Evidence for the interaction of the hereditary haemochromatosis protein, HFE, with the transferrin receptor in endocytic compartments

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HFE, the protein mutated in hereditary haemochromatosis type 1, is known to interact with the transferrin receptor (TfR) on the cell surface and during endocytosis [Gross, Irrinki, Feder and Enns (1998) J. Biol. Chem. **273**, 22068–22074; Roy, Penny, Feder and Enns (1999) J. Biol. Chem. **274**, 9022–9028]. However, whether they are capable of interacting with each other once inside the cell is not known. In the present study we present several lines of evidence that they do interact in endosome compartments. Cells expressing a chimaera of HFE protein with the cytoplasmic domain of lysosomal-associated membrane protein 1 (LAMP1) in place of its own (HFE–LAMP) show a decrease in the half-life of the TfR. This implies that the interaction between HFE and TfR in endosomes targets the TfR to lysosomal compartments. The interaction between TfR and HFE–LAMP was confirmed by immunoprecipitation, in addition to immunofluorescence studies. Addition of transferrin (Tf) to HFE–LAMP-expressing cells competes with HFE for binding to the TfR, thereby increasing the half-life of TfR and confirming that the HFE–LAMP–TfR complex reaches the cell surface prior to entering the endosomal vesicles and trafficking to the lysosome. These results raise the possibility that interaction of HFE and TfR in intracellular vesicles may play an important role in determining the function of HFE in iron homoeostasis, which is still unknown. Analysis of endosomal pH and the iron content of internalized Tf indicated that HFE does not appear to alter the unloading of iron from Tf in the endosome.

Key words: endosome, hereditary haemochromatosis, HFE, LAMP1, lysosome, transferrin receptor.

INTRODUCTION

Hereditary haemochromatosis is an autosomal recessive disease that, over time, causes an accumulation of iron in the parenchymal tissues of affected patients. HFE is the protein that is mutated in the majority of hereditary haemochromatosis cases. Since its identification in 1996 [1], many insights regarding HFE have been elucidated, although its true role in iron homoeostasis remains unclear. Previous studies showed that HFE interacts with the transferrin receptor (TfR). Transferrin (Tf) is a serum protein that binds up to two molecules of ferric iron and subsequently binds to the TfR on the surface of cells. Once this complex is internalized, the acidic milieu of the endosome induces a conformational change in the TfR, facilitating the release of iron from Tf [2–5]. The iron-free Tf (apo-Tf) stays bound to the TfR and is recycled to the cell surface where, in the neutral to slightly basic extracellular environment, the apo-Tf is released, allowing further binding of diferric-Tf to the receptor to continue the cycle.

When cells express HFE, the apparent affinity of the TfR for Tf is decreased [6,7]. The reduction in apparent affinity is due to the competition of HFE and Tf for overlapping binding sites on the TfR [8–10]. Our lab and others have shown previously that HeLa cells expressing HFE have reduced iron levels [7,11–13] and that such reduction occurs through the Tf-mediated iron-uptake pathway [14]. HFE expression does not affect either the kinetics of TfR cycling or the distribution of TfR within the cell [14]. Furthermore, we have shown that HFE and TfR are capable of interacting within 30 min of their synthesis. They are associated on the cell surface and also interact during endocytosis [6,7,14]. Whether or not HFE and TfR remain associated once in the endosome is not known. *In vitro* studies using the soluble forms of both TfR and HFE lacking the transmembrane and cytoplasmic domains have over a 2000-fold decrease in binding at pH less than or equal to 6.2 compared with pH 7.2 [10]. Whether the transmembrane and cytoplasmic domains play a role in the interaction, or whether the ionic environment of the endosome affects the interaction between these two proteins, has not been determined.

To study the possible interaction of HFE and TfR in cells, a chimaeric HFE that has its cytoplasmic domain replaced with that of lysosomal-associated membrane protein 1 (LAMP1) was generated. LAMP1 is a lysosomal protein and its cytoplasmic domain contains the lysosomal-targeting motif YQTI, allowing it to traffic from the cell surface to lysosomes. Both LAMP1 and TfR endocytose via a common endocytic compartment [15]; however, LAMP1 is targeted to lysosomes whereas TfR is recycled to the plasma membrane. An HFE–LAMP/tetracycline-transactivatable (tTA) HeLa cell line that can be induced to express HFE–LAMP was generated to determine whether TfR remains associated with HFE as it passes through the endosome. We show that induction of HFE–LAMP expression resulted in a decreased TfR half-life, due to its targeting to lysosomal compartments. Additionally, immunofluorescence showed TfR and HFE–LAMP co-localized in perinuclear compartments within the cell.

