferric Tf with ^{125}I-diferric Tf (open squares, r^2= 0.953) is not significantly different from that of human unglycosylated diferric Tf with ^{125}I-diferric Tf (closed circles, r^2= 0.933) on HFE- fWTHFE/tTA HeLa cells. The Ki calculated using the method described by Cheng and Prusoff for both curves was ~2.5 nM. This experiment was performed in triplicate, but was not repeated.

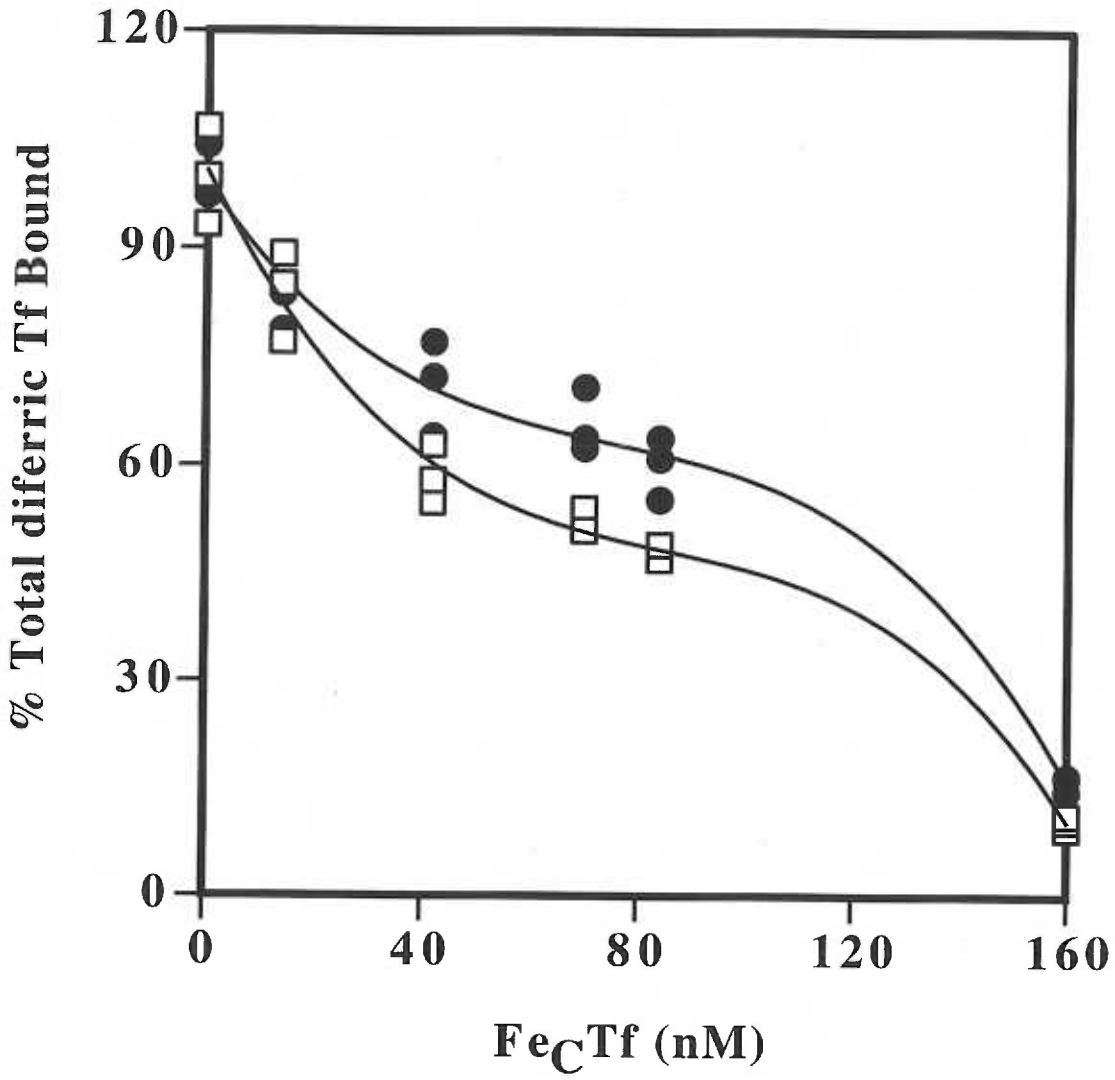

Figure 5.3. The affinity of the TfR for Fe_CTf is not increased by fHFE. Competition of 14 to 160 nM Fe_CTf with ^{125}I-diferric Tf on cells expressing only the TfR (open squares) is more efficient than competition of Fe_CTf with ^{125}I-diferric Tf on cells expressing the HFE-TfR complex (closed circles). The calculated Ki of Fe_CTf for TfR was 36 nM (see text for description of calculations), while the calculated Ki of Fe_CTf for the HFE-TfR complex was approximately four fold greater (136 nM). Curves were fit in the Cricket graph program using r^2 values. This experiment was performed in triplicate and is representative of two experiments without variation in the trend.

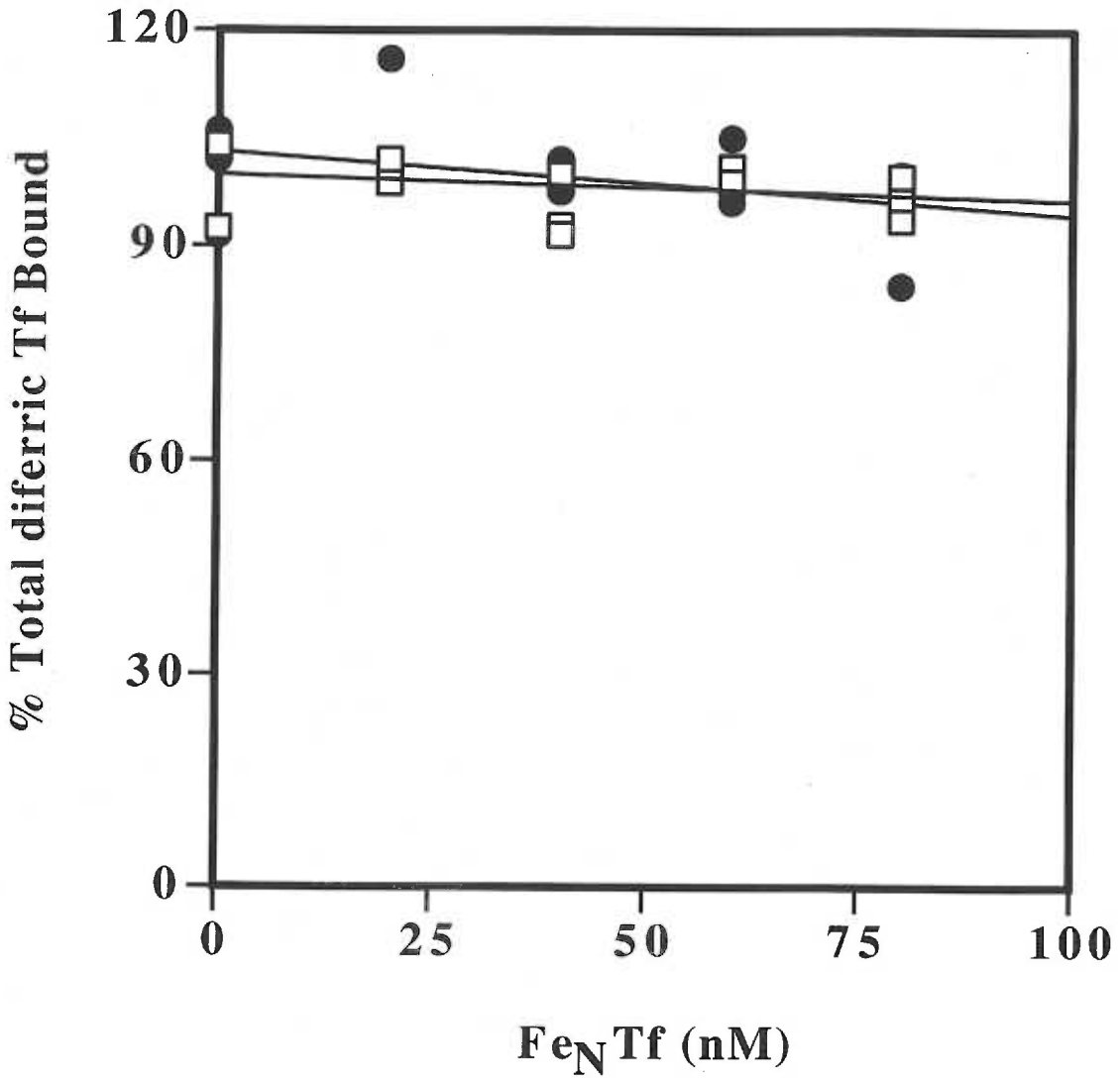

Figure 5.4. Lack of Fe_NTf binding to the TfR or the fHFE-TfR complex. Competition of 20 to 100 nM Fe_NTf with ^{125}I diferric Tf on the TfR (open squares) or the HFE-TfR complex (closed circles) does not significantly displace ^{125}I diferric Tf. While HFE does not increase the affinity of Fe_NTf for the TfR in this experiment, the data suggests that Fe_NTf does not bind with significant affinity to the TfR or the HFE-TfR complex. This experiment was performed in triplicate, but was not repeated.

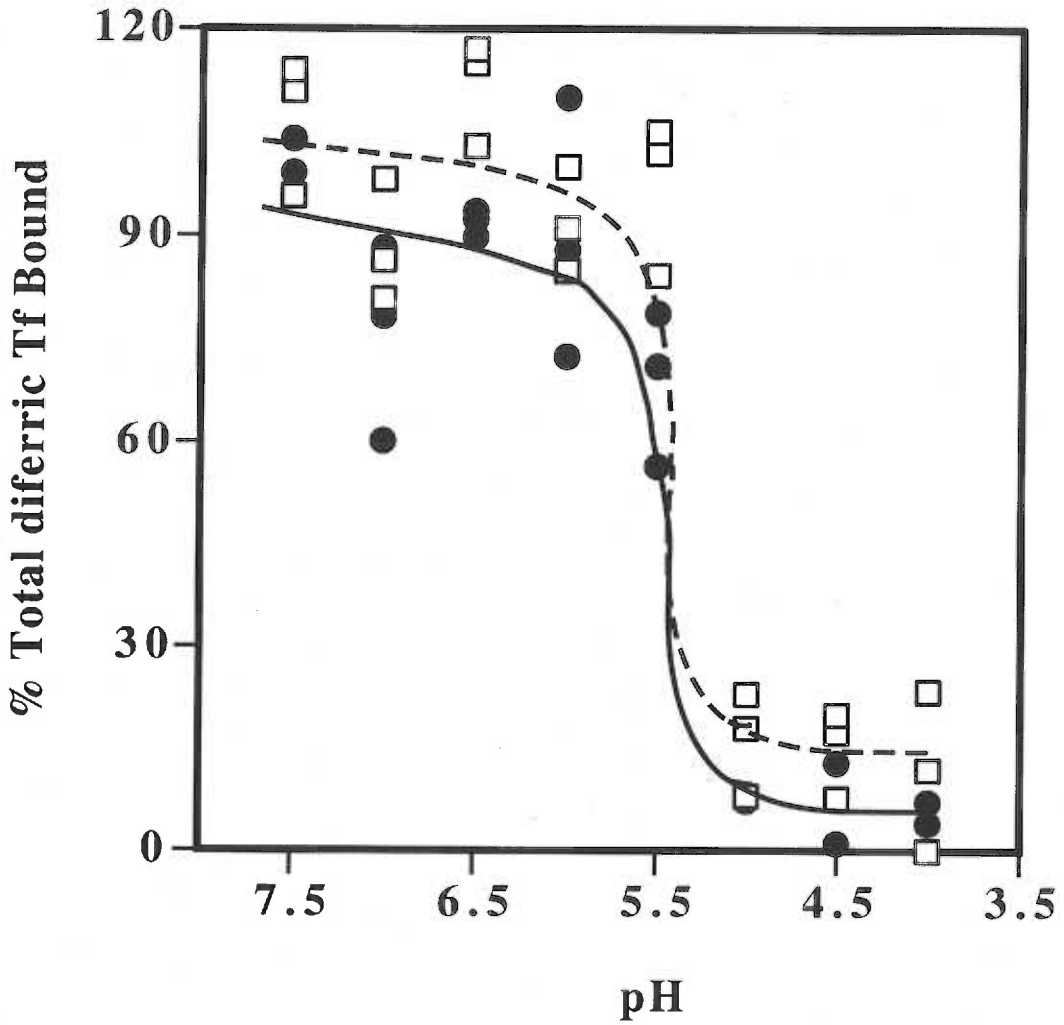

Figure 5.5. fHFE does not prevent pH dependent unloading of iron from Tf. ^{125}I-Tf was bound to cells at pH 7.4. Then, the binding buffer was changed to a lower pH, allowing conformational changes in Tf and the TfR that potentiate iron loss. After 10 minutes, cells were washed in neutral pH buffer. The ^{125}I-apo-Tf resulting from iron loss was removed at neutral pH. The amount of ^{125}I-diferric Tf that remains associated with the TfR (open squares, dotted line) or the HFE-TfR complex (closed circles, solid line) between pH 4.0 and pH 7.5 was quantitated in fWTHFE/tTA HeLa cells. ^{125}I-Tf loses high affinity binding for both the TfR and the HFE-TfR complex between pH 5.5 and 5.0, suggesting it has been converted to monoferric or apo-Tf forms that have low affinity for the TfR at near neutral pH. Curves were fit by inspection. This experiment was performed in triplicate, but was not repeated.

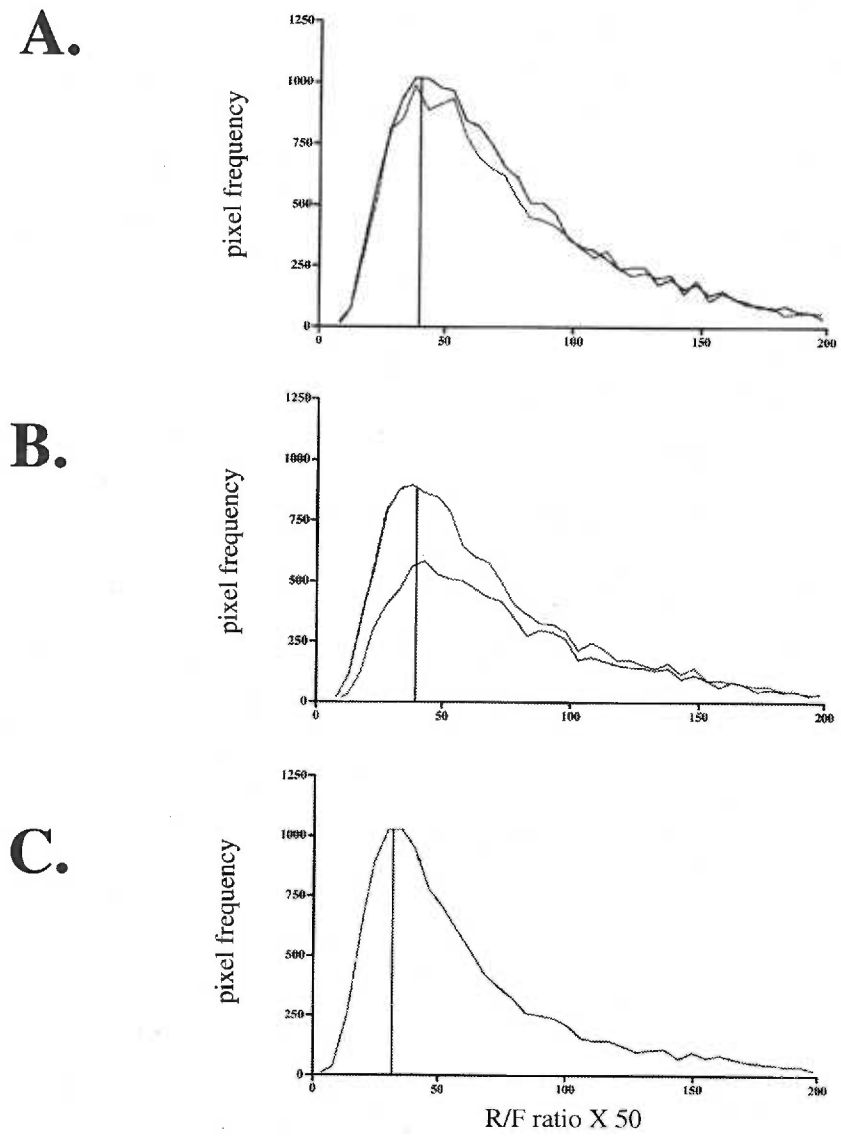

Figure 5.6. fHFE does not increase the pH of endosomal compartments. Endosomal pH was quantified in (A) 2 confocal planes of HFE- fWTHFE/tTA HeLa cells, or (B) 2 confocal planes of HFE+ fWTHFE/tTA HeLacells, or (C) 1 confocal plane of methylamine treated tTA HeLa cells. No significant difference was observed in the median pH distribution of HFE- versus HFE+ endosomes (represented by the vertical lines). Methylamine did significantly shift the endosomal pH distribution toward more alkaline pH (as represented by the shift in the vertical line along the abscissa, toward the origin). These experiments were performed in duplicate and are representative of three experiments without significant variation.

