Co-trafficking of HFE, a Nonclassical Major Histocompatibility Complex Class I Protein, with the Transferrin Receptor Implies a Role in Intracellular Iron Regulation*

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Cindy N. Gross‡§, Alivelu Irrinki¶, John N. Feder¶, and Caroline A. Enns‡¶

From the ‡Department of Cell and Developmental Biology, Oregon Health Sciences University, Portland, Oregon 97201-3098 and ¶Progenitor Inc., Menlo Park, California 94025

The mechanism by which a novel major histocompatibility complexity class I protein, HFE, regulates iron uptake into the body is not known. HFE is the product of the gene that is mutated in >80% of hereditary hemochromatosis patients. It was recently found to co precipitate with the transferrin receptor (Feder, J. N., Penny, D. M., Irrinki, A., Lee, V. K., Lebron, J. A., Watson, N., Tsuchihashi, Z., Sigal, E., Bjorkman, P. J., and Schatzman, R. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1472-1477; Parkkila, S., Waheed, A., Britton, R. S., Bacon, B. R., Zhou, X. Y., Tomatsu, S., Fleming, R. E., and Sly, W. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13198-13202) and to decrease the affinity of transferrin for the transferrin receptor (Feder et al.). 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 min of their synthesis with pulse-chase experiments. HFE and the transferrin receptor co immobiloprecipitate 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 the 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.

Positional cloning of HFE from the HLA-linked region of chromosome 6p and its subsequent crystallization (6) have revealed its significant sequence and structural homology (37% identity in the ectodomain) to MHC1 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 that the ancestral peptide-binding groove is too narrow for such a function (6). Emerging results suggest that the functional role of HFE may lie in its interaction with the transferrin receptor (TTR) (6–8). 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 (9–13). Antibodies against these MHC class I proteins decrease surface binding of insulin or the epidermal growth factor to their respective receptors, suggesting that these MHC class I proteins are involved in the formation of high affinity ligand-binding sites. The neonatal Fc receptor is a nonclassical MHC class I molecule that acts as an antibody transporter. The current model of neonatal Fc receptor function proposes binding of ligand at acidic pH and transport in acidic vesicles through the epithelial monolayer. Further evidence suggests the involvement of the neonatal Fc receptor in IgG homeostasis in the serum (for review, see Ref. 14). Likewise, HFE may be involved in regulated transport of molecules associated with iron homeostasis.

The molecular evidence concerning HFE mediation of iron homeostasis has been converging on its close association with TFR. Northern blot analysis indicated that HFE is widely distributed throughout the body, with more abundant message levels in organs that are major sites of iron metabolism, namely the liver and intestine (5). Its 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 intestine (15)) 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 intestinal epithelia and the identification of HFE in the placenta (7) are indicative of a more general association with TIR in regulating iron transport across epithelial barriers. It has long been suspected that individuals with hereditary hemochromatosis have a defect in the transport of iron across the mucosal barrier, i.e. transport into the blood rather than uptake from the lumen (4). The perinuclear staining of HFE in proliferating crypt cells is

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†To whom correspondence should be addressed: Dept. of Cell and Developmental Biology, L215, Oregon Health Sciences University, Portland, OR 97201-3098. Tel.: 503-494-5845; Fax: 503-494-4253; E-mail: ennsca@ohsu.edu.

†‡ The abbreviations used are: MHC, major histocompatibility complex; Tf, transferrin; TIR, transferrin receptor; hTIR, human TIR; HFE, FLAG epitope-tagged HFE; PAGE, polyacrylamide gel electrophoresis; tet, tetracycline; PBS, phosphate-buffered saline; endo H, endo-β-N-acetylglucosaminidase H; FITC, fluorescein isothiocyanate; Ft, ferritin.
indicate co-localization with recycling receptors, such as TIR (16), rather than with the hypothesized iron transporter on the apical surface of these cells. The regulatory influence of HFE on iron transport may therefore be either in internal cellular compartments or at the basolateral cell surface.