Abbreviations used: Tf, transferrin; TfR, transferrin receptor; LAMP1, lysosomal-associated membrane protein 1; Dox, doxycycline; Ft, ferritin; DMEM, Dulbecco's modified Eagle's medium; R/F, rhodamine/fluorescein; tTA, tetracycline-transactivatable.

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MATERIALS AND METHODS

Plasmids

To create the pUHD 10-3 HFE–LAMP1 construct, the pUHD 10-3 fWTHFE plasmid (described in [7]) was linearized with the restriction enzyme *Sca*I. Primers were designed to replace the cytoplasmic domain of HFE and the FLAG epitope (RKRQGSRGAMGHYVLAEREDYKDDDDK) with the cytoplasmic domain of LAMP1 (RKRSHAGYQTI), which includes an internalization and lysosomal sequence. Both of these proteins have a membrane anchor of RKR, so the LAMP1 domain was added in place of the HFE domain following this motif. The reverse primer included a termination codon (5′-GCG-GCC-GCT-TAT-CAG-ATA-GTC-TGG-TAG-CCT-GCG-TGA-CTC-CTC-TTC-CTT-AAT-ATT-ATG-3′) and the forward oligonucleotide was designed from a nearby *Bam*HI site (5′-CCC-GGG-GAT-CCT-CTA-GCG-3′). We utilized the above primer pair to amplify and loop in the desired sequence, creating the HFE–LAMP cDNA in pUHD 10-3.

Subcloning

The HFE–LAMP PCR product was ligated into the 3′ T-overhang of the pGEM-T vector (Promega). pGEM-T/HFE–LAMP and pUHD 10-3/fWTHFE plasmids were cut with *Not*I, gel purified and cut with *Bst*XI to yield the amplicon fragment (HFE–LAMP, 529 bp) and vector fragment (pUHD 10-3, 3698 bp). The amplicon and vector fragments were ligated, transformed and confirmed with *Not*I and *Bst*XI restriction digests in addition to sequence analysis.

The sequenced pUHD 10-3 HFE–LAMP plasmid revealed an A-to-T mutation in the LAMP1 domain, changing the amino acid sequence from RKRSHAGYQTI to RKRSHAGYQSI. The Tyr-Xaa-Xaa hydrophobic residue sequence at the C-terminus of the cytoplasmic domain of LAMP1 is necessary for internalization and essential for lysosomal targeting [16]. The mutation in our clone falls within this motif (YQTI to YQSI) but did not alter the ability of the protein to internalize or target to the lysosome.

Generation of the HFE–LAMP/tTA HeLa cell line

The generation of the tetracycline-repressible HeLa cells that express FLAG-epitope-tagged wild-type HFE (fWTHFE/tTA HeLa) was described previously [7]. The HFE–LAMP/tTA HeLa cell line was generated in an identical manner. Colonies were screened by Western analysis for expression of HFE using a rabbit anti-(human HFE) antibody (a gift from Dr Pamela Bjorkman, California Institute of Technology, Pasadena, CA, U.S.A.). The resulting HFE–LAMP/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) and 300 ng/ml puromycin, with or without 1 μg/ml doxycycline (Dox+ and Dox− respectively; Dox is an analogue of tetracycline). The HFE–LAMP protein was expressed in cells without Dox.

Western immunodetection

Cell extracts from $\approx 6 \times 10^5$ cells were diluted with 4 × Laemmli buffer [125 mM Tris/HCl (pH 6.8), 4 % SDS, 20 % glycerol and 10 % 2-mercaptoethanol] [17], or immunoprecipitates were eluted with 2 × Laemmli buffer and subjected to electrophoresis on SDS/polyacrylamide gels (8, 10 or 12 %) under reducing conditions. The proteins were transferred to nitrocellulose (Nitropure 0.45 μm; Osmonics). Immunoblot analysis was performed using sheep anti-TfR serum [18,19] (1:10 000 dilution), rabbit anti-HFE (EX1 #137; a gift from Dr John Feder, Bristol-Myers Squibb Co., Pennington, NJ, U.S.A.; 1:10 000 dilution) and rabbit anti-[human ferritin (Ft)] (1:500 dilution; Dako) followed by the appropriate secondary antibody conjugated to horseradish peroxidase and chemiluminescence (SuperSignal; Pierce) as per the manufacturer's directions.