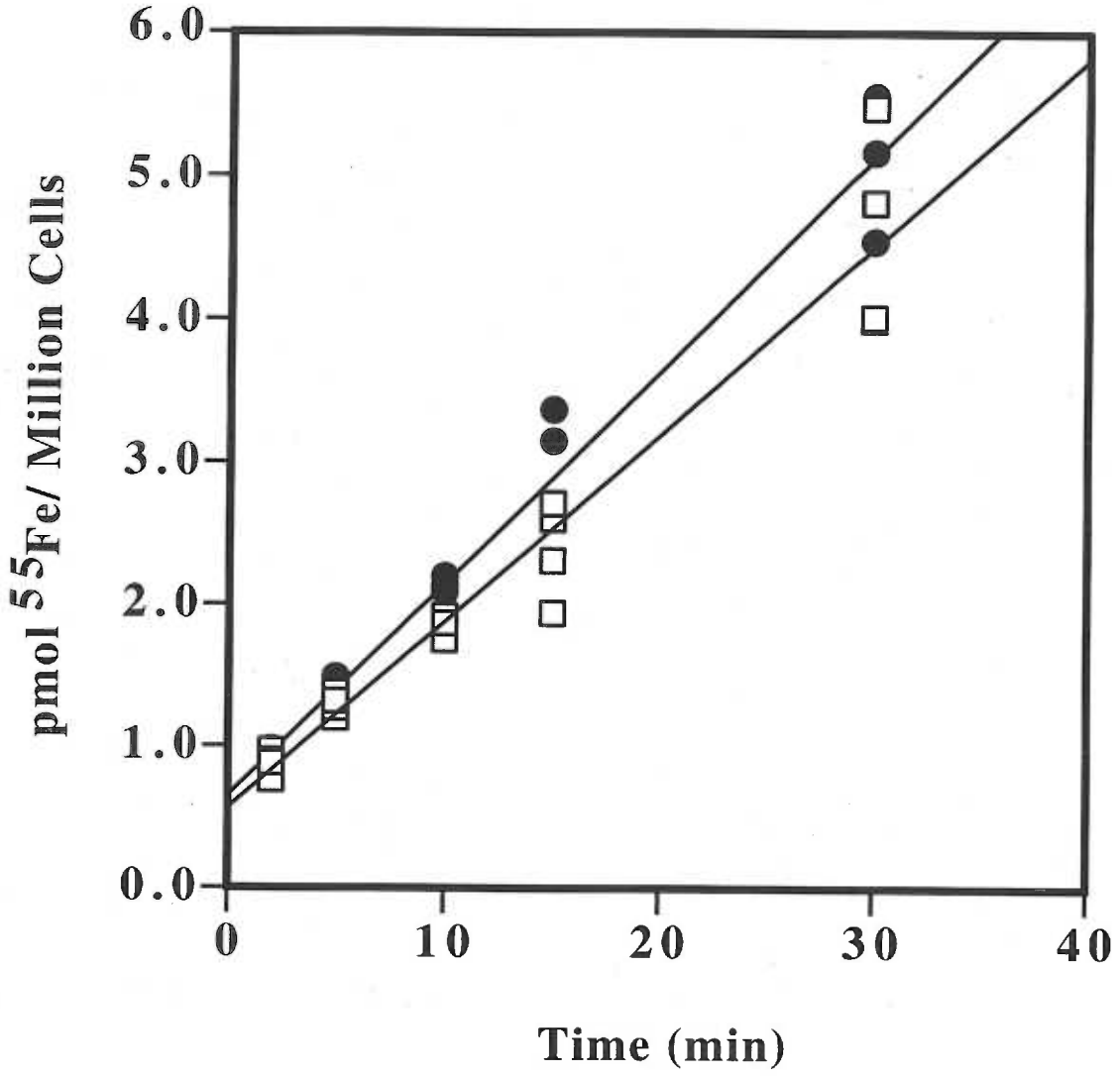

Figure 5.7. fHFE does not reduce ^{55}Fe-NTA uptake at pH 6.0. ^{55}Fe uptake from ^{55}Fe-NTA was measured in HFE- (open squares) or HFE+ (closed circles) fWTHFE/ tTA HeLa cells. The rate of ^{55}Fe uptake in pmoles per million cells per minute for HFE- and HFE+ was 0.131 (r^2= 0.941) and 0.150 (r^2= 0.978), respectively. Linear regression was determined in the Cricket Graph program. These results are representative of three experiments performed with quadruplicate data points without significant variation between experiments.

CHAPTER 6

Increased IRP1 and IRP2 Activity in HFE-Expressing Cells Implies Reduction in Labile Iron Pool

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SUMMARY

Iron regulatory proteins are cytosolic proteins involved in the maintenance of cellular iron homeostasis. Iron regulatory proteins bind to stem loop structures found in the mRNA of key proteins involved iron uptake, storage, and metabolism and regulate the expression of these proteins in response to changes in cellular iron needs. We have shown previously that HFE-expressing fWTHFE/tTA HeLa cells have slightly increased transferrin receptor levels and dramatically reduced ferritin levels when compared to the same clonal cell line without HFE. We have also shown that while HFE does not alter transferrin receptor trafficking or non-transferrin mediated iron uptake, it does specifically reduce ^{55}Fe uptake from transferrin. In this report, we provide evidence supporting the hypothesis that HFE reduces the labile iron pool. Consistent with increased iron regulatory protein activation, HFE-expressing cells synthesize less ferritin. Furthermore, iron regulatory protein RNA binding activity is increased almost 5 fold in HFE-expressing cells through the activation of both iron regulatory protein isoforms. This result was confirmed by supershift experiments with antibody specific to iron regulatory protein 1 or iron regulatory protein 2 and immunoblot of iron regulatory protein 1 and 2 protein levels. These results suggest that the labile iron pool is maintained at a lower concentration in HFE-expressing cells, despite up-regulation of transferrin receptor and down-regulation of ferritin.

INTRODUCTION

Biological systems maintain an appropriate iron balance through regulatory mechanisms that enable them to meet the iron demand, but prevent iron overload. Cytoplasmic iron regulatory proteins (IRPs) regulate the levels of several key proteins involved in iron uptake, storage and utilization by binding to iron responsive elements (IREs) in their respective mRNAs [reviewed in (Eisenstein and Blemings, 1998; Hentze and Kuhn, 1996; Theil, 1998)]. The activities of the two IRP isoforms are primarily dependent on an elusive cytoplasmic pool of intracellular iron referred to herein as the labile iron pool. The iron-dependent RNA binding activity exhibited by both IRPs equips them to communicate the iron status of the cell to the cellular machinery involved in iron homeostasis.

Hereditary hemochromatosis is an autosomal recessive genetic disease that results from a defect in regulated iron transport across the intestinal epithelial cells. The disease is characterized by increased transfer of iron from the enterocyte to the blood (McLaren et al., 1991). The transfer of iron across the duodenal epithelium is inversely proportional to the relative iron repletion of an organism [reviewed in (Bothwell et al., 1995)]. Increasing evidence points to the involvement of IRP machinery, or at the very least the labile iron pool, in the regulation of dietary iron uptake and transfer across the enterocyte (Garate and Nunez, 2000; Schumann et al., 1999). The specific effects of IRPs on enterocyte proteins of iron transport and homeostasis are just beginning to be investigated (Abboud and Haile, 2000; McKie et al., 2000; Wardrop and Richardson, 1999).

Individuals who are homozygous for the most common mutation, C282Y, in the hereditary hemochromatosis protein, HFE, (Feder et al., 1996) experience chronic increased dietary iron uptake, but the mechanism behind this increase is not yet known. HFE associates very tightly with the TfR in cultured cells (Bennett et al., 2000; Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998) as well as in the placenta and duodenum

(Parkkila et al., 1997; Waheed et al., 1999). Cells grown in culture that express HFE have increased TfR levels and decreased Ft levels suggesting an HFE-specific decrease in the labile iron pool (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999). Consistent with these results is the finding that HFE specifically reduces iron uptake from Tf (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999) and leads to activation of IRPs (Corsi et al., 1999; Riedel et al., 1999). Whether one or both IRP1 and IRP2 are activated has not been determined. Such information could shed light on whether increases in IRP activity are through an iron-mediated mechanism or by other pathways.

The ability of the two IRP isoforms to bind IREs is differentially regulated. IRP1 contains a [4Fe-4S] cluster when the labile iron pool is relatively high. The [4Fe-4S] cluster precludes binding to target IRE-containing mRNAs (Haile et al., 1992; Rouault et al., 1991; Tang et al., 1992). In contrast, IRP2 protein levels change with fluctuations in the labile iron pool. When the concentration of the labile iron pool is high, amino acid residues unique to IRP2 are oxidized leading to ubiquitination, and subsequent degradation of the protein (Guo et al., 1995; Guo et al., 1994; Iwai et al., 1998; Iwai et al., 1995). In addition to IRP regulation by iron, signal transduction (Brown et al., 1998; Schalinske and Eisenstein, 1996) and oxidative stress (Bouton et al., 1996; Drapier et al., 1993; Hanson et al., 1999; Pantopoulos et al., 1996; Weiss et al., 1993) pathways are all hypothesized to regulate IRP RNA binding activities (Hanson and Leibold, 1999). The differential binding of IRP1 and IRP2 to the assorted IREs of mRNAs could also impose differential regulation of the iron-related proteins (Ke et al., 1998). The physiological relevance of these pathways is only beginning to be realized.

To define the mechanism by which HFE expression leads to the activation of IRPs, we have characterized IRP activities in our cell line expressing wild type HFE under the control of the tetracycline (tet) transactivatable promoter (fWTHFE/tTA HeLa). We provide evidence supporting the hypothesis that HFE expression increases both IRP1 and IRP2 RNA binding activity by reducing the labile iron pool. We speculate that HFE

operates through the Tf-mediated iron uptake pathway to establish a setpoint for the transfer of iron across the enterocyte that is dependent on the labile iron pool of its precursor. During enterocyte differentiation, this set point may determine the relative activities of proteins involved in the export of iron to the blood (Andrews, 1999; Cox and Kelly, 1998).

MATERIALS AND METHODS

Cell lines- The fWTHFE/tTA HeLa cell line expressing FLAG epitope-tagged wild type HFE (fWTHFE) under the tet-responsive promoter has been previously described (Gross et al., 1998). Cells were grown in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% fetal bovine serum, 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE-) or without (HFE+) 2 μg/ml tet.

fHFE induction in the presence of saturating Tf- HFE- and HFE+ fWTHFE/tTA HeLa cells were resuspended in tet free medium. The cells were centrifuged at 5000 x g for five minutes and resuspended in tet free medium three times. HFE- cells were seeded into 35 mm dishes containing DMEM with 10% fetal bovine serum with tet to repress fHFE expression or without tet to induce fHFE expression and with or without 100 nM human Tf. HFE+ cells were also seeded into DMEM with 10% fetal bovine serum with or without 100 nM human Tf. After three days in culture, cells were put on ice, washed three times with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.4), and lysed for 5 minutes in NET-Triton (150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4). Immunoblot detection of fHFE and Ft from cell extracts was performed as described previously (Gross et al., 1998) using M2 anti-flag epitope antibody (Kodak) and sheep anti-human Ft antibody (The Binding Site, Ltd.), respectively.

^{35}S-Methionine/Cysteine Incorporation into Ft and IRP1- Subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cells in 35 mm dishes were washed two times with sterile PBS and preincubated for 10 minutes at 37° C in DMEM without methionine or cysteine (met-/cys- DMEM, Sigma) prior to labeling. After preincubation, the methionine and cysteine-free DMEM was removed and the cells were labeled for varying times with 100 μCi ^{35}S-Translabel (DuPont, New England Nuclear) in methionine and cysteine-free DMEM with

10% fetal bovine serum. After the indicated times cells were put on ice and lysed as described above. Cell extracts were subjected to immunoprecipitation as described below and analyzed with denaturing SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) under reducing conditions. Gels were fixed, treated with Amplify (Amersham, Pharmacia Biotech) for 30 minutes, dried and subjected to PhosphorImager analysis.

Immunoprecipitations- For Ft immunoprecipitations, subconfluent ^{35}S-methionine/cysteine labeled HFE- and HFE+ fWTHFE/tTA HeLa cells were washed two times with 2 ml of PBS and lysed with NET-Triton on ice. Lysates of ~10^6 cells were preadsorbed for 1 hour at 4°C with 30 µl of bovine serum albumin-conjugated agarose. Preadsorbed lysates were incubated for 1 hour at 4° C with 15 µl protein A Sepharose (50% suspension, Zymed) and 3 µl affinity purified rabbit anti-rat Ft antibody (Chen et al., 1997). The pellet was resuspended in 100 µl of NET-Triton and washed through 1 ml of radioimmune precipitation assay (RIPA) buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, and 0.2% sodium azide, pH 7.4) with 15% sucrose. Samples were eluted in 100 µl of 2X Laemmli buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) (Laemmli, 1970) and subjected to SDS-PAGE analysis on a 12% denaturing acrylamide gel under reducing conditions.

For IRP1 immunoprecipitations, HFE- and HFE+ subconfluent fWTHFE/tTA HeLa cells in 35 mm dishes were washed two times with 2 ml of sterile PBS (pH 7.4) and lysed with HEPES-citrate buffer (20 mM HEPES, 1 mM sodium citrate, 5 mM EDTA, 0.5% NP-40, 100 µg/ml leupeptin, 100 µg/ml pepstatin, 250 µg/ml soybean trypsin inhibitor, 200 µM phenylmethylsulfonyl fluoride, 10 µM MG132, pH 7.4) on ice. Lysates of ~10^6 cells were preadsorbed for 1 hour at 4°C with 30 µl of bovine serum albumin conjugated agarose. Preadsorbed lysates were incubated for 1 hour at 4° C with 15 µl protein A Sepharose (50% suspension, Zymed) and 30 µg rabbit anti-rat IRP1 antibody

affinity purified against purified rat liver IRP1 (Schalinske et al., 1997). The pellet was resuspended in 100 µl of NET-Triton, washed and eluted with Laemmli buffer as described above. Samples were subjected to SDS-PAGE analysis on a 6% denaturing acrylamide gel under reducing conditions.

Electrophoretic Mobility Shift Assay- RNA binding assays used the first 73 nucleotides of the rat L-Ft 5' untranslated region which contains the IRE. Internally ^{32}P labeled RNA was synthesized as described (Eisenstein et al., 1993). The RNA binding assay was performed as previously described (Eisenstein et al., 1993) with the following modifications. HFE- and HFE+ fWTHFE/tTA HeLa cells were harvested by trypsinization after they had been washed in Hanks balanced salt solution. Trypsinized cells were resuspended in Hanks and pelleted by centrifugation. The cell pellet was resuspended in 10 ml of Hanks and centrifuged again. The cell pellet was lysed in lysis buffer (Eisenstein et al., 1993) plus 2 mM DTT, 400 µM phenylmethylsulfonyl fluoride, leupeptin (40 µg/ml), pepstatin (4 µg/ml), soybean trypsin inhibitor (100 µg/ml) and MG132 (10 µM) by vortexing occasionally over a 10 minute period. Lysates were clarified by centrifugation at 14,000 rpm in an Eppendorf model 3415C centrifuge at 4° C for 10 minutes. For the RNA binding assay, protein lysates were included at a concentration of 1 to 1.5 µg/ 30 µl reaction in the presence of binding buffer (Eisenstein et al., 1993) containing 0.6 µg nuclease free bovine serum albumin, 2 mM DTT and 1 to 1.2 nM RNA. IRE binding activity which was inducible by 2-ME was determined in the presence of 8% 2-mercaptoethanol (2-ME). Addition of 2-ME to the cell lysates reduces the [4Fe-4S] cluster in IRP1 and converts it from the cytosolic aconitase to the RNA binding form of the protein. Lower concentrations (2%) had little effect on the activation of IRE binding activity of IRP in HeLa cell extracts and only partial effects on rat fibroblast (RF2) extracts. Heparin (3 µl) was added (final concentration 0.45 µg/ml) just before loading the gel.