More specific evidence for the interaction of HFE with TIR is demonstrated by the co-immunoprecipitation of HFE with TIR (7, 8) and more significantly by the ability of HFE to increase the dissociation constant of transferrin (Tf) with TIR (6, 8). The association of HFE with TIR 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 this study demonstrate that cells expressing HFE have decreased ferritin levels and increased TfR number, implying low intracellular iron levels. We also demonstrate that HFE is able to associate with TfR within 30 min of its synthesis and can associate with TIR in both the biosynthetic and metabolic pathways. HFE pools that are not associated with TIR are rapidly degraded. Immunofluorescent detection of fHFE in HeLa cells indicates that it co-localizes with TIR and Tf on the cell surface and in intracellular compartments. Localization of HFE on the cell surface and in endocytic compartments implicates HFE in the regulation of iron uptake, especially in light of indirect evidence suggesting that cells expressing HFE have decreased intracellular iron stores.

**Experimental Procedures**

**Plasmids**—The plasmid pcDNA3.1* containing HFE cDNA clone 24 fused to the octapeptide FLAG sequence (Progenitor Inc.) has been previously described (17). The tetracycline-repressible plasmid puHD10-3 was a kind gift from Drs. M. Gossen and H. Bujard (Zentrum für Molekulare Biologie, Universität Heidelberg) (18).

**Subcloning**—The HFE coding sequence is contained within a 3.0-kilobase XhoI/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 puHD15-1 were a gift from Dr. Sandra L. Schmid (Scripps Research Institute). They were cotransfected with the fWTHFE/puHD10-3 plasmid and the pBSpac plasmid containing the (Scripps Research Institute). They were cotransfected with the fWTHFE/puHD10-3 plasmid and the pBSpac plasmid containing the puromycin resistance gene (12) using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s directions. Colonies selected with puromycin (200 ng/ml) and recloned to ensure a pure cell line were subjected to SDS-PAGE analysis on a 10% denaturing acrylamide gel. Gels were fixed, treated with Amplify (Amersham Pharmacia Biotech) for 30 min, dried, and subjected to PhosphorImager analysis (Molecular Dynamics, Inc.).

**Endo-β-N-acetylglucosaminidase H Treatment**—Immunoprecipitated pellets were resuspended in 90 μl of denaturing buffer (0.5% SDS and 1% 2-mercaptoethanol) and boiled for 10 min at 95 °C. Samples were cooled and 10 μl of 10× citrate buffer (500 mM sodium citrate (pH 5.5)) was added to the pellet. Samples were incubated for at least 2 h at 37 °C in the presence or absence of endo-β-N-acetylglucosaminidase H (endo H; 8 units; New England Biolabs Inc.). Samples were eluted with 50 μl of 10× 2-μm buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE analysis on a 10% denaturing acrylamide gel. Gels were fixed, treated with Amplify, dried, and subjected to PhosphorImager analysis.

**Pulse-Chase Experiments**—Uninduced (tet+) and induced (tet−) subconfluent fWTHFE/TA HeLa cells in 35-mm dishes were washed three times with sterile PBS (pH 7.4) and preincubated for 15 min at 37 °C in RPMI 1640 medium minus iron (Life Technologies, Inc.) prior to labeling. Cells were pulsed for 10 min at various time points with 100 μCi of [35S]sodium metatetrathionate in RPMI–Met medium with 10% fetal bovine serum. The cells were then washed three times with PBS and chased at 30-min intervals between 0 and 120 min with complete medium. At the indicated times, cells were pulsed for 5 min on ice in NET-Triton. Cell extracts were subjected to immunoprecipitation with M2 or 4093 antibody as described above and analyzed by SDS-PAGE on a 10% denaturing acrylamide gel under reducing conditions. Gels were fixed, treated with Amplify, and subjected to PhosphorImager analysis.

**Western Immunodetection**—Cells extracts from ∼3 × 10^6 cells were diluted with 4× LaemmlI buffer, or immunoprecipitates were eluted with 2× LaemmlI buffer and subjected to electrophoresis on 10 or 12% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis was performed using sheep anti-hTfR serum (1:10,000 dilution) (19), mouse anti-FLAG antibody M2 (1:20,000 dilution), and/or sheep anti-human ferritin antibody (1:100 dilution; The Binding Site, Ltd.) followed by the appropriate secondary antibody conjugated to horseradish peroxidase and chemiluminescence (SuperSignal, Pierce) per the manufacturer’s directions.