Immunoprecipitation

HFE–LAMP/tTA HeLa Dox− and Dox+ cells were washed three times with 2 ml of PBS (pH 7.4) and lysed with NET-Triton [150 mM NaCl, 5 mM EDTA and 10 mM Tris (pH 7.4) with 1 % Triton X-100]. Cell lysates were adsorbed for 1 h at 4 °C with 50 μl of donkey anti-mouse antibody (Jackson Laboratories)-coated Gamma Bind Plus Sepharose (Protein G–Sepharose; Amersham Biosciences) and 4 μl of monoclonal anti-HFE antibody (8C10 mouse anti-hHFE) [20]. After centrifugation in a microfuge for 2 min, the Protein G–Sepharose pellet was resuspended in 100 μl of 2 × Laemmli buffer and subjected to SDS/PAGE analysis on an 8 % acrylamide gel. Gels were transferred to nitrocellulose and immunodetected with sheep anti-TfR antibody or rabbit anti-HFE antibody as described above.

Immunofluorescence

Subconfluent HFE–LAMP/tTA HeLa (Dox− and Dox+) cells grown on coverslips were washed three times with 2 ml of Hank's balanced salts modified medium (without sodium bicarbonate; pH 7.4; Sigma) at room temperature. All cells were fixed for 15 min in 4 % paraformaldehyde (Electron Microscopy Sciences) at room temperature. They were washed twice for 1 min each with Hank's medium, permeabilized with 0.2 % Triton X-100 in Hank's medium for 10 min at room temperature, and washed three times, for 5 min each, with Hank's medium. The cells were blocked for 1 h at room temperature in Hank's medium supplemented with 10 % fetal bovine serum. The cells were incubated for 1 h at room temperature in sheep anti-TfR (1:800 dilution in blocking solution) or 8C10 mouse anti-HFE (1:1000 dilution). Coverslips were then washed three times with Hanks and incubated again for 1 h at room temperature with Alexa 488 donkey anti-sheep secondary antibody (1:500 dilution) or Alexa 594 donkey anti-mouse secondary antibody (1:250 dilution; Molecular Probes). Cells were mounted with ProLong Antifade (Molecular Probes) and imaged by deconvolution microscopy (Deltavision) on a Nikon microscope (60 × oil immersion lens).

Half-life experiments

Induced (Dox−) and uninduced (Dox+) subconfluent HFE–LAMP/tTA HeLa cells in 35 mM dishes were washed three times with sterile PBS (pH 7.4) and labelled with 50 μCi of [^{35}S]methionine/cysteine (Perkin Elmer Life Sciences) in DMEM minus methionine/cysteine (Invitrogen) medium supplemented with 20 % fetal bovine serum overnight. The cells were washed three times with PBS and chased from 0 to 24 h with complete medium. At the completion of the chase, all cells were lysed for 5 min on ice in NET-Triton. Cell lysates were preadsorbed for at least 45 min at 4 °C with 50 μl of Protein A–Sepharose 4B (Zymed) per $\approx 6 \times 10^5$ cells to reduce precipitation of non-specific protein. Preadsorbed lysates were incubated for 1 h at 4 °C with 50 μl of Protein A–Sepharose and 1.4 μl of sheep

anti-TfR serum. After incubation, the sample was spun down and the pellet was resuspended in 200 μl of NET-Triton and washed through 1 ml of NET-Triton containing 15 % sucrose. Samples were eluted in 100 μl of 2 × Laemmli buffer and subjected to SDS/PAGE analysis on 8 % denaturing gel. Gels were fixed, treated with Amplify (Amersham Biosciences), dried and subjected to PhosphorImager analysis.