Twenty-five microliters of each reaction was loaded onto a gel shift (Eisenstein et al., 1993) and electrophoresed at 300 volts constant voltage for 35 minutes at 4°C.

Human IRP1 and IRP2 co-migrate in gel shift assays. To determine the contributions of IRP1 and IRP2 to the binding activity present in HeLa cells, antibody supershift experiments were performed. For supershift analysis 1 μg of HeLa cell lysate was incubated with 10 μg of antibody specific for IRP1 and IRP2 (Schalinske et al., 1997) for 2 hours at 4° C. The antibody/lysate preparations were then incubated with IRE RNA and the RNA binding activity was determined as described above. Cytosol from the rat fibroblast cell line RF2 was used as standard for antibody specificity since rodent IRP1 and IRP2 migrate separately in gel shift assays. The contributions of IRP1 and IRP2 to the total binding activity in HeLa cell extracts was determined by the amount of binding activity that was not supershifted by a saturating concentration of antibody. This approach was taken because the polyclonal antibody against IRP2 is capable of both supershifting the RNA protein complex and it also inhibits RNA binding by IRP2. Hence it is not possible use the supershifted complex to determine the amount of IRP2 RNA binding activity.

PhosphorImager Quantitation- IP Lab Gel 1.5 (Molecular Dynamics, Inc.) was used to quantitate immunoprecipitations by determining the volume within a region of fixed pixel number at each band of interest. A region of the same pixel number from an area within the same lane was subtracted from the band of interest as background. RNA binding activity was also quantified using phosphoimaging. The phosphoimage was calibrated (digital light units per fmol RNA) using a standard curve of ^{32}P RNA in the phosphoimage coupled with scintillation counting of the RNA sample. Background was determined by running a lane with RNA alone. Data were analyzed by one-way analysis of variance using SAS (Cary NC) version 8.0. Differences were considered significant at $P<0.05$. Data are expressed as the mean plus or minus the standard error of the mean for three independent experiments.

Immunodetection of IRPs- Subconfluent HFE- and HFE+ fWTHFE/tTA HeLa cells were grown in 35 mm dishes. After 2 days, cells were treated with either 50 μM desferoxamine or 100 nM human holotransferrin (Intergen Co.) overnight. Cells were lysed in NET-Triton after at least 12 hours of treatment. Cell extracts from $1X10^6$ HFE- or HFE+ cells were generated with IRP lysis buffer (20 mM HEPES, 1 mM sodium citrate, 5 mM EDTA, 0.5% NP-40, 100 μg/ml leupeptin, 100 μg/ml pepstatin, 250 μg/ml soybean trypsin inhibitor, 200 μM phenylmethylsulfonyl fluoride, 10 μM MG132, pH 7.4). Samples were diluted with 4X Laemmli buffer (Laemmli, 1970) and subjected to electrophoresis on denaturing 6% SDS-polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis for IRP1 was performed using chicken anti-rat IRP1 serum (1:10,000 dilution), followed by 150 ng/ml biotinylated goat anti-chicken antibody (Vector Labs) and 43 ng/ml horse-radish peroxidase conjugated strepavidin (TAGO Immunologicals). Immunoblot analysis for IRP2 was performed using 780 ng/ml rabbit anti-rat IRP2 antibody (Schalinske et al., 1997) followed by goat anti-rabbit antibody conjugated to horse-radish peroxidase (1:10,000 dilution, Roche Molecular Biochemicals). Immunoblots were developed using chemiluminescence (Super Signal, Pierce) per the manufacturer's directions.

RESULTS

Decreasing Ft levels in the presence of saturating Tf- The reduction observed in Ft levels of cells expressing HFE has been reported to occur either via a reduced binding of Tf to the TfR (Lebron et al., 1999; Salter-Cid et al., 1999) or a reduction in the amount of iron released from Tf (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). Ft levels were assayed in cells with varying amounts of fHFE in the presence and absence of Tf in order to distinguish between these two possibilities. To be certain that the decrease in Ft levels in fHFE-expressing cells was not completely attributable to the decrease in the TfR affinity for Tf, fHFE was induced in the presence of 100 nM human diferric Tf. This concentration is well below circulating concentrations of diferric Tf in the plasma, but is sufficient to saturate the HFE-TfR complex (Feder et al., 1998; Gross et al., 1998; Roy et al., 1999). To be certain that the observed Ft levels in fHFE-expressing cells were independent of a previously established set point by HFE, fHFE expression was induced in HFE- cells by withdrawal of tetracycline. Using this method, cells with the comparable levels of Ft (HFE- and HFE±) at the beginning of treatment were generated. Cell extracts were obtained and normalized for protein from cells after three days of fHFE induction. Immunoblots of these extracts were used to analyze the relative amount of Ft for each cell type and treatment (Figure 6.1).

Cells expressing fHFE had lower Ft levels than cells not expressing HFE. After three days in culture, the Ft levels in cells induced to express fHFE (HFE±) were less than that of HFE- cells (compare Figure 6.1B, lanes 1 versus 2 and lanes 4 versus 5). The Ft levels in HFE± cells dropped from the level of HFE- cells to a level between HFE- and HFE+ cells. At saturating levels of human Tf, cells expressing fHFE still showed decreased levels of Ft compared to cells grown in the absence of human Tf indicating that the lower levels of Ft were not due to less binding of Tf to the TfR. The presence of 100 nM human Tf in the medium sufficiently saturates the TfR despite its reduced affinity for Tf

with HFE binding (Roy et al., 1999). We have shown previously that HFE+ cells take up the same amount of Tf per surface TfR at 50 and 100 nM Tf (Roy et al., 1999). Combined with our previous observation that HFE reduces ^{55}Fe uptake from Tf (Roy et al., 1999), these results support the hypothesis that HFE decreases Ft levels by reducing iron uptake from Tf, rather than merely by reducing the affinity of Tf for the HFE-TfR complex.

fHFE expression reduces Ft synthesis- If HFE lowers the amount of iron taken up into the cell then the amount of Ft translated should decrease. Ft expression is highly regulated at the translational level by IRP proteins. To verify that the reduced Ft expression observed in fHFE-expressing cells was consistent with translational regulation by IRPs, we tested for a reduction in Ft synthesis in fHFE-expressing cells (Figure 6.2). Under low intracellular iron concentrations, IRPs bind the 5' IRE of Ft mRNA and prevent recruitment of the eIF3/40S ribosomal complex to the eIF4F cap-binding complex, thereby reducing the rate of Ft synthesis (Muckenthaler et al., 1998).

Cells were labeled for 15, 30 or 45 minutes in the presence of 100 μCi of ^{35}S methionine/cysteine, then immunoprecipitated with antibody against Ft. The incorporation of ^{35}S methionine/cysteine into total protein as measured by trichloroacetic acid precipitable counts did not significantly differ between HFE- and HFE+ cells (data not shown), indicating that the rates of protein synthesis in the presence or absence of HFE are similar. The rate of ^{35}S methionine/cysteine incorporation into Ft was approximately 40% less for cells that express fHFE (Figure 6.2B). A similar time course was performed at 5, 10 and 15 minutes with the same result (data not shown). These results suggest that the reduced Ft levels detected on immunoblot at steady state are the result of decreased Ft synthesis, which is consistent with an increase in IRP RNA binding activity.

IRP RNA binding activity is increased in fHFE-expressing fWTHFE/tTA HeLa cells- The decreased rate of ^{35}S methionine/cysteine incorporation into Ft in fHFE-expressing cells suggested that the IRP RNA binding activity in these cells might be increased. To determine the relative RNA binding activity by IRPs in fHFE-expressing cells, we performed gel shift analysis on HFE- and HFE+ fWTHFE/tTA HeLa cell extracts.

IRE RNA binding activity was determined in extracts of HFE- and HFE+ fWTHFE/tTA HeLa cells (Figure 6.3). Initially, the combined contribution of IRP1 and IRP2 to the IRE binding activity was determined since the two human IRPs co-migrate in gel shift assays. Cells expressing fHFE had an RNA binding activity of 2910 ± 270 fmol/mg protein. This was 4.6 fold higher than the IRE binding activity measured in fWTHFE/tTA HeLa cells not expressing HFE (640 ± 60 fmol/mg protein) (Figure 6.3). The addition of 2-ME to extracts from cells not expressing HFE increased IRE binding activity by about 2-fold (1200 ± 60 fmol/mg protein). A rat fibroblast cell line, RF2, was used as a control to show a similar effect of 2-ME on IRP activation (Figure 6.3). However, there was no effect of 2-ME on IRE RNA binding activity in extracts from cells expressing fHFE (Figure 6.3), indicating that all 2-ME inducible IRPs were already present in the RNA binding form. These results imply that HFE activates IRPs, but does not identify the mechanism behind IRP activation.

IRP1 and IRP2 RNA binding activity is increased in fHFE-expressing fWTHFE/tTA HeLa cells- Gel shift analysis indicated that fHFE-expressing cells had increased IRP activity. We used gel supershift analysis to determine the relative activity changes in IRP1 and IRP2 isoforms (Figure 6.4). Saturating concentrations of antibodies specific for IRP1 or IRP2 were used (Schalinske et al., 1997). The intensity of the supershifted band for both IRP1 and IRP2 in HFE+ cells was increased over that of HFE- cells (Figure 6.4A, lane 2 versus 5 and lane 3 versus 6). The antibody specific for IRP2 is directed against the 73 amino acid sequence unique to this IRP. This polyclonal antibody can both supershift the

IRP2/IRE complex as well as inhibit IRP2 RNA binding activity. Hence, we quantitated the amount of RNA binding activity that was not supershifted by an IRP-specific antibody and used that value as an indication of the contribution of the other IRP to the IRE binding activity present in these cells. Using this method, HFE+ cells had between 2 and 3 fold increased RNA binding activity over that of HFE- cells for IRP1 and IRP2 (Figure 6.4B).

Expression of fHFE was associated with an alteration of the relative contribution of IRP1 and IRP2 to the total spontaneous IRE binding activity present in the fWTHFE/tTA HeLa cells. In HFE- cells, IRP1 contributed slightly more than 50% of the total binding activity and the IRP1/IRP2 ratio was 1.2 (Table 1). However, in HFE+ cells, IRP1 contributed about two-thirds of the total binding activity such that the IRP1/IRP2 ratio increased to 1.8 (Table 1).

Citrate and other aconitase substrates block the ability of high levels of 2-ME to convert cytosolic aconitase into a high affinity RNA binding form of IRP1(Haile et al., 1992). In lysates from HFE- cells, citrate partially inhibited the effect of 2-ME (data not shown) suggesting that some of the 2-ME inducible activity in these extracts was due to activation of cytosolic aconitase into a high affinity RNA binding form. About half of the 2-ME inducible activity was not affected by citrate addition suggesting that 2-ME was also activating a latent form of IRP2 (data not shown) (Schalinske and Eisenstein, 1996).

IRP protein levels are consistent with activity- Gel shift assays indicated that both IRP1 and IRP2 RNA binding activities were increased in fHFE-expressing cells. IRP1 changes in RNA binding activity are most commonly modulated by the presence or absence of the [4Fe-4S] cluster, keeping IRP1 protein levels constant (Haile et al., 1992). IRP2 RNA binding activity is regulated by the stability of the IRP2 protein which is degraded when the labile iron pool is high and stabilized when the labile iron pool is low (Guo et al., 1995; Iwai et al., 1998). We tested HFE- and HFE+ cells for changes in steady state levels of the

IRPs to determine whether the expression of IRP1 and IRP2 were consistent with the observed IRP RNA binding activity.

Immunoblot analysis was used to determine IRP1 and IRP2 protein levels in HFE- and HFE+ cells and their regulation with iron treatments. HFE- and HFE+ fWTHFE/tTA HeLa cells were treated overnight with standard growth medium (DMEM with 10% fetal calf serum), medium containing 50 μM of the iron chelator desferoxamine, or medium containing 100 nM human Tf as an iron source. Immunoblots of cell extracts normalized for protein concentration were used to analyze relative amounts of IRP1 and IRP2 (Figure 6.5). IRP1 levels did not change significantly with iron treatments or with fHFE expression (Figure 6.5A), but IRP2 levels did respond to iron treatment and fHFE expression (Figure 6.5B). In the presence of the iron chelator desferoxamine, IRP2 levels increased (Figure 6.5B, lane 1 versus 2 and lane 4 versus 5) while the addition of iron to the cells through Tf decreased IRP2 levels (Figure 6.5B lane 1 versus 3 and lane 4 versus 6). IRP2 levels responded to iron treatments regardless of HFE expression, however IRP2 levels were increased in HFE+ cells over that of HFE- cells with each treatment (Figure 6.5B lanes 4-6 versus 1-3). Because the stability of IRP2 is iron regulated, these results suggest that increased IRP activity in HFE+ cells is the result of a reduction in the labile iron pool rather than through iron independent activation of IRP RNA binding activity such as phosphorylation of the IRP or modification of the [4Fe-4S] cluster by reactive oxygen species (Bouton et al., 1996; Brown et al., 1998; Pantopoulos et al., 1996).

Synthesis of IRP1 is not significantly increased with fHFE expression- Immunoblot analysis of IRP levels did not indicate a significant change for the IRP1 protein in fHFE expressing cells. To use a more sensitive assay for changes in protein level, the rate of ^{35}S methioniné/cysteine incorporation into IRP1 was determined for HFE- and HFE+ cells (Figure 6.6). Cells were labeled for 45, 90, 135, or 180 minutes in the presence of 100 μCi of ^{35}S methionine/cysteine, then immunoprecipitated with antibody against IRP1

(Figure 6.6A). The rate of ^{35}S methionine/cysteine incorporation into total cellular protein did not differ significantly between HFE- and HFE+ cells (data not shown).

Quantitation of ^{35}S methionine/cysteine incorporation into IRP1 did not show a significant difference between HFE- and HFE+ cell lines (Figure 6.6B). These results are consistent with immunoblot analysis of IRP1 levels and confirm that fHFE increases IRP activity through posttranslational modification of the IRP proteins.

DISCUSSION

We have previously reported that HFE alters the iron status of cells (Gross et al., 1998; Roy et al., 1999). It reduces the steady-state levels of Ft (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999), reduces the amount of iron absorbed from Tf (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999), and leads to the activation of IRP RNA binding activity (Corsi et al., 1999; Riedel et al., 1999). IRPs are regulated by the labile iron pool and by stimuli from other processes such as kinase cascades (Brown et al., 1998; Schalinske and Eisenstein, 1996) and in response to oxidative stress (Bouton et al., 1996; Drapier et al., 1993; Hanson et al., 1999; Pantopoulos et al., 1996; Weiss et al., 1993). A direct relationship between HFE expression, the labile iron pool and IRP RNA binding activity has not been thoroughly examined. Therefore, we characterized the activities of IRP1 and IRP2 in fWTHFE/tTA HeLa cells to test whether the mechanism by which HFE regulates iron homeostasis works through the labile iron pool or independent of it. Our results are consistent with HFE acting to lower the intracellular pool of labile iron.

Although HFE binding decreases the affinity of the TfR for Tf (Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998), HFE's activity is hypothesized to be specific to the acquisition of iron from Tf rather than the uptake of Tf itself (Riedel et al., 1999; Roy et al., 1999). HFE reduces Tf-mediated iron uptake under concentrations where the binding of Tf to the TfR is saturated (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999). Saturating concentrations of Tf are still well below the observed plasma Tf concentrations and are, therefore, physiologically relevant. To determine whether the low iron phenotype described in the HFE-expressing cells is explained simply by the change in the affinity of the HFE-TfR complex for Tf, we examined the effect of fHFE expression on Ft levels in the presence of 100 nM Tf. HFE does not reduce Tf uptake at this concentration (Roy et al., 1999). Thus, our observation that Ft levels in fHFE-expressing

cells still decrease in the presence of 100 nM human Tf argues that HFE affects steady state Ft levels independent of its affect on the TfR affinity for Tf.