**Tunicamycin Treatments**—Subconfluent fWTHFE/TA HeLa cells (tet+ and tet−) in 35-mm dishes were washed three times with sterile PBS (pH 7.4) and preincubated for 1 h at 37 °C with complete medium. The next day, the cells were lysed for 5 min on ice in NET-Triton. Cell extracts were subjected to immunoprecipitation with antibody M2 to immunoprecipitate HFE or with antibody 4093 to immunoprecipitate TIR as described above and analyzed by SDS-PAGE on a 10% denaturing acrylamide gel under reducing conditions. Gels were fixed, treated with Amplify, dried, and subjected to PhosphorImager analysis.

**Western Blotting with monoclonal anti-transferrin receptor antibody (H8G4, Zymed Laboratories, Inc.) as described (19).**

**Immunoprecipitation**—fWTHFE/TA HeLa cells were washed three times with 2 ml of PBS (pH 7.4) and lysed with NET-Triton (150 mM NaCl, 50 mM Tris-HCl, pH 7.4 with 1% Triton X-100). Cell lysates were preadsorbed for at least 45 min at 4 °C with 50 μl of Pansorbin (Calbiochem) per 10^6 cells to reduce precipitation of non-specific protein. Preadsorbed lysates were incubated for at least 45 min at 4 °C with 30 μl of rabbit anti-mouse antibody (Miles Scientific)-coated Pansorbin and 2 μg of either mouse anti-FLAG antibody (M2, Eastman Kodak Co.) or mouse anti-transferrin receptor antibody (4093, gifts from Vonnie Landt, Washington University, St. Louis, MO) or with 50 μl of Pansorbin and 3 μl of sheep anti-hTfR serum per 10^6 cells. The Pansorbin pellet was resuspended in 200 μl of NET-Triton and washed through 1 ml of radioimmunoprecipitation assay buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM Tris, 150 mM NaCl, and 0.2% sodium azide) with 15% sucrose. Samples were eluted in 100 μl of 2× LaemmlI buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol) (20) with 10% 2-mercaptoethanol and subjected to SDS-PAGE analysis on a 10% denaturing acrylamide gel.

**TF-Agarose Precipitation**—Lysates of 10^6 tet+ or tet− fWTHFE/TA HeLa cells were preadsorbed for 1 h at 4 °C with 100 μl of bovine serum albumin covalently linked to agarose (50% suspension in PBS). Supernatants were incubated for 1 h at 4 °C with 200 μl of transferrin covalently linked to agarose (50% suspension in PBS). The pellet was resuspended in NET-Triton and eluted twice with 75 μl of 2× LaemmlI buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE on a 12% denaturing acrylamide gel as described above. Gels were transferred to nitrocellulose and subjected to Western blotting as described above.

**PhosphorImager Quantitation**—IP Lab Gel 1.5 (Molecular Dynamics, Inc.) was used to quantitate images by determining the volume within a region of fixed pixel number at each band of interest within the
anti-fHFE  TfR  Ft

tet +  -  -

0 25 50

FIG. 1. Inducible expression of fHFE in fWTHFE/TA HeLa cells. Lysates of \(3 \times 10^5\) fWTHFE/TA 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-hTfR serum, 100 μg/ml anti-FLAG antibody M2, or sheep anti-ferritin (1:100) antibodies and the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000). Chemiluminescence detected \(\sim 94\), \(\sim 43\), and 19/21-kDa bands representing TfR, HFE, and Ft, respectively. The increased TfR expression and decreased Ft expression in HFE-expressing cells are indicative of a decrease in intracellular iron load. These results are representative of three experiments without significant variation between experiments.