Tf competition

Subconfluent HFE–LAMP/tTA HeLa cells (Dox−) in 35 mm dishes were washed three times with sterile PBS (pH 7.4) and labelled with 50 μCi of [^{35}S]methionine/cysteine in DMEM minus methionine/cysteine medium with 20 % fetal bovine serum overnight. After washing, the cells were incubated with or without 3 mg/ml diferric-Tf in complete medium for 0–24 h. Cells were washed and solubilized and the cell extracts were incubated with sheep anti-TfR to immunoprecipitate TfR as described above and analysed by SDS/PAGE on an 8 % acrylamide gel under reducing conditions. Gels were fixed, treated with Amplify, dried and subjected to PhosphorImager analysis.

PhosphorImager quantification and calculation of half-lives

IP Lab Gel 1.5 (Molecular Dynamics) was used to quantify images by determining the area within a region of fixed pixel number at each band of interest on the gel. Background of each lane was subtracted from the band of interest to obtain comparable values. Half-lives were calculated from analysis of the radioactive bands using a Molecular Dynamics PhosphoImager and Image 1.5 software. The values were plotted on a semi-log plot and slopes calculated using Cricketgraph.

Tf-^{55}Fe uptake and ^{125}I-labelled diferric-Tf (^{125}I-Tf) preparation

The procedures for ^{55}Fe loading on to human apo-Tf and Tf-^{55}Fe uptake were essentially the same as described previously [7]. The effect of monensin on Tf-^{55}Fe was measured as described in the relevant figure legend. Human diferric-Tf was labelled with Na^{125}I using lactoperoxidase as described previously [15,19].

Urea/PAGE

Urea/PAGE has the advantage that it can separate apo-, monoferric- and diferric-Tf based on their different rates of migration under this specific condition. It was performed as described previously [5,21] with the following modifications. Subconfluent Dox+ and Dox− fWTHFE/tTA HeLa cells in 35 mm wells were first incubated with 1 ml of prewarmed incubation medium (DMEM with 20 mM Hepes and 2 mg/ml ovalbumin, pH 7.4) containing 100 nM ^{125}I-labelled diferric-Tf at 37 °C for 30 min to allow ^{125}I-Tf internalization and cycling. The externally bound Tf was then stripped with an acidic buffer (0.5 M acetic acid and 0.5 M NaCl) for 2 min at 4 °C, followed by four washes with 2 ml of NET. Cells were lysed in 80 μl of NET-Triton. After mixing with an equal amount of 2 × loading buffer [7 M urea, 1.25 × TBE (100 mM Tris/10 mM boric acid/5 mM EDTA), pH 8.5, and 10 % sucrose], samples were subjected to urea/acrylamide-gel electrophoresis (6 M urea, 6 % acrylamide and 1 × TBE, pH 8.5), and run at 4 °C at 100 V for 20 h. After fixation, the gel was dried and exposed to X-ray film at − 80 °C. To avoid any residual amount of iron contamination, all the above solutions were pretreated with a de-ionization mix (AG 501-X8 resin; Bio-Rad, Hercules, CA, U.S.A.) for at least 3 h with constant stirring at the ratio of 5 g of resin/100 ml of solution.

To show the efficacy of urea/PAGE in the separation of different forms of Tf and to ensure that the above procedure truly reflects the status of Tf within the endosome, we also ran samples prepared by incubating cells with ^{125}I-Tf at 4 °C without or with subsequent acid wash as controls, of which the former was expected to show only the cell-surface bound ^{125}I-Tf and the latter was expected to show no ^{125}I-Tf signal in the urea/PAGE. We also performed several positive controls by pre-incubating the cells with different concentrations of monensin (0, 20, 50 and 100 μM) for 30 min at 37 °C to inhibit the endosomal lumen acidification. ^{125}I-Tf (100 nM) was added and incubated for 30 min with the same concentrations of monensin as above. Cell lysates were subjected to urea/PAGE.