Steady state levels of Ft are decreased in cells expressing HFE. IRP binding to the Ft IRE prevents translation by excluding the binding of the eIF3/40S ribosomal complex to the eIF4F cap-binding complex (Muckenthaler et al., 1998). To test whether Ft levels in HFE+ cells were reduced as a result of translational control, the rate of incorporation of ^{35}S methionine/cysteine into Ft was determined in HFE- and HFE+ cells. Translation of Ft is reduced in fHFE-expressing cells by approximately 40% in a 45 minute time course. This result suggests that decreased Ft levels in HFE+ cells are the result of decreased translational efficiency, which is consistent with increased IRP RNA binding activity and a reduction in the labile iron pool.

Direct analysis of IRP RNA binding activity in fWTHFE/tTA HeLa cells indicated that fHFE expression leads to the activation of IRP RNA binding activity. HFE+ fWTHFE/tTA HeLa cells exhibited an IRE RNA binding activity almost 5 times more than HFE- cells. In our cell line, the difference between the IRP activities of HFE- and HFE+ cells was not equalized with 2-ME as previously reported (Corsi et al., 1999; Riedel et al., 1999). This is probably due to the increase in IRP RNA binding activity of both IRP1 and IRP2. This discrepancy with previously reported results may be due to the difference in levels of IRP2 induction between HeLa cell strains.

In the absence of HFE, fWTHFE/tTA HeLa cells contain latent forms of IRP1 and IRP2. Only 50% of 2-ME inducible activity in HFE- fWTHFE/tTA HeLa cells was blocked by citrate, suggesting HFE- cells also contain latent IRP2 (Haile et al., 1992; Henderson and Kuhn, 1995; Henderson et al., 1993; Schalinske and Eisenstein, 1996). However, when fHFE was expressed, neither of these latent forms were present, indicating full activation of IRP RNA binding activity in fHFE-expressing cells. The enhanced activity of IRP1 in HFE+ cells was due to a relative increase in the proportion of IRP1 in the RNA binding form, rather than an increase in the amount of IRP1 protein.

IRP2 protein, however, was increased in HFE+ cells over HFE- cells. This result is consistent with IRP2 regulation by a depleted labile iron pool and may explain why RNA binding activity is greater in HFE+ cells, even in the presence of 2-ME.

Expression of fHFE was associated with a significant shift in the IRP1 to IRP2 ratio due to differences in the degree to which the activity of each binding protein was increased. The IRP1/IRP2 ratio increased from 1.2 in HFE- cells to 1.8 in HFE+ cells, suggesting IRP2 RNA binding activity was not as efficiently induced as IRP1. HFE may reduce the labile iron pool to a concentration range that provides more efficient activation of IRP1 than IRP2. The induction of RNA binding activity by these two proteins occurs through two different pathways, but they are dependent on the same labile iron pool. Recent evidence indicates that both IRPs bind well to the Ft IRE but that IRP2 does not bind as well to non-Ft IRE (Ke et al., 1998). In light of this observation it is interesting that in the presence of HFE, Ft expression appears more dramatically affected than does that of the TfR (Roy et al., 1999). This may permit cells to balance the reduction in the labile iron pool due to reduction in iron-uptake of HFE-expressing cells with their iron-storage capacity without having to greatly increase expression of the TfR. Like the differential regulation of the expression of Ft and mitochondrial aconitase in liver (Chen et al., 1997), the dissimilar regulation of Ft and TfR in HFE expressing cells would appear to provide another example of differential regulation of IRE-containing mRNA by IRPs. The molecular basis for the difference in the magnitude of activation of IRP1 and IRP2 in HFE+ fWTHFE/tTAHeLa cells awaits further investigation.

In this study we have investigated HFE's role in the coordination of intracellular iron sensing machinery, in particular through modulation of the labile iron pool. The results we report support the hypothesis that HFE establishes a new, lower steady state for the cellular labile iron pool. This reduced concentration of iron exists despite the increase in TfR numbers and the decrease in Ft protein. This HFE-specific reduction of the labile iron pool may be important for regulation of organismal iron homeostasis because, despite

the exquisite regulation afforded by IRPs, HFE's role in the maintenance of iron homeostasis remains indispensable. Individuals suffering hereditary hemochromatosis have reduced levels of functional HFE (Feder et al., 1996; Levy et al., 1999; Rothenberg and Voland, 1996; Santos et al., 1996; Zhou et al., 1998) and cannot appropriately regulate iron uptake despite functional IRPs (Cairo et al., 1997; Santos et al., 1998).

We have shown that HFE reduces the labile iron pool. While the activity of IRPs and the expression of their target proteins are affected by this change in the labile iron pool, HFE-specific regulation of iron homeostasis may not be limited to regulation through the IRPs. We speculate that HFE's control of the labile iron pool may have further reaching effects on iron homeostasis through other regulatory mechanisms dependent on the labile iron pool such as iron-dependent transcription or iron-dependent protein trafficking. Experiments designed to test for the influence of HFE on the expression and activity of proteins involved in enterocyte-specific iron transport may clarify the purpose for HFE's hypothesized modulation of the labile iron pool in the enterocyte precursor cells of the crypt (Andrews, 1999; Cox and Kelly, 1998).

Table 1.

Relative Contributions of IRP1 and IRP2 to Total IRP Binding Activity as a Function of HFE Expression

Cell Sample	IRP1[a] (Percent of Total)	IRP2[a] (Percent of Total)	Ratio of IRP 1/ IRP2 RNA Binding Activities[a]
HFE -	55 ± 1	45 ± 1	1.2 ± 0.07
HFE +	64 ± 2[b]	36 ± 2[b]	1.8 ± 0.14[b]

a. The relative contribution of IRP1 and IRP2 to the total IRP binding activity in HeLa cell extracts was determined as follows. Saturating amounts of antibodies specific for IRP1 or IRP2 were used to individually supershift the binding activity for a given IRP. The amount of activity not supershifted by the IRP1 antibody was judged to be due to IRP2. The amount of activity not shifted by each antibody was summed and this was used to determine the percentage that each IRP contributed to the total activity. These values were determined from 3 independently prepared extracts of HeLa cells expressing or not expressing HFE. All values are reported as mean ± S.E.M.

b. Significantly different from HFE- cells ($P < 0.05$).

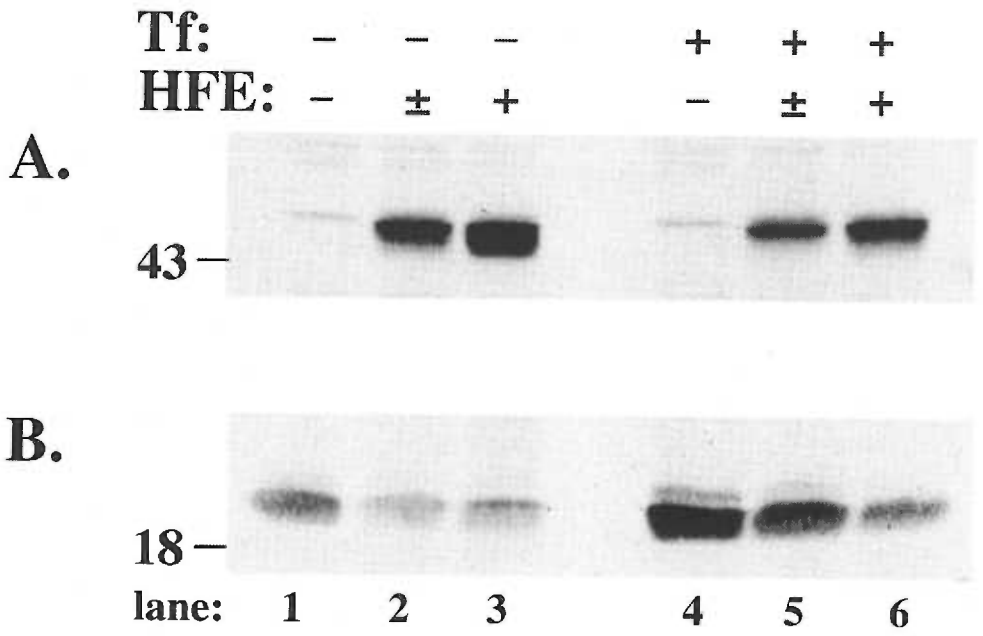

Figure 6.1. fHFE reduces Ft expression in the presence of 100 nM Tf. Approximately 350 μg of cellular protein was run on a 12% denaturing polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with (A) mouse anti-FLAG antibody (M2, 1:10,000 for HFE) and HRP-conjugated rabbit anti-mouse antibody (1:10,000) or (B) sheep anti-human Ft antibody (1:200) and HRP-conjugated swine anti-goat antibody (1:20,000). Chemiluminescence detected (A) HFE at 43-45 kDa and (B) the Ft light and heavy chains at 19 and 21 kDa. Ft levels in cells that have been expresing HFE (HFE+) or were induced to express HFE (HFE±) are less than that of HFE- cells in all cases. HFE expression leads to the reduction of Ft levels even in the presence of saturating Tf. These results are representative of three independent experiments without significant variation between experiments.

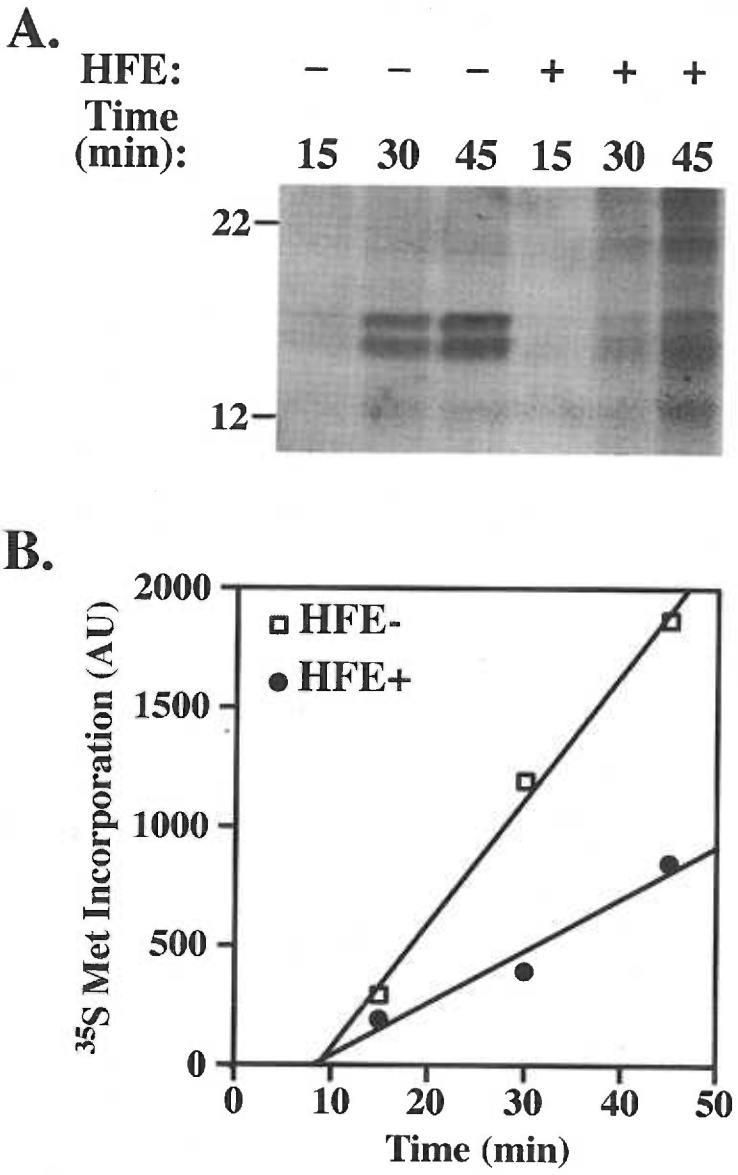

Figure 6.2. The rate of ^{35}S-methionine incorporation into Ft is reduced in fHFE-expressing cells. Approximately 6.5X10^5 HFE- or HFE+ fWTHFE/tTA HeLa cells were labeled over a 45 minute timecourse with 100 μCi ^{35}S methionine. Ferritin was immunoprecipitated and run on a 12% polyacrylamide gel under reducing conditions. (A) Autoradiograph shows a significant reduction in ^{35}S methionine incorporation in HFE-expressing cells. (B) Quantitation of ^{35}S methionine incorporation in HFE- (open squares) and HFE+ (closed circles). These results are representative of three independent experiments without significant variation between results.

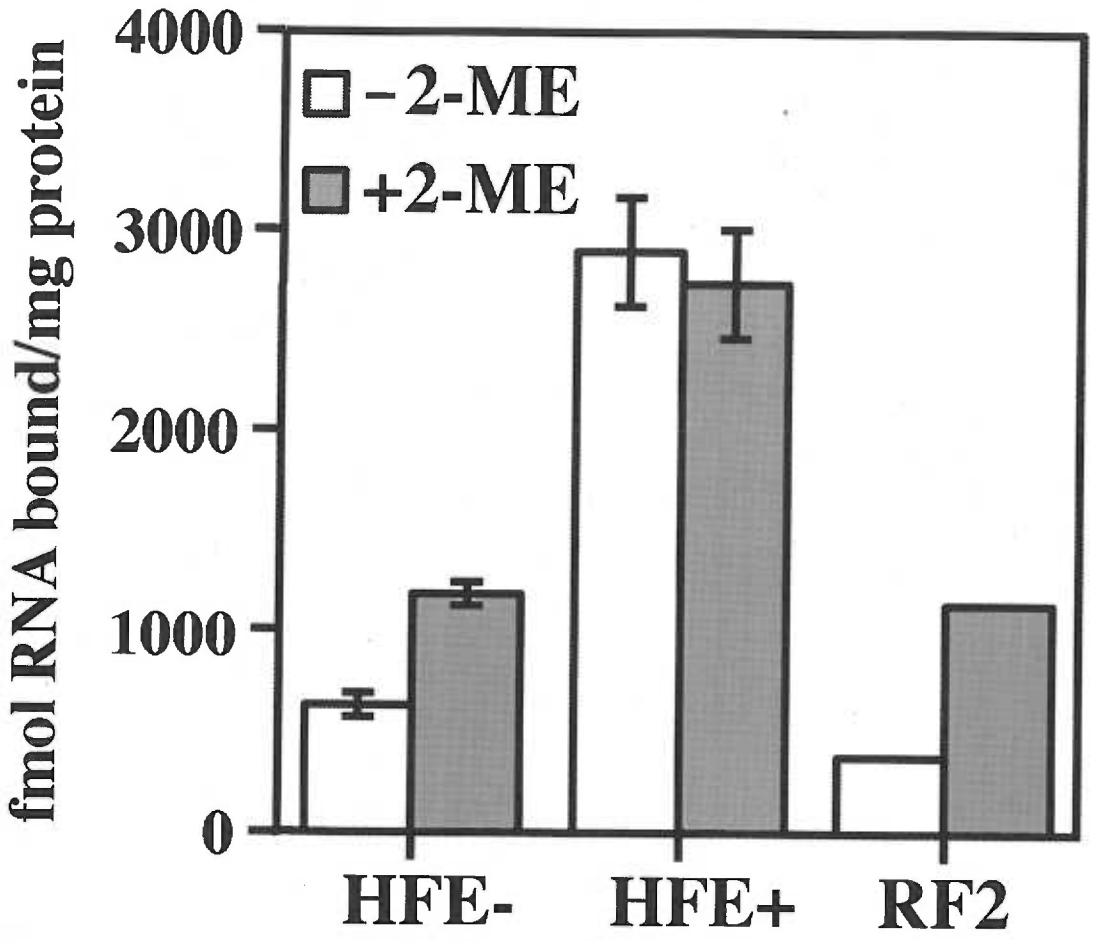

Figure 6.3. Total IRP RNA binding activity is increased in fHFE-expressing cells. Between 1 and 1.5 μg clarified cellular protein was assayed for IRP RNA binding activity as described under "Materials and Methods". RNA binding was performed without 2-ME (open bars) or with 2-ME (filled bars) in the assay buffer. HeLa IRP RNA binding activity was comparable to that of RF2 cells. HFE-expressing cells had increased IRP RNA binding activity in the presence or absence of 2-ME and, unlike RF2 or HFE- cell lysates, were not induced in the presence of 2-ME. These results are representative of those obtained in at least three independent experiments.