RESULTS

Identification of a Clone That Expresses Epitope-tagged HFE under Control of the Tetracycline-regulated Promoter—The tetracycline-responsive promoter system developed by Gossen and Bujard (18) was used to create a cell line in which HFE expression could be tightly controlled. tTA HeLa cells stably express a tetracycline-transactivable (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/pUHD10-3 multiple cloning region, promoting expression of HFE. In the presence of tetracycline-supplemented (tet−) medium, the tTA fusion protein binds tetracycline and releases from the tetracycline-responsive promoter, preventing HFE transcription. Fig. 1 shows fHFE, TfR, and ferritin (Ft) expression in fWTHFE/TA HeLa cells that have been uninduced (tet−) or induced (tet+) for at least 10 days. Western blotting of lysates of \(3 \times 10^9\) cells detected no HFE (\(\sim 43\) kDa) in tet− cells with anti-FLAG antibody M2. fHFE was easily detected in lysates of tet+ cells. TfR (94 kDa) levels increased slightly (<50%) in cells expressing HFE. A large decrease (>10-fold) in the levels of Ft (19 and 21 kDa) was seen in cells expressing HFE. These results imply that overexpression of HFE reduces intracellular iron load. The iron regulatory protein modulates changes in TfR and Ft levels. At low intracellular iron concentrations, the iron regulatory protein binds to the iron response element 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 iron regulatory protein to the 5'-untranslated stem loop of Ft mRNA, blocking translation and thus decreasing Ft levels.

HFE Lowers the Affinity of TfR for Tf in fWTHFE/TA HeLa Cells—Scatchard analysis of Tf binding to \(1.38 \times 10^5\) uninduced (tet−; open squares) or \(1.17 \times 10^5\) induced (tet+; closed squares) fWTHFE/TA HeLa cells shows elevation of the dissociation constant from 1.2 to 11 nM when fHFE is overexpressed. Changes in the Scatchard plot show that the affinity of Tf for TfR decreases when fHFE is present. Increased Tf-binding sites (52%) in keeping with the increased expression of TfR on Western blots (Fig. 1). These data also show that HFE expression moderately increased Tf-binding sites (52%), in keeping with the increased expression of TfR on Western blots (Fig. 1).
nprecipitation of fHFE shortly after synthesis indicated little association between HFE and TfR (Fig. 3, 0 min chase). TfR was capable of coprecipitating with HFE within 30 min of chase when a significant portion of TfR was completely sensitive to endo H (Fig. 3, 30 min chase, lower bands of doublet at ~87 kDa (∼Endo H) and ~80 kDa (+Endo H)). Thus, HFE can associate with TfR in the endoplasmic reticulum/cis-Golgi compartments. Between 30 and 120 min of chase, the amount of TfR that coprecipitated with HFE did not change, indicating that HFE does not gain any more ability to bind HFE over this period of time. In addition, within the first 120 min of chase, most of the HFE was rapidly degraded in that the levels of HFE decrease 2.5-fold. PhosphorImager analysis of the amount of TfR that immunoprecipitated with HFE (taking into consideration the relative abundance of Met in each protein) indicated that after an overnight label, the ratio of TfR to HFE was 1.7:1.

HFE Associated with TfR Is More Stable—The relative stabilities of HFE and TfR were compared over 8 h (Fig. 4, A and B) to further investigate the turnover of free and complexed HFE. Cells were fully induced to express fHFE by withdrawal of tetracycline from the medium for at least 3 days. Quantitation of the absolute amount of HFE synthesized indicated that initially four times more HFE was synthesized than TfR (Fig. 4, A and C, 0 chase after 1-h label). fHFE was rapidly degraded in the first 4 h of pulse-chase (Fig. 4, A and B). Degradation continued at a slower rate over the next 4 h, whereas the amount of TfR that co-immunoprecipitated with fHFE was unchanged (Fig. 4B). The 35S quantitation shown here is specific to the experiment shown and is representative of several experiments.

TfR immunoprecipitation showed constant levels of TfR for 8 h (Fig. 4C). Previous studies indicated that half-life of the TfR in the absence of HFE is ~24 h (21, 22). Surprisingly, fHFE levels co-immunoprecipitating with TfR were virtually unchanged over this time course (Fig. 4D). Quantitation of the relative amounts of HFE and TfR demonstrated that one HFE bound per TfR dimer (Fig. 4D), consistent with earlier reports (6). These experiments indicate that when more HFE is synthesized than TfR, HFE is rapidly degraded, whereas the complex between TfR and HFE is stable. Two possibilities could account for these results. β2-Microglobulin might be limiting in this system. In this case, if HFE did not complex with β2-

microglobulin, it would be rapidly degraded. Alternatively, TfR might stabilize the HFE-β2-microglobulin complex.