Immunofluorescence analysis and measurement of endosomal pH

The methods for measuring pH of endosomes have been described previously [22]. Briefly, subconfluent Dox+ or Dox− fWTHFE/tTA HeLa cells and Dox+ or Dox− (mock-induced) tTA HeLa cells were seeded on a coverslip on the bottom of a dish and grown for 2 days. Cells were incubated with medium containing 10 μg/ml (125 nM) Tf labelled with both rhodamine and fluorescein. Fluorescence images were collected between 5 and 15 min of labelling with a Zeiss 63 × planapochromat NA 1.4 oil immersion objective. Images were collected with a Zeiss Axiovert microscope using a Bio-Rad MRC-600 confocal attachment, with 515–545 nm fluorescein and 575 nm rhodamine emission filter sets, and 488 nm excitation. The rhodamine/fluorescein (R/F) ratio increases as the pH becomes more acidic because rhodamine fluorescence is pH-independent and fluorescein fluorescence is quenched as the pH decreases. Image processing was performed as described previously [22]. For each optical plane the ratios of R/F fluorescence were calculated for each pixel whose intensity for both fluorophore channels was above background. Histograms of pixel frequency of R/F ratio values were constructed. To confirm the pH-sensitivity of the probe, data were collected from cells in which the endosomes were alkalinized by incubating with 40 mM methylamine.

RESULTS

Identification of the HFE–LAMP/tTA HeLa clone

The tetracycline-responsive promoter system developed by Gossen and Bujard [23] was used to create a cell line in which HFE expression could be tightly controlled. The expression of TfR, HFE and Ft in this new cell line was compared against the previously characterized cell line fWTHFE/tTA HeLa [7,14,24] to confirm that the HFE–LAMP was functional; that is, its ability to decrease intracellular Ft levels. HFE expression was induced in cells in the absence of Dox (Dox−; Figure 1). HFE migrates as a broad heterogeneous band presumably due to heterogeneity in glycosylation, which has been noted previously [7,13,20,25]. The apparent molecular mass of HFE–LAMP is slightly lower than fWTHFE because the LAMP1 cytoplasmic domain is only 11 amino acids compared with the 27 amino acids comprising the HFE cytoplasmic domain (19 amino acids) with a FLAG-tag epitope (8 additional amino acids) of the fWTHFE. It too migrates as a broad band that appears even broader than the wild-type HFE, perhaps due to the increased turnover of the lysosomally directed chimaera. Both cell lines showed a decrease

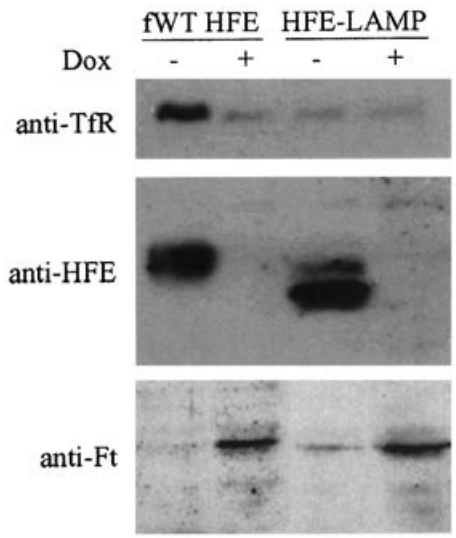

Figure 1 Inducible expression of HFE in fWTHFE/tTA HeLa and HFE–LAMP/tTA HeLa cells

Lysates of $\approx 6 \times 10^5$ fWTHFE/tTA HeLa and HFE–LAMP/tTA HeLa cells, induced (Dox−) or uninduced (Dox+) for HFE expression, were run on a 12% denaturing acrylamide gel under reducing conditions. Proteins were transferred to nitrocellulose and detected with sheep anti-TfR (1:10 000), rabbit anti-HFE (1:10 000) or rabbit anti-Ft (1:500), and the appropriate horseradish peroxidase-conjugated secondary antibody (1:10 000). Chemiluminescence detected $\approx$94-, $\approx$43- and $\approx$19/21-kDa bands representing TfR, HFE and Ft respectively. The increased TfR expression (fWTHFE cells only) and decreased Ft expression in HFE-expressing cells (Dox−) are indicative of a decrease in intracellular iron load. The difference in molecular mass of HFE in the two different cell lines is due to the removal of its cytoplasmic domain (19 amino acids) and the FLAG tag (eight amino acids) in the fWTHFE cells and the addition of only 11 amino acids of the LAMP1 cytoplasmic domain, resulting in a decrease in molecular mass compared with fWTHFE. These results are representative of three experiments without significant variation between experiments.