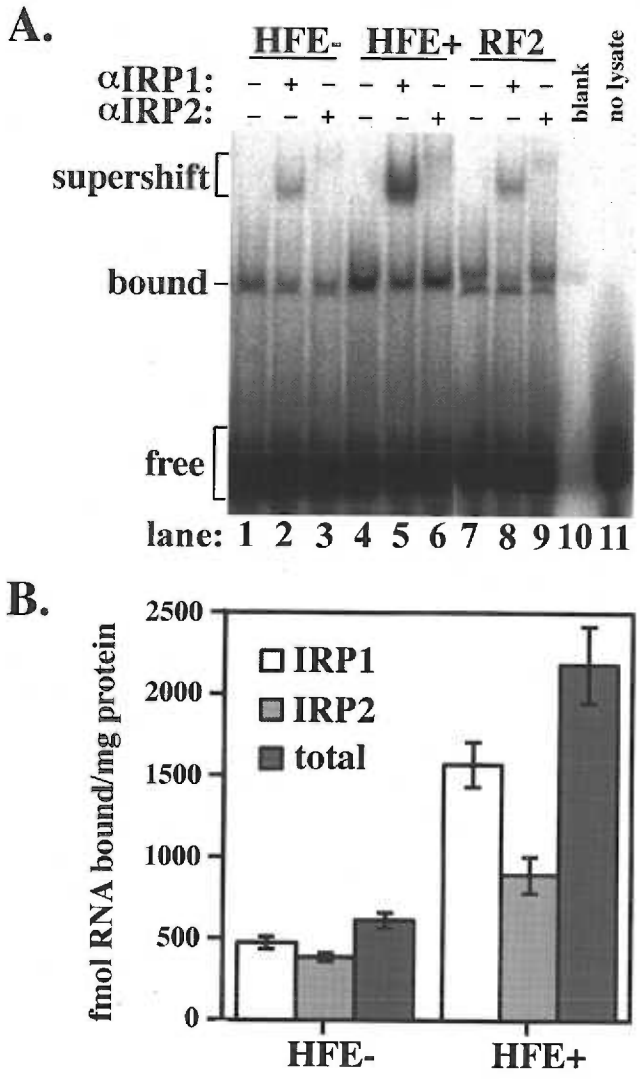

Figure 6.4. IRP1 and IRP2 RNA binding activity are increased in fHFE-expressing cells. One μg of clarified cellular protein was incubated with 10 μg IRP1 or IRP2 antibody, then assayed for IRP RNA binding activity as described under "Materials and Methods". Binding of IgG to the protein-RNA complex results in "supershifted" RNA probe (panel A). HFE+ cells had increased IRP1 and IRP2 RNA binding activity. Quantitation of the IRP bound probe that was not supershifted by IgG indicates that HFE-expressing cells had approximately 3.3 fold more IRP1 activity (open bars) and 2.3 fold more IRP2 activity (light gray bars), with total IRP activity increasing approximately 3.6 fold (dark gray bars) (panel B). These results are representative of those obtained in at least three experiments.

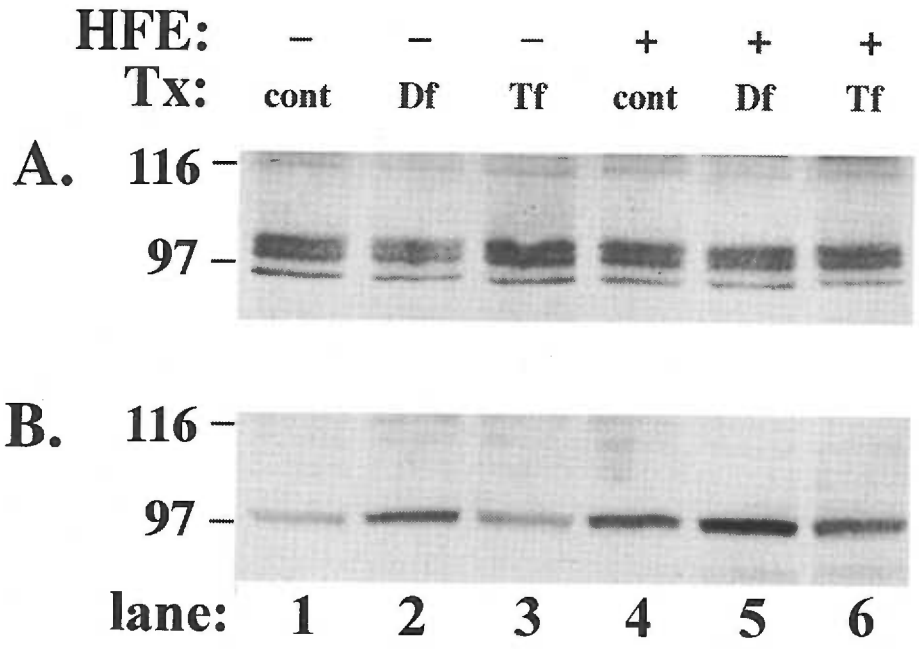

Figure 6.5. Steady state levels of IRP proteins are consistent with reduction of the labile iron pool in fHFE-expressing cells. Approximately 500 μg of cellular protein was run on a 6% polyacrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with (A) chicken anti-rat IRP1 antibody(1:5000), biotin-conjugated goat anti-chicken antibody (1:10,000) and HRP-conjugated strepavidin (1:20,000) or (B) rabbit anti-rat IRP2 antibody (1:5000) and HRP-conjugated goat anti-rabbit antibody (1:10,000). Chemi-luminescence detected IRP1 and IRP2 at greater than 97 kDa. (A) While IRP1 levels did not change significantly with Tf iron treatment or HFE-expression, (B) IRP2 levels decreased with the addition of iron and increased under conditions which depleted the labile iron pool such as in the presence of Df, the iron chelator or with HFE-expression. These results are representative of those obtained in multiple experiments.

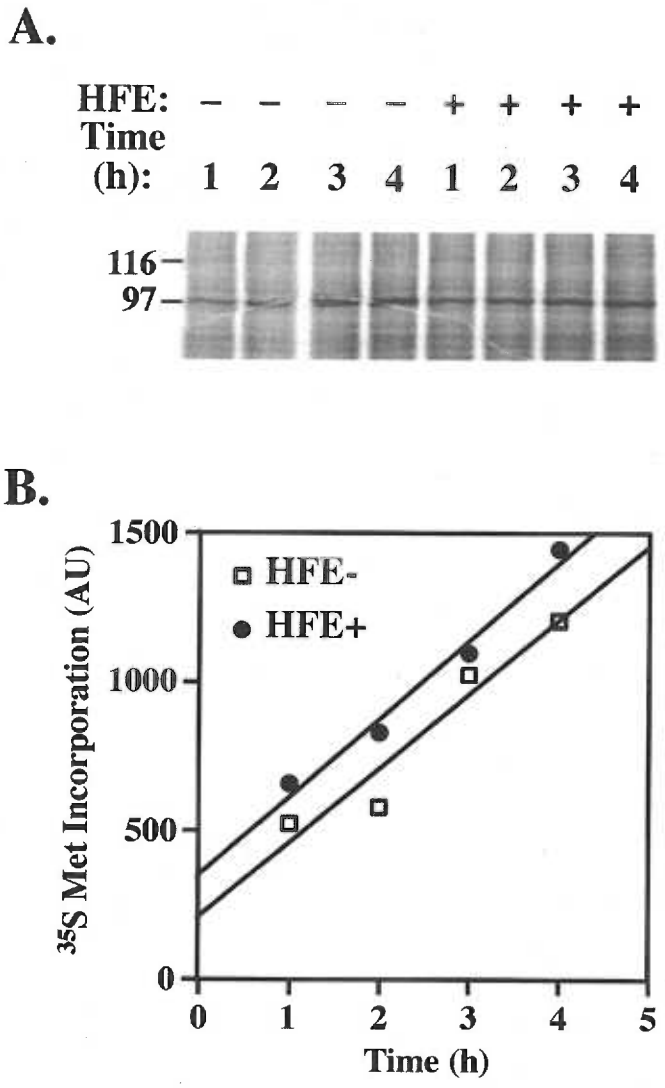

Figure 6.6. The rate of ^{35}S methionine incorporation into IRP1 does not change in fHFE-expressing cells. Approximately 1X10^6 HFE- or HFE+ fWTHFE/tTA HeLa cells were labeled over a four hour timecourse with 100 μCi ^{35}S methionine. IRP1 was immunoprecipitated and run on a 6% polyacrylamide gel under reducing and denaturing conditions. (A) Autoradiograph shows no significant change in ^{35}S methionine incorporation in HFE-expressing cells. (B) Quantitation of ^{35}S methionine incorporation in HFE- (open squares) and HFE+ (closed squares) shows no significant change in the rate of ^{35}S-methionine incorporation into IRP1 for HFE-expressing cells. Linear regression was performed in the Cricket graph program. These results are representative of multiple experiments without significant variation between results.

CHAPTER 7

Iron Homeostasis: New Tales from the Crypt

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SUMMARY

The enterocyte is a highly specialized cell of the duodenal epithelium that coordinates iron uptake and transport into the body. Until recently, the molecular mechanisms underlying iron absorption and iron homeostasis have remained a mystery. This review focuses on the proteins and regulatory mechanisms known to be present in the enterocyte precursor cell and in the mature enterocyte. The recent cloning of a basolateral iron transporter and investigations into its regulation provide new insights into possible mechanisms for iron transport and homeostasis. The roles of proteins such as iron regulatory proteins, the hereditary hemochromatosis protein (HFE)-transferrin receptor complex, and hephaestin in regulating this transporter and in regulating iron transport across the intestinal epithelium are discussed. A speculative, but testable, model for the maintenance of iron homeostasis, which incorporates the changes in the iron-related proteins associated with the enterocyte's life cycle as it journeys from the crypt to the villar tip, is proposed.

INTRODUCTION

Iron is required by almost every organism. The ability of this transition metal to exist in two redox states makes it useful at the catalytic center of fundamental biochemical reactions. DNA synthesis, transport of oxygen and electrons, and respiration all require iron. The same properties that make iron useful in each of these reactions also make it toxic. Free iron has the ability to generate oxidative radicals that damage essential biological components such as lipids, proteins, and DNA. An organism must sense its internal iron load and respond appropriately by altering iron uptake and storage processes. In the case of humans, the amount of iron that is absorbed, rather than the amount that is excreted, is controlled. Inappropriate responses or lack of a response lead to anemia or iron overload.

The most important checkpoint of iron homeostasis in higher organisms is contained in the epithelial cell layer of the duodenum, which is responsible for sensing changes in body iron demands and then adapting to meet them. Within the crypts of the intestine are multipotent precursor cells, some of which migrate onto the villus and differentiate into enterocytes (Daniele and D'Agostino, 1994; Hocker and Weidenmann, 1998). The enterocytes are specialized for absorption and transport of iron (Figure 7.1). The precursor cells differ from enterocytes in their expression of proteins related to iron uptake and transport (Canonne-Hergaux et al., 1999; Donovan et al., 2000; Hodin et al., 1995; Pothier and Hugon, 1980; Vulpe et al., 1999) (Figure 7.2). While its precursor acts only as a sensor of body iron needs, upon differentiation the enterocyte is capable of transporting iron. New proteins required for the absorption, storage, and export of the organism's total dietary iron requirement are expressed in the enterocyte.

Regulation of iron uptake into the body occurs at two interfaces of the intestinal epithelium; the apical and basolateral membranes. The apical membrane of the differentiated enterocyte, which faces the intestinal lumen, is specialized for transport of

heme and ferrous iron into the cell. At least three pathways have been observed for this transport process. The most extensively characterized uptake pathway is via the divalent metal transporter, DMT1 (previously named Nramp2 and DCT1). The DMT1 sequence (Gruenheid et al., 1995; Pinner et al., 1997), function (Fleming et al., 1998; Fleming et al., 1997; Fleming et al., 1999; Garrick et al., 1999; Gruenheid et al., 1999; Gunshin et al., 1997; Su et al., 1998; Tandy et al., 2000), and regulation (Canonne-Hergaux et al., 1999; Trinder et al., 2000; Wardrop and Richardson, 1999; Zoller et al., 1999) have been reviewed recently (Andrews, 1999; Andrews and Levy, 1998). DMT1 is a proton symporter that transports ferrous iron and other divalent metals from the intestinal lumen into the enterocyte (Fleming et al., 1998; Fleming et al., 1997; Gunshin et al., 1997). Its expression is regulated by body iron stores (Gunshin et al., 1997; Zoller et al., 1999), but it may also be susceptible to regulation by dietary iron (Crosby, 1966; O'Neil-Cutting and Crosby, 1987), or through a recently observed post-translational mechanism (Trinder et al., 2000). Iron can also be absorbed by the heme-iron uptake pathway which functions as a very efficient means of iron uptake, but the molecular mechanisms of transport into or across the intestinal epithelium have not yet been elucidated (Bezwoda et al., 1983; Monsen, 1988; Wyllie and Kaufman, 1982). Finally, a mucin-integrin-mobilferrin pathway has also been identified as a possible means of iron uptake (Conrad and Umbreit, 1993).

The basolateral membrane mediates the transfer of the iron transported into the intestinal epithelial cells to the rest of the body. Iron that is not exported into the plasma is lost with exfoliation of the intestinal epithelium (Geyer, 1979; Hocker and Weidenmann, 1998; Lombard et al., 1997). The proteins related to iron metabolism on the basolateral membrane of the precursor and mature enterocyte either sense body iron stores, or facilitate regulated iron transport into the plasma. These include the TfR-HFE complex (Feder et al., 1996; Feder et al., 1998; Waheed et al., 1999); the basolateral iron transporter (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000); and the ceruloplasmin

homologue, hephaestin (Vulpe et al., 1999). While the TfR structure (Lawrence et al., 1999), function (Enns et al., 1996; Lok and Loh, 1998), and regulation (Kuhn, 1998) have been well described, the remaining proteins have only recently been identified. Advances toward the understanding of their individual function and regulation are the subjects of this review.

COMMUNICATION AND REGULATION

The enterocyte receives signals from various tissues as to the relative repletion of iron stores. Though none have been clearly identified, several "regulators" for iron homeostasis have been hypothesized based on the segregation of iron requirements within an organism. When the amount of iron found in body stores such as the liver, skeletal muscle, and blood drops below a critical level, the stores regulator increases iron uptake until the reserves are replete again (Cox and Peters, 1980; Gavin et al., 1994; Hunt and Roughead, 2000; Nathanson et al., 1985). The stores regulator acts on a pathway that facilitates a slow accumulation of non-heme dietary iron (about 1 mg/day) (Sayers et al., 1994). It does not seem to significantly regulate heme-iron uptake (Bezwoda et al., 1983; Hunt and Roughead, 2000; Skikne and Cook, 1992). The stores regulator also has the important task of preventing iron overload after ensuring iron needs are met. It reconditions the intestinal epithelium such that iron absorption is reduced in the face of enlarged iron stores and it reduces the average uptake of iron per day in adulthood when growth requirements decrease (Finch, 1994). Because this regulator must signal between the liver, muscle, and intestine, a soluble component is hypothesized. Serum Ft (Flowers et al., 1986; Taylor et al., 1988), Tf (Raja et al., 1999; Taylor et al., 1988), and the serum TfR (Cook et al., 1990; Feelders et al., 1999) have all been proposed as candidate molecules.

The erythropoietic regulator is a second hypothesized regulator of iron absorption that communicates the erythropoietic demand of the organism rather than directly reflecting iron stores. In support of this hypothesis, individuals with normal, or even increased, iron stores up-regulate iron absorption as marrow iron requirements increase (Andrews, 1999; Finch, 1994). An increase in erythropoiesis alone is not enough to increase iron absorption. Rather, the imbalance between the marrow's rate of erythropoiesis and its iron supply is thought to induce iron absorption (Andrews, 1999; Kooistra et al., 1991; Schiffer et al., 1966). The absorptive pathway targeted by the erythropoietic regulator is probably distinct from the pathway targeted by the stores regulator, as evidenced by the rate of iron uptake. Anemic individuals can absorb between 20 and 40 mg of iron per day: an increase much greater than that which the stores regulator is capable of producing (Finch, 1994). Like the stores regulator, the erythropoietic regulator is hypothesized to be a soluble component of the plasma as it must signal between the erythroid marrow and the intestine (Cazzola et al., 1999).

Recent insights into the regulation of the machinery required for iron absorption have confirmed earlier hypotheses that hypoxia may play a role as an independent regulator that induces intestinal iron absorption (McKie et al., 2000; Mukhopadhyay et al., 2000; Raja et al., 1986). Whether this regulatory pathway is truly distinct from the one induced by the erythropoietic regulator is uncertain. The signaling pathways and molecular components involved in the up-regulation of iron absorption through any of these regulators remain to be determined.