HFE Recognizes a Site on TfR Other Than That of Tf—The close association of HFEF and TfR over time and the lowered affinity of Tf for TfR in fHFE-expressing cells led us to investigate whether HFE has the same TfR-binding site as Tf. Earlier studies examining the association of Tf and TfR indicated that the unglycosylated form of TfR does not fold correctly and does not bind Tf (23–25). We investigated whether Asn-linked glycosylation was necessary for the association of fHFE with TfR. tf’ and tet’ fWTHFE/TA HeLa cells were treated with tunicamycin to inhibit Asn-linked glycosylation. Western blotting showed co-immunoprecipitation of unglycosylated TfR (~80 kDa) with HFE (Fig. 5, eighth lane, arrow). The discrepancy in the amount of unglycosylated TfR in tet’ and tet’ fWTHFE/TA serum was 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 HFE is independent of glycosylation, the structural elements necessary for TfR binding to Tf are different than for Tf binding to TfR.

To confirm that HFE does not exclude Tf from the binding site of TfR, cell lysates of ~1 × 10⁶ cells grown in tet’ or tet’ medium were precipitated with Tf-agarose and analyzed by Western blotting as described under “Experimental Procedures.” Simultaneous isolation of fHFE with TfR on Tf-agarose (Fig. 6) confirmed that HFE recognizes a site on TfR other than that of Tf.

HFE Co-localizes with TfR—Immunocytochemical staining of fHFE and TfR was performed to determine their intracellular localization.
localization of HFE and Tf (Fig. 8, E and F, respectively). Combined with results from Fig. 7, Tf, TIR, and HFE all appear to traffic through the same endocytic perinuclear compartment.

**DISCUSSION**

To continue to define the relationship among iron, Tf, TIR, and HFE, we have investigated the biosynthesis and trafficking of HFE in relation to TIR and have shown that HFE may regulate intracellular iron levels since its expression greatly decreases the levels of ferritin and modestly increases the levels of Tf. Both Ft and TIR are encoded by mRNAs containing iron response elements in their untranslated regions (for a review, see Ref. 27). Tf mRNA is stabilized by iron regulatory protein binding in a low cytoplasmic iron environment, whereas the translation of Ft mRNA is blocked. Modulation of TIR and Ft by HFE in this manner implicates HFE in the regulation of intracellular iron load.

Scatchard analysis of ¹²⁵I-Tf binding to tet" and tet" cell lines shows that Tf-binding sites increase from 1.8 × 10⁷/uninduced cell to 2.8 × 10⁷/induced cell, confirming the elevated level of TIR seen on Western blotting. The Scatchard data also show that HFE decreases TIR affinity for Tf as seen in the increase in Kₐ from 1.2 to 11 nm. This is comparable to the 5–75 nm increase in Kₐ upon addition of 1 mM soluble HFE-β₂-microglobulin heterodimer to HeLa cells reported earlier (8). The previous report showed no significant increase in TIR 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 HFE-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 HFE and TIR and susceptibility to endo H digestion demonstrate that these proteins are capable of associating with each other within 30 min of synthesis while the proteins are in the endoplasmic reticulum/medium-Golgi compartment. The pulse-chase analysis also indicated that a significant amount of HFE is degraded between 90 and 120 min, the remainder of which is further processed by addition of complex carbohydrates less sensitive to endo H digestion. This pool of higher molecular mass HFE is protected from degradation by its association with TIR and/or β₂-microglobulin.

The kinetics of degradation of the HFE pool associated with TIR are much slower than the degradation rate of the non-TIR-associated pool. Approximately 50% of the HFE expressed in our system was degraded within 4 h of synthesis, whereas the amount of TIR remained steady. The pool of HFE that associated with the TIR, however, did not change significantly over the course of 8 h. These results suggest that the complex is much more stable than HFE alone. Quantitation of the steady-state amount of HFE co-immunoprecipitated with TIR yielded a stoichiometry of two TIRs for every HFE, or one transferrin receptor dimer for every HFE. The same stoichiometry has been reported by Lebron et al. (6) using gel filtration.