in the levels of Ft (19 and 21 kDa) in cells expressing fWTHFE and HFE–LAMP (Figure 1). These results imply that expression of HFE reduces the intracellular iron load. The iron-regulatory proteins ('IRPs') modulate changes in TfR and Ft levels. At low intracellular iron concentrations, the iron-regulatory protein binds to the iron response element ('IRE') stem loop structure in the 5′ untranslated region of Ft mRNA, blocking translation and lowering Ft levels. The same reduction in intracellular iron load results in the binding of the iron-regulatory protein to the 3′ untranslated portion of the TfR mRNA, stabilizing the message and thus increasing the amount of TfR protein. In keeping with a low-iron phenotype generated by HFE, TfR levels increased in cells expressing fWTHFE. However, TfR levels did not increase in cells expressing HFE–LAMP. The lack of change of TfR expression in Dox− HFE–LAMP/tTA HeLa cells is probably due to increased lysosomal degradation of TfR, as demonstrated in subsequent experiments.

Association of TfR and HFE in HFE–LAMP/tTA HeLa cells

We and others have shown previously that HFE and TfR can interact in cultured cells [6,7]. To confirm this interaction in the HFE–LAMP/tTA HeLa cell line, immunoprecipitation experiments were carried out. Cells were immunoprecipitated with an HFE antibody (8C10 mouse anti-human HFE [20]) followed by immunodetection with sheep anti-TfR and rabbit anti-HFE antibodies (Figure 2). In cells expressing the HFE–LAMP (Dox−), TfR was co-precipitated with HFE, providing evidence for the association of HFE and TfR in this cell line

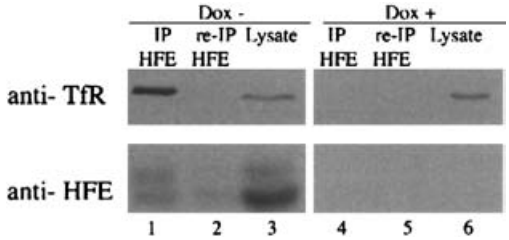

Figure 2 Association of TfR and HFE in HFE–LAMP/tTA HeLa cells

Lysates from $\approx 6 \times 10^5$ HFE–LAMP/tTA HeLa Dox− or Dox+ cells were incubated with Protein G–Sepharose in the presence of 8C10 mouse anti-HFE antibody for 1 h at 4 °C. After pelleting and eluting the samples with Laemmli buffer, the eluants were subjected to SDS/PAGE. Immunodetection was carried out with sheep anti-TfR and rabbit anti-HFE (EX1 #137) antibodies followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescence detected $\approx$97 kDa (TfR) and $\approx$43 kDa (HFE) species upon immunoprecipitation (IP) with the HFE antibody, providing evidence for the association of HFE–LAMP and TfR. Re-IP, re-immunoprecipitation control; these samples were immunoprecipitated a second time with the same antibody to confirm that all of the protein was pulled down in the initial immunoprecipitation.

(Figure 2, lane 1). As expected, in cells that did not express HFE–LAMP (Dox+) no HFE–LAMP could be detected, no TfR was co-precipitated (Figure 2, lane 4) and TfR was only detected in the cell lysate (Figure 2, lane 6).

Half-life of the TfR in the presence of HFE–LAMP

To test our hypothesis that TfR and HFE interact in the endosome, we examined the half-life of TfR in the HFE–LAMP-expressing cells. If TfR is in fact being targeted to the lysosome by interacting with the HFE–LAMP protein, the half-life of TfR should be less than its half-life in the absence of HFE–LAMP. The half-life of TfR was reduced from 14.7 to 7.9 h in the presence of HFE–LAMP (Figures 3A and 3C). This reduction in half-life for the TfR supports the idea that HFE–LAMP is sorting TfR to the lysosome where it is being degraded, and this process is faster than the degradation rate of the typical recycling TfR. Such a reduction in the half-life for TfR is not due to the association of TfR with the wild type HFE. We observed the opposite of what we measured in HeLa cells expressing wild-type HFE. In the fWTHFE/tTA HeLa cells, the half-life of TfR was doubled compared with these cells with fWTHFE turned off (Figures 3B and 3C). The latter was a surprising result, in that we have previously shown that the expression of HFE in these cells has no effect on endocytosis, exocytosis or cellular distribution of the TfR [14]. This result implies, however, that the interaction of HFE and TfR stabilized the TfR protein in some way.