The stores and erythropoietic regulators are humoral factors that maintain iron homeostasis for the entire organism. Other regulatory mechanisms are in place for the maintenance of iron homeostasis for a single cell. Briefly, IREs are stem loop structures in the 3' or 5' untranslated region of several key mRNAs encoding proteins of iron metabolism. IRPs work in conjunction with these elements to sense and respond to changes in the amount of chelatable iron in the intracellular environment, or the "labile iron

pool". Through the interaction of IRPs with IREs, Tf uptake increases by stabilizing the TfR mRNA, while Ft storage of iron decreases by blocking translation of Ft mRNA. These events result in an increase in the labile iron pool. Conversely, Tf uptake decreases and Ft levels increase when intracellular iron concentrations rise. The reciprocal regulation of these two proteins has been extensively reviewed for non-polarized cells elsewhere (Eisenstein and Blemings, 1998; Hentze and Kuhn, 1996).

The labile iron pool of the enterocyte appears to regulate a subset of proteins involved in iron homeostasis. TfR expression in duodenal crypts responds to increases or decreases in body iron stores (Anderson et al., 1990; McKie et al., 1996; Pietrangelo et al., 1992; Pountney et al., 1999). Similarly, duodenal Ft levels decrease with increased transfer of iron to the plasma (Pountney et al., 1999). Much attention has been focused on the role of IRPs in the modulation of enterocyte-specific iron transport proteins, especially proteins with IRE containing messages such as DMT1 (Gunshin et al., 1997; Wardrop and Richardson, 1999) and the newly identified basolateral iron transporter, Ferroportin1/Ireg1/MTP1 (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). DMT1 has an IRE in the 3' untranslated region of its message, suggesting it would be degraded, like the TfR message, in the context of a high labile iron pool. Ferroportin1/Ireg1/MTP1 has an IRE in the 5' untranslated region of its message, suggesting it would be translated more efficiently, like the Ft message, in the context of a high labile iron pool. Experiments designed to test for the control of DMT1 and Ferroportin1/Ireg1/MTP1 by IRPs (Abboud and Haile, 2000; Gunshin et al., 1997; McKie et al., 2000; Wardrop and Richardson, 1999) have suggested other regulatory mechanisms may be involved in addition to regulation through IRPs. While the labile iron pool and IRPs influence iron transport across the enterocyte (Eisenstein, 1998; Garate and Nunez, 2000; Schumann et al., 1999), other forms of regulation through the labile iron pool such as iron regulated transcription (McKnight et al., 1980; White and Munro, 1988),

degradation (Guo et al., 1995; Iwai et al., 1998), and intracellular trafficking events (Abboud and Haile, 2000; Trinder et al., 2000) could also be involved.

THE HFE-TRANSFERRIN RECEPTOR COMPLEX

HFE-associated HH is a disorder of enterocyte iron regulation characterized by increased dietary iron uptake and resulting in iron overload. Though an increase in Tf saturation may be observed within the first two decades, the complications due to iron overload usually do not present until the fourth or fifth decade of life (Bothwell et al., 1995). The defect has been described as an increase in the iron regulatory "set point" (Gavin et al., 1994) because affected individuals experience a chronic increase in the rate of iron transfer from the enterocyte to the blood (McLaren et al., 1991). The HH defect has been attributed to a decrease in the amount of functional HFE protein (Feder et al., 1996; Feder et al., 1997; Levy et al., 1999; Rothenberg and Voland, 1996; Santos et al., 1996; Waheed et al., 1997; Zhou et al., 1998).

The first indication of how HFE might regulate iron metabolism came with the discovery that it associates with the TfR (Bennett et al., 2000; Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998; Parkkila et al., 1997; Waheed et al., 1999). The TfR is expressed at the cell surface and binds the serum iron transport protein, Tf, with high affinity. Tf releases iron in endosomes following their acidification, after which the iron is transported across the endosomal membrane and targeted for use in iron containing enzymes, or for storage in Ft (Enns et al., 1996; Lok and Loh, 1998). HFE binds the TfR with an affinity close to that of Tf, reducing the TfR affinity for Tf and competing with Tf binding (Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998; Lebron et al., 1999).

HFE's close association with the Tf-mediated iron uptake pathway (Corsi et al., 1999; Riedel et al., 1999; Roy et al., 1999) and its location in endosomes (Gross et al., 1998) and on the basolateral side of enterocyte precursor cells (Parkkila et al., 1997)

(Figure 7.2) implicates a role for HFE in sensing body iron stores. Tf (Raja et al., 1999; Taylor et al., 1988), and the serum TfR (Cook et al., 1990; Feelders et al., 1999), two proteins with which HFE may directly interact, have both been hypothesized as stores regulators. The mechanism by which HFE might facilitate the sensing of body iron stores remains unknown, however. Alternatively, HFE's role in establishing the set point for iron transport may be independent of body iron stores. HFE-deficient mice are capable of changing the rate of dietary iron uptake with changes in body iron stores (Santos et al., 1998; Zhou et al., 1998).

Indirect evidence implies that the relative concentrations of HFE and the TfR are important in iron loading in mice. An elegant study used the HFE and TfR knock out mice to determine the relative contributions of these two proteins to iron loading. The authors found that mice lacking HFE and one TfR allele experienced more iron loading than HFE knock out mice with two normal TfR alleles. The same trend was seen with the mice that have the HH C282Y HFE allele and only one TfR allele (Levy et al., 2000). Since mice with only one copy of the TfR cannot increase TfR levels to that of wild type mice (Levy et al., 1999), this result suggests that the ratio between HFE and the TfR is critical for maintenance of iron homeostasis. The actual HFE:TfR stoichiometry is not clear, nor are the relative amounts of each protein in intestinal precursor cells. Studies of HFE and the TfR in transfected cells and in solution suggest the stoichiometry is 1:2, or one HFE per TfR dimer (Gross et al., 1998; Lebron et al., 1998). However, the crystal structure indicates a 2:2 stoichiometry is also possible under very high concentrations of HFE (~8 mM) (Bennett et al., 2000). Though the iron loading results of the compound HFE-/- and TfR+/- knock out mice are informative, they must be interpreted cautiously. The HFE knock out mice with only one TfR allele also have iron deficient erythropoiesis. The compound knockout mice may take up more iron than the HFE knockout mice due to the response of the erythroid regulator rather than the stores regulator (Levy et al., 2000).

Surprisingly, overexpression of HFE in cells grown in culture reduces iron uptake and lowers intracellular Ft levels (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999; Roy et al., 1999). This result was initially unexpected because the enterocytes of individuals with HH, who posses little or no functional HFE, appear to have lower Ft levels than normal individuals (Pietrangelo et al., 1995). While the effect on intracellular Ft is a universal finding, the mechanism by which this might be accomplished is more controversial. Initial studies established that HFE does not reduce Tf uptake at saturating Tf concentrations or alter the cycling of the TfR (Roy et al., 1999). Other reports attributed differences in iron uptake to a reduction in Tf uptake (Salter-Cid et al., 1999) or a reduction in the rate of Tf recycling to the cell surface (Ikuta et al., 2000).

Consistent with its effect on Ft and TfR regulation (Gross et al., 1998), HFE has been shown to increase the RNA binding activity of IRPs (Corsi et al., 1999; Riedel et al., 1999). The involvement of IRPs is of great interest due to their seemingly universal control over cellular iron regulation including pathways of iron uptake, storage, and utilization (Hentze and Kuhn, 1996; Kuhn, 1998). IRPs are functional in individuals with HH in that they can respond to fluctuations in iron levels (Cairo et al., 1997; Flanagan et al., 1995). These results suggest that IRP RNA binding activity, though necessary, is not sufficient for the maintenance of iron homeostasis in an individual. Whether HFE has direct effects on IRP RNA binding activity as do protein kinase C and nitric oxide, or whether HFE increases IRP RNA binding activity indirectly through a reduction of the labile iron pool remains to be seen (Bouton et al., 1996; Brown et al., 1998; Pantopoulos et al., 1996).

FERROPORTIN1/IREG1/MTP1

An exciting addition to our understanding of iron metabolism has come with the cloning and characterization of a putative basolateral iron transporter. Several groups have

used multiple systems by which to isolate and characterize the gene referred to as *Ireg1* (McKie et al., 2000) or *MTP1* (Abboud and Haile, 2000) in mice and *weissherbst (weh)* in zebrafish (Donovan et al., 2000). The protein product is a putative multiple membrane spanning transporter that has been shown to function as an iron exporter (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). Two of the aforementioned studies used a xenopus oocyte expression system to characterize iron export in oocytes expressing Ferroportin1/Ireg1/MTP1 (Donovan et al., 2000; McKie et al., 2000). Both groups co-expressed DMT1 to load oocytes with iron and then assayed for iron efflux in the presence of apo-Tf. One study also indicated that ceruloplasmin was necessary for iron efflux while apo-Tf was not. These results are consistent with the ability of hypotransferrinemic mice to facilitate mucosal iron transfer despite the lack of Tf expression (Bernstein, 1987; McKie et al., 2000).

The localization of Ferroportin1/Ireg1/MTP1 in cells and tissues is consistent with its proposed function of exporting iron from cells. In the duodenum, Ferroportin1/Ireg1/MTP1 localizes to mature enterocytes and is absent from the crypts (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000) (Figure 7.2). The protein is also found in the liver, predominantly in Kupffer cells where iron is scavenged from red blood cells, but some immunostaining also localizes to the hepatocytes (Abboud and Haile, 2000; Donovan et al., 2000). The specific intracellular localization of Ferroportin1/Ireg1/MTP1 in polarized cells is on the basolateral membranes of placental trophoblasts (Donovan et al., 2000) and MDCK cells overexpressing the transporter (McKie et al., 2000).

Investigations aimed at understanding the regulation of this iron exporter have identified several potential levels of regulation. The Ferroportin1/Ireg1/MTP1 mRNA possesses an IRE in the 5' UTR which binds IRPs (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). This stem loop structure is similar to other IREs found at the 5' end of mRNAs encoding Ft, erythroid 5-aminolevulinate synthase, mitochondrial

aconitase, and succinate dehydrogenase (McKie et al., 2000). The protein products of these messages are not translated under low iron conditions due to binding of IRPs, suggesting Ferroportin1/Ireg1/MTP1 would be down-regulated under conditional of low intracellular iron (Hentze and Kuhn, 1996). While this stem loop structure confers iron-dependent regulation of luciferase in desferoxamine treated COS-7 cells (Abboud and Haile, 2000) and binds IRPs (Abboud and Haile, 2000; McKie et al., 2000), the protein levels found *in vivo* under varying conditions of iron repletion are not entirely consistent with this type of regulation. Immunohistochemical staining for the iron exporter revealed strong reactivity in the Kupffer cells of iron replete mice with weaker staining in iron depleted mice, consistent with regulation through the IRPs. However, the reciprocal result was found in the duodenal epithelium; immunohistochemical staining was strong in iron depleted mice and weaker in iron replete mice (Abboud and Haile, 2000).

Other levels of regulation must also exist because the Ferroportin1/Ireg1/MTP1 message is increased in the duodenum of hypotransferrinemic mice (McKie et al., 2000). If translational control of protein synthesis was the only mode of regulation, message levels should not fluctuate. Up-regulation of the message was also observed under hypoxic conditions (McKie et al., 2000). A hypoxic response is in keeping with some studies that have observed a connection between the erythroid regulator and the mucosal oxygen supply in regulation of iron uptake (Raja et al., 1988; Sauer et al., 1969). The rapid loading of hepatic iron stores in the hypotransferrinemic mice (Bernstein, 1987) to levels above those of the HFE knock out mouse (Levy et al., 1999; Zhou et al., 1998) implicates up-regulation of pathways that can increase iron uptake many times that of the stores regulator, such as that induced by the erythroid regulator (Finch, 1994). As hypotransferrinemic mice suffer severe anemia (Bernstein, 1987; Raja et al., 1999), either an erythropoietic or a hypoxic response may be the mechanism by which the Ferroportin1/Ireg1/MTP1 message is up-regulated in the hypotransferrinemic mice. In fact, transfusion of these mice with erythrocytes of wild type littermates reduced transfer of iron across the basolateral

membrane without changing iron uptake across the apical membrane (Buys et al., 1991; Raja et al., 1999). While specific iron transporters were not addressed by these experiments, the data suggests that apical iron uptake may be unaffected by alleviation of anemia, but that transport of iron across the basolateral membrane was down-regulated with an increase in hematocrit. These results are consistent with what we now know concerning regulation of DMT1 and Ferroportin1/Ireg1/MTP1. While DMT1 regulation would not be expected to change rapidly, Ferroportin1/Ireg1/MTP1 activity would be expected to diminish with an increase in hematocrit and the subsequent increase in oxygen delivery.

Recent findings argue for additional Ferroportin1/Ireg1/MTP1 regulation at the level of protein trafficking. Abboud and coworkers observed that this exporter has significant intracellular localization in enterocytes of iron replete mice, while iron depleted mice have more pronounced localization of Ferroportin1/Ireg1/MTP1 to the basolateral membrane (Abboud and Haile, 2000). Similarly, an iron-dependent regulation of DMT1 subcellular localization has been observed in the duodenal epithelium. In this case, control and iron loaded rats showed predominant staining of DMT1 in intracellular sites of the enterocyte while iron depleted rats showed predominant staining of DMT1 at the apical membrane (Trinder et al., 2000).

HEPHAESTIN

Sex linked anemia (sla) is a disorder that highlights the selectivity of the enterocyte basolateral iron transport machinery and emphasizes its role in the regulation of iron absorption. Sla mice suffer the paradox of iron-overloaded enterocytes and insufficient amounts of plasma iron for the production of red blood cells. While dietary iron absorption by the enterocyte is relatively normal, efflux of iron through the basolateral membrane and into the plasma is inhibited (Anderson et al., 1998; Bannerman, 1976). The iron that

remains trapped within the sla enterocyte is hypothesized to be lost with exfoliation of these cells (Lombard et al., 1997). Postnatal anemia is corrected as the mouse matures, but body iron stores remain depleted throughout the lifetime of the mouse, suggesting the total iron demand is never met (Edwards et al., 1977; Manis, 1970).

The sequence for hephaestin, the protein product of the gene mutated in the sex-linked anemia mouse (Vulpe et al., 1999), has significant homology to the serum protein, ceruloplasmin (Harris et al., 1995; Yoshida et al., 1995). Ceruloplasmin is a serum multi-copper ferroxidase required for efficient recycling of iron between storage and donation sites in the liver, reticuloendothelial system, and the blood (Harris et al., 1999; Mukhopadhyay et al., 1998; Osaki et al., 1971). The cloning of hephaestin emphasizes the role of copper in the transfer of iron from the enterocyte to the plasma. Copper is required for efficient iron transport in biological systems from yeast to mammals and may even be considered to have a regulatory role due to the selectivity of iron transporters for ferrous or ferric iron forms (Askwith and Kaplan, 1998). The 194 amino acid deletion in hephaestin that is responsible for the sex linked anemia phenotype is expected to prevent oxidation of iron and transport across the basolateral membrane of the enterocyte (Vulpe et al., 1999). Whether hephaestin's proposed oxidase activity is necessary for the selectivity of a specific basolateral iron transporter, the selectivity of another iron transport protein, or to maintain a concentration gradient of ferrous iron across the basolateral membrane is not known.

Unlike ceruloplasmin, the C-terminal portion of the hephaestin sequence has a predicted membrane-spanning domain that would orient the multicopper ferroxidase activity on the cell surface or in the lumen of vesicles to act in concert with an iron exporter. *In situ* hybridization has localized hephaestin to the enterocytes of the villus, excluding the crypt cells (Vulpe et al., 1999) which is consistent with its proposed role in the transport of iron through these cells (Figure 7.2). Immunostaining has localized hephaestin to perinuclear compartments (Kuo et al., 1999), indicating that it may be part of a biosynthetic compartment like the trans-Golgi, or that it cycles, like the TfR, between the basolateral

membrane and endocytic compartments. The vesicular localization of hephaestin raises the possibility that iron derived from the cytoplasm could be loaded onto a specific carrier such as apo-Tf (Alvarez-Hernandez et al., 2000; Alvarez-Hernandez et al., 1998). Alternatively, the vesicular localization may prevent interaction of the iron targeted for export with undesired intracellular iron targets. While hephaestin certainly plays a crucial role in iron efflux from the enterocyte, the actual transporter that shares the iron efflux pathway with hephaestin is not yet known. Future experiments that co-express Ferroportin1/Ireg1/MTP1 and hephaestin in tissue culture or *Xenopus* expression systems may identify complementary roles for these proteins.