Not only does HFE have a different stoichiometry of binding to TIR, but it binds to a different region of TIR than Ft. Tunicamycin-treated tet" and Ft-HeLa cells are capable of associating unglycosylated TIR and HFE, as shown by the co-immunoprecipitation of TIR with HFE. Tf binding to TIR is glycosylation-dependent because Tf does not show measurable binding to unglycosylated TIR (23, 24). Tf and HFE binding is not mutually exclusive since HFE coprecipitates with TIR bound to Tf-agarose. The original observation (8) that HFE decreases Tf binding affinity for TIR, but not the number of binding sites, is also in agreement with the present results.

Immunocytochemical staining of HFE in tet" and Ft-HeLa cells is punctate and perinuclear and exhibits co-localization...
The pattern is similar to TfR immunostaining in tet^1 fWTHFE/tTA HeLa cells that do not express fHFE. These results show that HFE does not drastically alter the trafficking of TfR. TfR is, traditionally, an excellent marker for recycling endosomes. Once a clathrin-coated vesicle containing 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 apotransferrin remain bound to each other and recycle to the cell surface. At neutral pH, apotransferrin is released, and TfR is free to bind more diferric Tf (for further discussion, see review in Ref. 28). HFE has no identifiable internalization motif in its cytoplasmic domain, yet fHFE co-localizes with Tf and TfR in perinuclear and cytoplasmic intracellular vesicles. The intracellular location of HFE may therefore be dependent on its association and endocytosis with TfR. The co-trafficking of HFE and 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 TfR. This finding is important in that it indicates that the function of HFE 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 HFE 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 that regulation of internal iron stores by HFE may occur within the endosome.

Hereditary hemochromatosis is a disease characterized by an increase in the set point for bodily iron load. Hereditary hemochromatosis patients have elevated levels of Tf iron saturation. The degree of Tf saturation is an indicator of bodily iron load. The association of HFE 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 num-

**FIG. 7. Co-localization of fHFE with TfR in fWTHFE/tTA HeLa cells.** A, permeabilized tet^1 cells stained with mouse anti-hTfR (4093) and FITC-labeled anti-mouse antibodies show predominant TfR localization in a perinuclear compartment, with some at the cell surface. B, permeabilized tet^1 cells stained with rabbit anti-HFE (CT1) and tetramethylrhodamine isothiocyanate-labeled anti-rabbit antibodies show no specificity for HFE, as tetracycline turns off expression of HFE and HeLa cells do not express endogenous HFE. C, permeabilized tet^1 cells stained with mouse anti-hTfR (4093) and FITC-labeled anti-mouse antibodies show no change in TfR localization despite fHFE expression in these cells. D, permeabilized tet^1 cells stained with rabbit anti-HFE (CT1) and tetramethylrhodamine isothiocyanate-labeled anti-rabbit antibodies show that 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 for 18 h in tetracycline) stained with antibody CT1 and tetramethylrhodamine isothiocyanate-labeled anti-rabbit antibody show a staining pattern identical to cells in D. These results are representative of four experiments without significant variation between experiments.

**FIG. 8. HFE localizes to same compartment as Tf.** A, permeabilized tet^1 cells stained with mouse anti-hTfR (4093) and FITC-labeled anti-mouse antibodies show TfR localization at the cell surface and in a perinuclear compartment. B, Texas Red-labeled Tf localizes to the same internal compartment as TfR in tet^1 cells. C, permeabilized tet^1 cells stained with mouse anti-hTfR (4093) and FITC-labeled anti-mouse antibodies show that TfR localization does not change with induction of HFE. D, Texas Red-labeled Tf continues to traffic through the same TfR-labeled perinuclear compartment despite fHFE expression. E, permeabilized tet^1 cells stained with mouse anti-FLAG (M2) and FITC-labeled anti-mouse antibodies show fHFE localization at the cell surface and in the same perinuclear compartment as TfR. F, Texas Red-labeled Tf localizes to the same perinuclear compartment as in C–E. TfR, Tf, and fHFE occupy the same compartment in tet^1 fWTHFE/tTA HeLa cells. These results are representative of three experiments without significant variation between experiments.
ber of ways. HFE may act by itself, regulate other proteins involved in iron metabolism, or 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 Tfr and iron uptake at the cellular level and in organs such as the liver, pancreas, pituitary, and heart that are damaged by iron overload in hereditary hemochromatosis 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 hemochromatosis patients.

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