Incubation of the HFE–LAMP/tTAHeLa cells with super-physiological levels of diferric-Tf (3 mg/ml) was used to determine whether Tf could increase the half-life of the TfR by competing for binding to TfR. Tf competes with HFE for binding to TfR in BIAcore binding studies using the ectodomains of these proteins [8–10,26]. Our approach would also address whether the HFE–LAMP–TfR complex was getting to the cell surface prior to trafficking to the lysosome, and not being directly targeted to the lysosome after exit from the *trans*-Golgi network. If Tf competes with HFE–LAMP, then an increase in TfR half-life should occur. The addition of Tf resulted in an increase in the half-life of TfR from $\approx$9.1 to 13.6 h (Figure 4). This shift in half-life upon Tf addition demonstrates that HFE and TfR are interacting on the cell surface and within intracellular compartments, and co-trafficking to the lysosome.

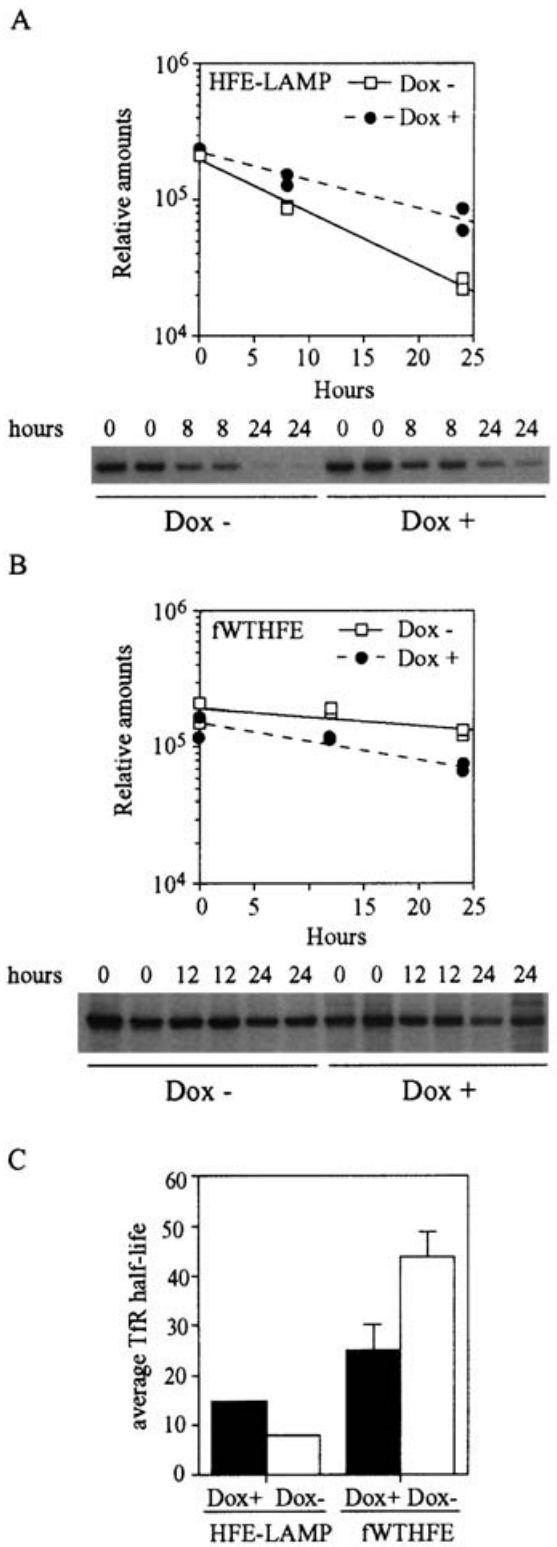

Figure 3 Effect of HFE on TfR half-life

(**A**) HFE expression decreases TfR half-life in HFE–LAMP/tTA HeLa cells. Approx. 6×10^5 cells expressing HFE–LAMP (Dox−, □) or lacking HFE–LAMP expression (Dox+, ●) were labelled overnight with 50 μCi of [^{35}S]methionine/cysteine and chased for 0, 8 or 24 h with complete medium. Cell lysates were immunoprecipitated with anti-TfR antibody and subjected to SDS/PAGE on 8 % acrylamide gels under reducing conditions. HFE–LAMP expression lowers the half-life of TfR from 15.1 to 7.7 h in this representative experiment as per the corresponding gel shown below. (**B**) HFE expression increases TfR half-life in fWTHFE/tTA HeLa cells. Approx. 6×10^5 cells expressing fWTHFE (Dox−, □) and lacking fWTHFE expression (Dox+, ●) were labelled overnight with 50 μCi of [^{35}S]methionine/cysteine and chased for 0, 12 or 24 h with complete medium. Cell lysates were treated as described in (**A**). fWTHFE expression increases the TfR half-life from 23 h (Dox+) to 50 h (Dox−) in this representative case as per the corresponding gel shown below. (**C**) The half-lives of TfR were measured for HFE–LAMP and fWTHFE/tTA HeLa cells. These experiments were repeated three times each and the half-lives for each cell line under each condition were averaged (14.7 h for HFE–LAMP Dox+ and 7.9 h for HFE–LAMP Dox−; 25 h for fWTHFE Dox+ and 44 h for fWTHFE Dox−). Error bars cannot be seen for the HFE–LAMP samples because they were so slight. In all cases the half-life of TfR decreased when HFE–LAMP was expressed and increased when fWTHFE was induced.

Localization of HFE–LAMP and TfR in HFE–LAMP/tTA HeLa cells