The mutated gene products in both sex linked anemia and HH have been shown recently to modulate the same iron absorption pathway. HFE knock out mice bred to *sla* mice have higher hepatic iron levels than wild type mice, but much less than HFE knock out mice (Levy et al., 2000). While these experiments suggest that the additional iron transported in HH utilizes the hephaestin pathway, HFE and hephaestin are not expected to physically interact. HFE is localized to epithelial cells located in the duodenal crypts while hephaestin is localized to epithelial cells located on the duodenal villus. How HFE might modulate hephaestin is not known, but the iron set point established by HFE in the cells of the crypt may determine the activity of hephaestin in the enterocyte.

MODEL FOR IRON HOMEOSTASIS

Based on these recent developments in the identification of iron transport machinery we propose a speculative, but testable, model for the maintenance of iron homeostasis (Figure 7.3). Intestinal regulation of iron homeostasis begins in the crypt where the regulatory set point for iron uptake is established. Temporal and spatial separation of regulatory machinery is required for the appropriate regulation of what will be the final level of iron transport across the mature enterocyte. Because the HFE- and TfR-expressing

cells of the intestinal crypt are not fully differentiated (Jeffrey et al., 1996; Levine and Woods, 1990) and do not express the DMT1 protein (Canonne-Hergaux et al., 1999; Trinder et al., 2000), they are not sensitive to iron levels in the lumen of the intestine. However, the body stores regulator and possibly the erythropoietic regulator can communicate the status of iron repletion and erythropoiesis through the plasma to these undifferentiated cells. By establishing the absorptive set point of the enterocyte in the undifferentiated state, the duodenum can isolate the signals received from the plasma without the confounding influence of the dietary iron pool derived from the lumen of the intestine, or the iron pool in transit across the epithelial layer.

HFE reduces the amount of iron absorbed from Tf (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999; Roy et al., 1999; Salter-Cid et al., 1999), stepping down the concentration of the labile iron pool (Figure 7.3). The set point established by the labile iron pool will determine IRP RNA binding activity (Hentze and Kuhn, 1996), iron regulated transcriptional activity (McKnight et al., 1980; White and Munro, 1988), and the modulation of iron-dependent post-translational trafficking (Abboud and Haile, 2000; Trinder et al., 2000) and degradation events (Guo et al., 1995; Iwai et al., 1998). Thus, even before differentiation to an enterocyte, the set point for intestinal regulation of iron transport is determined by the labile iron pool of its precursor. The regulatory activities are set in place and will perform their respective functions when proteins specific to the differentiated enterocyte are expressed.

Differentiation occurs in a gradient along the crypt/villus axis, turning off the expression of proteins specific to the crypt and inducing the expression of enterocyte-specific proteins (Daniele and D'Agostino, 1994; Hodin et al., 1995; Pothier and Hugon, 1980). With the initial synthesis of enterocyte-specific proteins, the set point of the labile iron pool that was established in the crypt determines the relative amounts of those proteins that are iron regulated. After differentiation induces expression of the iron transport machinery, changes in the labile iron pool are likely to occur. Whether iron-dependent

regulation of these proteins will affect transport through the enterocyte will depend upon the stability of the individual proteins. Iron-dependent transcriptional and translational regulation will not affect the activity of proteins with long half-lives, while iron-dependent degradation will.

The identification of IREs on the mRNA of DMT1 and Ferroportin1/Ireg1/MTP1 (Abboud and Haile, 2000; Gunshin et al., 1997; McKie et al., 2000; Zoller et al., 1999) suggest they are iron regulated through IRPs and the labile iron pool. Regulation of these two proteins will establish the transport capacity of the fully differentiated enterocyte. Their balance is best exemplified by the HH defect (Figure 7.3). In the enterocyte precursors of individuals with HH, the concentration of the labile iron pool is increased as compared to individuals with functional HFE. Upon differentiation of the hereditary hemochromatotic enterocyte, the increased labile iron pool increases the amount of Ferroportin1/Ireg1/MTP1 and the capacity for iron transport into the plasma, consistent with the HH defect observed by McLaren and colleagues (McLaren et al., 1991). At the same time, this increased regulatory iron pool reduces DMT1 protein levels.

The enterocytes of individuals with HH have low Ft levels (Pietrangelo et al., 1995) and increased DMT1 message levels (Fleming et al., 1999; Zoller et al., 1999) in the face of saturated body iron stores. While the latter finding may seem to conflict with our model for regulation, we speculate that the levels of DMT1 and Ft in the enterocytes of hereditary hemochromatotic individuals reflect the final, iron depleted, steady state achieved by the increased activity of Ferroportin1/Ireg1/MTP1. If Ferroportin1/Ireg1/MTP1 transport surpasses that of DMT1 in individuals with HH, intracellular iron stores from Ft and the labile iron pool will be depleted, activating IRP RNA binding activity. The increased IRP RNA binding activity may lead to an increase in DMT1 protein, or the decrease in the labile iron pool may alter protein trafficking events and redistribute DMT1 to the apical membrane. Similar regulation leading to increased Ferroportin1/Ireg1/MTP1 protein activity in macrophages may explain why the Kupffer cells of hereditary

hemochromatotic individuals are relatively free from iron deposits (Brink et al., 1976; Valberg et al., 1975) and have increased IRP RNA binding activity (Cairo et al., 1997) while the rest of the liver is iron loaded. Experiments that quantitate relative DMT1 and Ferroportin1/Ireg1/MTP1 levels and activity in control and HH samples and in HFE deficient mouse models will reveal the suitability of this model.

Enterocytes may continue to be influenced after differentiation is complete by external signals such as the erythropoietic or hypoxic regulator which has been shown to up-regulate mucosal iron transfer in the hypotransferrinemic mouse (Buys et al., 1991; Raja et al., 1999). Up-regulation of Ferroportin1/Ireg1/MTP1 has been observed in the hypotransferrinemic mouse (McKie et al., 2000) and may be the direct result of changes in transcriptional activity induced by either regulator. Because the iron loading of the hypotransferrinemic mouse is more severe than that of the HFE knock out mouse, the regulation of Ferroportin1/Ireg1/MTP1 is likely to differ between these two mouse models. A hierarchy of regulation through transcriptional, translational and degradation events, as well as the regulation of accessory proteins like hephaestin is likely to fine-tune the enterocyte iron export machinery that has been described. Experiments that test for differences in Ferroportin1/Ireg1/MTP1 activity in hypotransferrinemic and HFE deficient mice may reveal differences in its regulation.

Finally, key to further understanding the details of iron homeostasis in the intestine will be the identification of the erythroid and body iron stores regulators and the mechanisms by which they regulate iron transporters, and possibly other iron-related proteins such as HFE, and hephaestin.

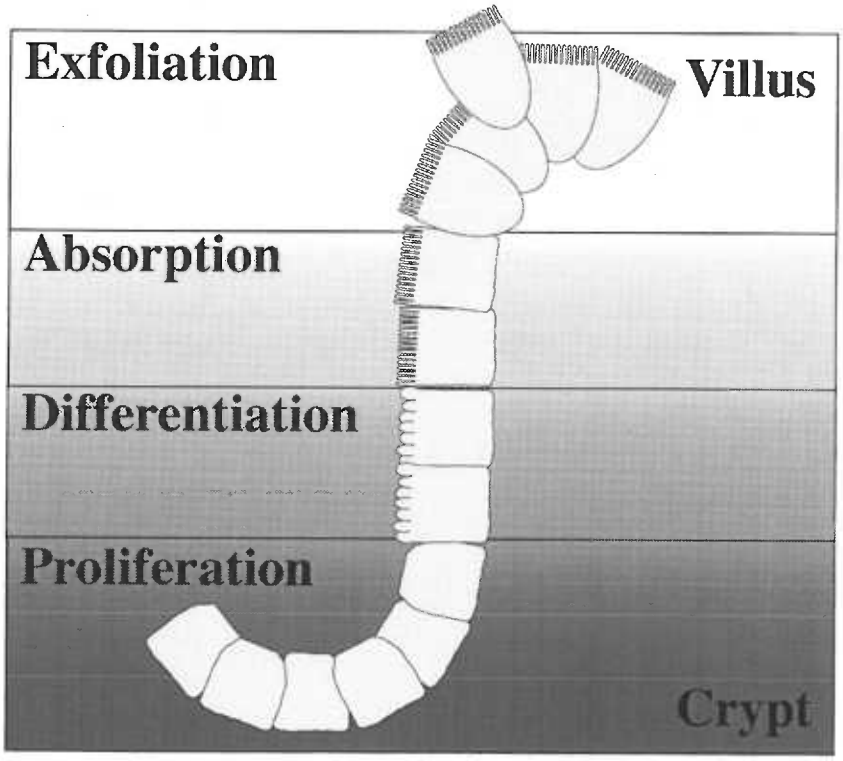

Figure 7.1. Life cycle of the duodenal enterocyte. Stem cells present in the crypts of the duodenum proliferate and give rise to precursor cells that further differentiate into absorptive cells or "enterocytes". These enterocytes are highly specialized for absorption of micronutrients from the intestinal lumen. Their apical membrane is structured with microvilli and contains enzymes that facilitate transport of nutrients through the epithelial cell layer. The majority of iron absorption is facilitated by these specialized cells. The process of differentiation occurs simultaneously with migration out of the crypt and onto the villus. "Zones" of cells in similar stages are evident when the crypt/villus junction is viewed in cross-section. After the cells have migrated to the villar tip, they are exfoliated and excreted.

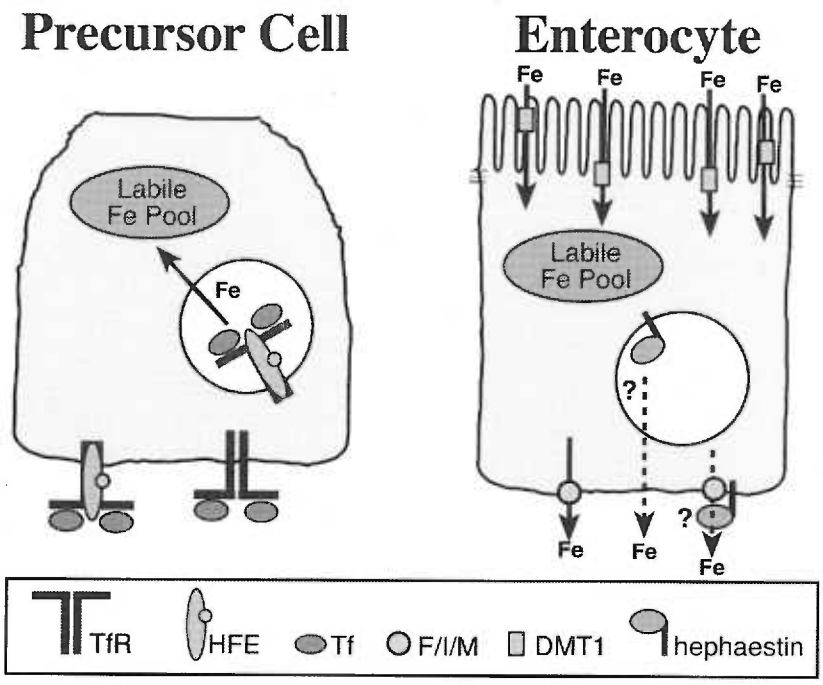

Figure 7.2. A model for establishment of the enterocyte iron absorptive setpoint in the crypt. Precursor cells (left) present in the duodenal crypts express a complement of iron metabolic proteins that are distinct from those of the differentiated enterocyte (right). In the crypt, the TfR-HFE complex facilitates iron uptake from Tf in the plasma and contributes to the labile iron pool. The labile iron pool directly influences IRP RNA binding activity and may affect iron regulated transcription, translation, protein trafficking, and degradation processes. In this way, the iron absorptive capacity, or "setpoint" for the enterocyte may be established by the labile iron pool in the crypt. When the precursor cell differentiates, the expression of proteins specific to the enterocyte, such as apical and basolateral iron transporters and hephaestin is induced. While the enterocyte precursor absorbs, but does not transport iron, the differentiated enterocyte takes up iron from the duodenum at the apical membrane through DMT1 and transports it into the plasma. The basolateral iron transporter, Ferroportin 1/Ireg1/MTP1 (F/I/M) has been shown to act independently as an iron exporter. The multicopper oxidase, hephaestin, is also required for efficient iron efflux from the enterocyte, but whether it acts through F/I/M,or another pathway is not yet known.

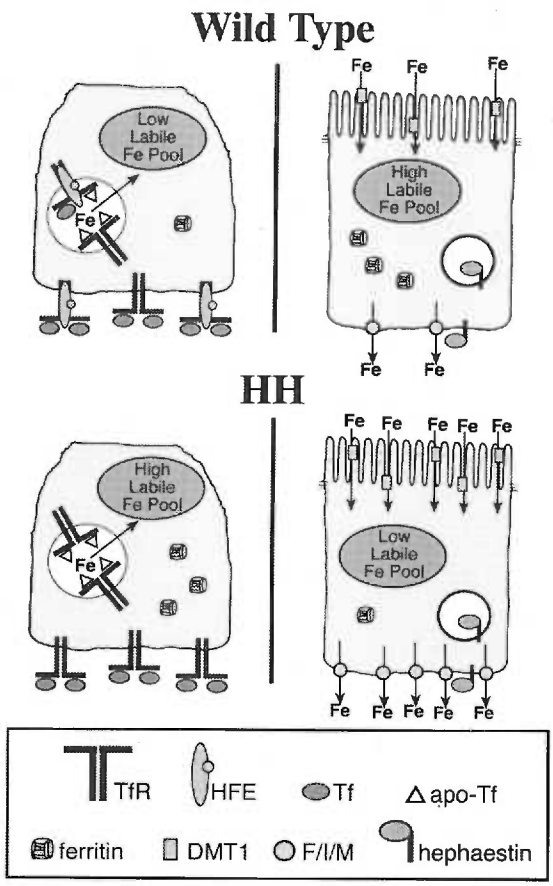

Figure 7.3. Hereditary hemochromatosis exemplifies inappropriate regulation of the iron absorptive setpoint in the crypt. Individuals with hereditary hemochromatosis (HH) have decreased levels of functional HFE. This contributes to a higher labile iron pool in the precursor cells of an individual with HH than an individual with functional HFE (wild type). When the precursor cell of a HH individual differentiates, the labile iron pool will initially be high. We speculate that this increase in the labile iron pool up-regulates machinery responsible for basolateral iron transfer such as Ferroportin1/Ireg1/MTP1 (F/I/M). The increased amount of basolateral iron efflux will lead to a depletion of the labile iron pool, which will increase levels of the apical iron transporter, DMT1, keeping intracellular iron levels low. This model explains why individuals with HH have higher levels of DMT1 and lower Ft levels in their duodenum and can be tested further by determining the activity of F/I/M in HH individuals and controls as well as in HFE deficient mice.

SUMMARY AND CONCLUSIONS

Within this thesis, we have detailed our efforts to investigate the role of HFE in cellular iron homeostasis. HFE is hypothesized to have a role in the maintenance of organismal iron homeostasis because of the iron overload pathology associated with HH individuals who inherit a founder mutation (C282Y) in the HFE gene (Feder et al., 1996). This mutation is a loss of function mutation that prevents efficient expression of the HFE protein product (Feder et al., 1997; Levy et al., 1999; Rothenberg and Voland, 1996; Santos et al., 1996; Zhou et al., 1998). Individuals with HH experience a chronic increase in iron absorption across the intestinal enterocyte (McLaren et al., 1991). Because the HH defect is likely to originate within cells that become specialized for iron transport, we hypothesized that a study of cellular iron metabolism in HFE-expressing cells would reveal a functional role for this curious protein of iron homeostasis.

HFE Expression Alters Cellular Iron Homeostasis

We have shown that expression of wild type HFE in fWTHFE/tTA HeLa cells induces a low iron phenotype (Figure 1.1). This result was the first report of a direct effect of HFE on cellular iron metabolism (Gross et al., 1998). The same observation in HeLa cells has been reported, independently, by two other groups (Corsi et al., 1999; Riedel et al., 1999). We have also reported the same low iron phenotype in HEK 293 cells (Figure 2.3). The most severe mutation in HFE, C282Y, does not reduce Ft expression in cultured cells (Figure 2.4), indicating that this low iron phenotype may correlate with the ability of HFE to function *in vivo*. However a less severe mutation in HFE, H63D, that correlates with HH does reduce Ft expression in cultured cells (Figure 2.4). Unlike the C282Y mutant, the H63D mutant HFE retains its ability to co-immunoprecipitate with the TfR (Feder et al., 1998). The difference between the cultured cell phenotypes of the HH

disease alleles indicated that the association of HFE with the TfR is an important part of HFE function.

HFE Association with the TfR Correlates with the Low Iron Phenotype in Cultured Cells

The association of HFE with the TfR is a critical part of HFE function that is closely regulated in cells. Wild type HFE and the TfR can associate with high affinity very early in the biosynthetic pathway (Figure 1.3) forming a stable complex whose stochiometry may be regulated by the cell (Figure 1.4). This complex cycles to Tf-positive compartments (Figure 1.7 and 1.8) that are assumed to be endosomes. Since endosomes are functionally defined by the presence of the TfR and Tf, it is impossible to determine whether HFE expression disrupts the localization of these proteins to endosomes. However, since HFE does not alter the cycling kinetics or distribution of the TfR (Figure 3.1-3.3) and since the HFE/Tf/TfR positive compartments have the same pH range as Tf/TfR positive compartments, (Figure 5.6) it is expected that, at least functionally, the compartment remains an endosome.

Co-localization of HFE and the TfR (Figures 1.7 and 2.2), like the ability to co-immunoprecipitate HFE and the TfR (Feder et al., 1998), correlates well with the ability of HFE to reduce Ft expression in cultured cells. The C282Y HFE mutant does not co-localize (Figure 2.2) or co-immunoprecipitate with the TfR (Feder et al., 1998), nor does it reduce Ft expression in HEK 293 cells (Figure 2.4). In contrast, the H63D mutant does co-localize (Figure 2.2) and co-immunoprecipitate with the TfR (Feder et al., 1998) and it reduces Ft expression in HEK 293 cells (Figure 2.4). The H63D HFE is hypothesized to be less stable than the wild type HFE (Lebron et al., 1998). The stable association of H63D HFE with the TfR and the reduction in Ft levels that we observed in cultured cells may result from over-expression of this mutant.

The association of HFE with the TfR depends heavily on the W103 residue of HFE (Lebron and Bjorkman, 1999). Mutation of this tryptophan to alanine decreases association of HFE with the TfR 3000 to 8800 fold (Lebron and Bjorkman, 1999), but unlike the C282Y HFE mutant, the W103A mutation still allows HFE to fold correctly and to be expressed at the cell surface (Ramalingam et al., 2000). This mutant does not reduce Ft expression (Ramalingam et al., 2000), indicating that the association of HFE with the TfR is important for HFE function, either for proper localization to the endosomal compartment, or for its direct effect on the TfR.

HFE Specifically Reduces Tf-Mediated Iron Uptake

HFE's close association with the TfR betrays its role in the generation of the low iron phenotype in cultured cells. Although the mechanism is disputed, several independent investigations agree that HFE specifically reduces iron uptake through the Tf-mediated pathway of iron uptake (Corsi et al., 1999; Ikuta et al., 2000; Lebron et al., 1999; Riedel et al., 1999; Roy et al., 1999). We have shown that HFE does not alter diferric Tf uptake (Figure 3.1), but still reduces iron uptake from Tf by 33% (Figure 3.4). We concluded that this effect on iron uptake is specific to the Tf-mediated pathway of iron uptake, because HFE did not reduce iron uptake through non-Tf iron uptake pathways (Figures 3.5 and 5.7).

Other groups that assayed for iron uptake from Tf (between 50 and 500 nM) also observed a reduction in the amount of iron absorbed from Tf (Corsi et al., 1999; Ikuta et al., 2000; Riedel et al., 1999). In contrast to our results, Corsi and colleagues observed an increase in non-Tf mediated iron uptake (Corsi et al., 1999) that was not observed in our cells. The IRE-containing DMT1 splice form would be expected to be upregulated like the TfR (Figure 1.1) in HFE-expressing cells. Our fWTHFE/tTA HeLa cells apparently do not contain this regulatable DMT1, because the small levels that are detected on immunoblot are not significantly different between HFE- and HFE+ fWTHFE/tTA HeLa cells (CNR-372

unpublished results). However, an iron regulated form of DMT1 may be expressed in our HEK 293 cells since it is upregulated in wild type and H63D HFE-expressing cells, but not in parental or C282Y HFE-expressing cells (CNR-372 unpublished results). Iron-dependent regulation of DMT1 in another HeLa strain might explain this difference between our results and those reported by Corsi and colleagues (Corsi et al., 1999).

The Mechanism by which HFE Reduces Tf-Mediated Iron Uptake Remains Unclear

The nature of the interaction between HFE and the TfR is the most important -- and most controversial -- link to the mechanism behind HFE's maintenance of iron homeostasis. Three hypotheses have been proposed concerning HFE's reduction of Tf-mediated iron uptake.

Hypothesis #1: HFE alters cycling of the TfR

The first hypothesis suggests that HFE affects trafficking of the TfR (Cox and Kelly, 1998). Ikuta and colleagues (Ikuta et al., 2000) have reported that an HFE-green fluorescent protein fusion construct slows recycling of ^{125}I-Tf from internal compartments to the cell surface. We did not see a significant decrease in ^{125}I-Tf efflux (Figure 3.2), or a redistribution of the TfR that would be expected to accompany such changes in TfR cycling (Figure 3.3). However, none of these experiments measured TfR cycling independent of Tf. Experiments that follow TfR cycling using an ^{125}I-labeled monoclonal antibody that does not interfere with HFE binding to the TfR would be best suited to determine whether such differences exist. We attempted to ^{125}I-label the anti-TfR monoclonal antibody 4093 for this purpose. However, after iodination, the 4093 antibody did not seem to bind specifically to the TfR (CNR-266 and -267 unpublished results).

Hypothesis #2: HFE competes with Tf for binding to the TfR

The second hypothesis concerning the mechanism behind HFE's specific reduction of Tf-derived iron uptake suggests that HFE competes directly with diferric Tf for binding to the TfR (Bennett et al., 2000; Lebron et al., 1999). Some of the experiments recorded herein support this hypothesis. First, we have shown that both the membrane bound form of HFE (Figure 1.2) and soluble HFE (Figure 4.1) increase the dissociation constant of the TfR for diferric Tf without reducing the number of diferric Tf binding sites. While this may reflect a change in the TfR affinity for diferric Tf, it might also reflect competition of HFE with the diferric Tf binding site on the TfR. We have also shown that soluble HFE competes directly with Tf for binding to the TfR (Figure 4.2).

Despite the fact that some experiments support the competition model of HFE action, several experiments we have reported argue against this hypothesis as the sole mechanism for decreased iron uptake from Tf. First, the HFE-TfR complex takes up the same amount of Tf per surface TfR as the TfR alone when Tf is present at a concentration sufficient to saturate the TfR in the presence of HFE (Figure 3.1). However, these experiments were performed in the presence of enough diferric Tf to overcome the affinity change induced by HFE. In a competition model diferric Tf would displace HFE from the TfR. The experiments represented by Figure 3.1 would characterize the endocytic rate of the TfR alone, rather than the HFE-TfR complex. This complication also merits an investigation of TfR endocytosis with an ^{125}I-labeled monoclonal antibody as described previously. Despite these complications with experimental protocol, it remains unclear how a competition model would function physiologically, since diferric Tf is present in the blood plasma at concentrations sufficient to saturate the TfR in the presence of HFE (Feder et al., 1998; Lane, 1975; Roy et al., 1999).

A second series of experiments that argues against a competitive mechanism for HFE are those that show that HFE is not significantly redistributed in the presence of saturating diferric Tf (Figures 4.3 and 4.4) as it should be if HFE cannot localize to the endosome independent of the TfR (Ramalingam et al., 2000). Again, in a competition

model when diferric Tf is present at concentrations sufficient to saturate the TfR in the presence of HFE, diferric Tf should displace HFE from the TfR. Unless the association of HFE with the TfR is independent of the Tf binding site on the TfR, HFE should not internalize. Unfortunately, the redistribution experiments with biotin are only qualitative and may not be sensitive enough to detect a difference in the distribution of the HFE protein.

A modification of the competition hypothesis, that may be supported by our data, is the hypothesis that HFE increases the efficiency of uptake of unsaturated Tf species. This hypothesis is consistent with the results generated from competition between diferric Tf and Fe_CTf on the TfR or HFE-TfR complex (Figure 5.3). While HFE increased the dissociation constant of the TfR for diferric Tf 10 fold (Figure 1.2), it only increased the dissociation constant of the TfR for Fe_CTf 4 fold (Figure 5.3). This relationship between HFE and the TfR could create an environment in which a greater percentage of the Tf species internalized by the HFE-TfR complex would be Fe_CTf than that internalized by the TfR alone. The reduction in ^{55}Fe absorbed from Tf by HFE+ fWTHFE/tTA HeLa cells in Figure 3.4 could be explained by this mechanism. The ^{55}Fe-Tf used in these experiments was only 85% saturated, indicating that as much as 30% of the Tf species could be monoferric, and selectively internalized by the HFE-TfR complex.

Hypothesis #3: HFE reduces iron uptake from Tf

A third hypothesis for the HFE-specific reduction in iron uptake is that HFE reduces the amount of iron absorbed from Tf in the endosome. HFE could perform its function in the endosome through its interaction with the TfR itself, by reducing iron release from Tf, or by down-regulating some other component of the endosomal iron uptake pathway such as the endosomal iron transporter or ferrireductase. To test between these hypotheses, Ramalingam and colleagues have mutated HFE to contain the low density lipoprotein receptor (LDLR) internalization motif in its cytoplasmic domain

(Ramalingam et al., 2000). With its own internalization motif, the W103A-LDLR HFE cycles to the recycling compartment without binding the TfR. Ft levels did not decrease in cells expressing the endosomally localized W103A HFE, suggesting HFE is likely to modulate cellular iron homeostasis through its interaction with the TfR rather than another component of endosomal iron uptake.

We have also investigated several potential components of endosomal iron uptake that might be down-regulated by HFE (chapter 5), but we could not identify any means by which components other than the TfR were affected by HFE. Recommendations for future experiments in this area have been suggested in chapter 5.

Concerns for future experiments that test models for HFE-TfR iron uptake

The first assay we developed for HFE function tested for a reduction in Ft expression in HFE+ cells. An important caveat for the Ft assay of HFE function arises with respect to the hypothesis that HFE reduces iron uptake from Tf. While HFE binds the TfR and reduces its affinity for Tf, the more important observation is that the HFE-TfR complex decreases the amount of iron absorbed from Tf. When applied to our observations in cell culture, where bovine Tf is the only Tf source, the changes in Ft levels may only reflect the ability of HFE to bind the TfR and compete with diferric Tf or to reduce its affinity for diferric Tf. The best experiment by which to assay the function of a mutant form of HFE may be to test for a reduction of iron uptake from the same amount of internalized Tf.

Of future interest, is a better characterization of the interaction of the HFE-TfR complex with monoferric Tf species. We have already discussed in chapter 5 experiments that are better designed to characterize the interaction of the HFE-TfR complex with multiple Tf species. Additionally, future experiments should test the effects of changes in Tf iron saturation on iron uptake by the HFE-TfR complex. The limits in the ability of HFE to diminish the labile iron pool as the saturation of Tf species change may have

important physiological implications for sensing changes in plasma Tf iron saturation. The experiments we report concerning ^{55}Fe uptake from Tf were performed with 85% iron saturated Tf. Repeating these experiments in the presence of 100%, 50%, or 25% saturated Tf may bring to light differences in HFE's sensitivity to unsaturated Tf species.

HFE Expression Decreases the Cellular Labile Iron Pool

Despite the fact that the mechanism for HFE's reduction of Tf-mediated iron uptake is not resolved, the reduction in iron uptake explains the HFE-induced low iron phenotype in cell culture. Several investigators have also observed that HFE induces IRP RNA binding activity (Corsi et al., 1999; Riedel et al., 1999), but these reports did not include the mechanism for IRP activation. The RNA binding activity of IRPs can be modulated through the labile iron pool (Guo et al., 1995; Guo et al., 1994; Haile et al., 1992; Iwai et al., 1998; Iwai et al., 1995; Rouault et al., 1991; Tang et al., 1992), signal transduction pathways (Brown et al., 1998; Schalinske and Eisenstein, 1996), or oxidative stress pathways (Bouton et al., 1996; Drapier et al., 1993; Hanson et al., 1999; Pantopoulos et al., 1996; Weiss et al., 1993). HFE could have an effect on any of these pathways.

We have provided evidence for an HFE-specific increase in the RNA binding activity of both IRP1 and IRP2 (Figure 6.4). Additionally, we have shown that IRP2 levels are increased in HFE+ fWTHFE/tTA HeLa cells (Figure 6.5), which is consistent with an HFE-specific decrease in the labile iron pool. That HFE can specifically reduce the labile iron pool is important for cellular iron-dependent regulatory mechanisms. While these regulatory mechanisms are dependent on the labile iron pool, they are not necessarily limited to regulation through IRPs, as iron-dependent transcription, degradation, and protein trafficking events may also be affected.

Our investigation of the labile iron pool has been restricted to the level and activity of IRPs. Experiments that investigate the status of the labile iron pool independent of IRPs would be valuable in that they would confirm that HFE regulates the labile iron pool rather

than IRPs. Calcein is a fluorescent probe that binds ferrous or ferric iron with dissociation constants (10^{-14} and 10^{-24} M, respectively) less than that of EDTA (Breuer et al., 1995; Breuer et al., 1995; Cabantchik et al., 1996). When calcein binds iron, its fluorescence is quenched (Cabantchik et al., 1996). Experiments that use calcein to quantitate the relative amounts of iron in the labile iron pool of HFE- versus HFE+ cells should therefore reveal more quenching of calcein fluorescence by HFE- cells than HFE+ cells because HFE- cells are hypothesized to have more iron in their labile iron pool. Preliminary investigations of the calcein reagent with a fluorescent cell sorter (CNR-342 and -357, unpublished results) and a fluorescent plate reader (Anderson, 2000) in our fWTHFE/tTA HeLa cells did not reveal a consistent correlation between Ft expression and calcein fluorescence. Future studies that image calcein fluorescence changes in single cells may provide more consistent results.

A Testable Model for HFE-Specific Regulation of Organismal Iron Homeostasis

We have used a nonpolarized cell system to pinpoint HFE's control over iron homeostasis to the Tf-mediated iron uptake pathway. The fWTHFE/tTA HeLa cell system is not likely to express all the components of iron metabolism that are represented in an enterocyte from its undifferentiated state in the crypt to its differentiated state on the villus (Figure 7.1). However, because the fWTHFE/tTA HeLa cell system has allowed us to follow in step-wise fashion HFE's control over the Tf-mediated pathway of iron uptake, it has provided a useful starting point for further investigation.

We have proposed a model for HFE-controlled regulation of iron homeostasis (Figure 7.3) which is contingent upon HFE's control over the labile iron pool of enterocyte precursor cells. Future experiments should test this model on polarizable cultured cells, such as the colon carcinoma cell lines, HuTu-80 or HT-29. Our model proposes that the transepithelial transport of iron by a differentiated enterocyte will be reduced if the

undifferentiated cells have a lower labile iron pool. This should be tested by decreasing the labile iron pool of undifferentiated cells prior to their differentiation on Transwell inserts. Quantitating transepithelial iron transport and the regulation of messages of selected iron metabolic proteins (such as DMT1, hephaestin, and Ferroportin1/Ireg1/MTP1) will reveal whether these proteins are regulated in a manner that is consistent with our model. Treating undifferentiated polarizable cells with the iron chelator Df, with Tf of varying iron saturation, and with soluble HFE will reveal whether these reagents have different effects on transepithelial iron transport and the regulation of enterocyte-specific iron metabolic proteins. Such differences might separate HFE-controlled regulation of iron homeostasis from independent regulators such as hypoxia and the erythropoietic regulator.

The isolation and characterization of the HFE gene promised new insights into the regulation of iron homeostasis. Four years later, if nothing else, we have a better understanding of the complexities of iron homeostasis for an organism. HFE may be a part of just one of many pathways that maintain iron homeostasis, but an understanding of the limits of HFE-regulated iron homeostasis will direct our attention toward new pathways as well.

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