The intracellular localization of TfR and HFE–LAMP was examined by immunofluorescence to determine the extent of co-localization of these two proteins (Figure 5). In control fWTHFE-expressing cells, co-localization of HFE and TfR in punctate vesicles was visible (Figure 5A), which has been seen previously [7]. Redistribution of HFE and TfR was seen in HFE–LAMP/tTA HeLa cells, whereby TfR and HFE–LAMP were detected co-localizing in perinuclear compartments (Figure 5B). This shift in distribution of both proteins confirms that TfR and HFE–LAMP are interacting within these cells and that the complex has a different trafficking pattern than control cells. Efforts to visualize TfR in lysosomal compartments show limited overlap, most likely due to the rapid degradation of the TfR once it reaches the lysosome (results not shown).

HFE does not increase the pH of the endosome

Previous studies indicate that the expression of HFE in HeLa cells lowers the intracellular iron levels [7,11–13]. Since HFE was capable of interacting with TfR in endosomal compartments, we wanted to determine which step of Tf-mediated iron uptake and release was altered by HFE. Tf-mediated Fe uptake by cells involves the binding of diferric-Tf to TfR on the cell surface followed by internalization of the complex into a low-pH endosomal compartment. In the endosome, iron dissociates from Tf and is reduced and transported across the endocytic vesicle. The apo-Tf–TfR complex is recycled back to the cell membrane. The pH of the endosome regulates the efficiency of iron release from Tf [5]. HFE could alter iron homoeostasis of the cell by decreasing the acidification of the endosome, thereby lowering the efficiency of iron release from Tf.

The pH-sensitive fluorophore, fluorescein, bound to diferric-Tf in combination with a pH-insensitive probe, tetramethylrhodamine-labelled Tf, was used to measure intracellular pH of the HFE/TfR-positive compartments. For these experiments, fWTHFE/tTA HeLa cells were used under both Dox− and Dox+ conditions, corresponding to with and without HFE expression, respectively. The R/F ratio of Tf-containing endosomes in both conditions was subsequently measured using microscopy [22]. To examine the pH of Tf-containing recycling endosomes, which accumulate in the pericentriolar region of cells, the R/F ratio of the pericentriolar endosomes was measured in two separate confocal planes from HFE-expressing and non-expressing cells (Figure 6A). The distribution of R/F ratios in these endosomes was unchanged by expression of HFE, indicating that HFE expression does not significantly alter endosome acidification. The rate-limiting step in return of Tf to the plasma membrane is movement from the recycling endosomes to the cell surface, and therefore Tf accumulates in recycling endosomes. To assess the pH of all endosomal compartments containing R/F-labelled Tf (e.g. early, sorting and recycling endosomes),

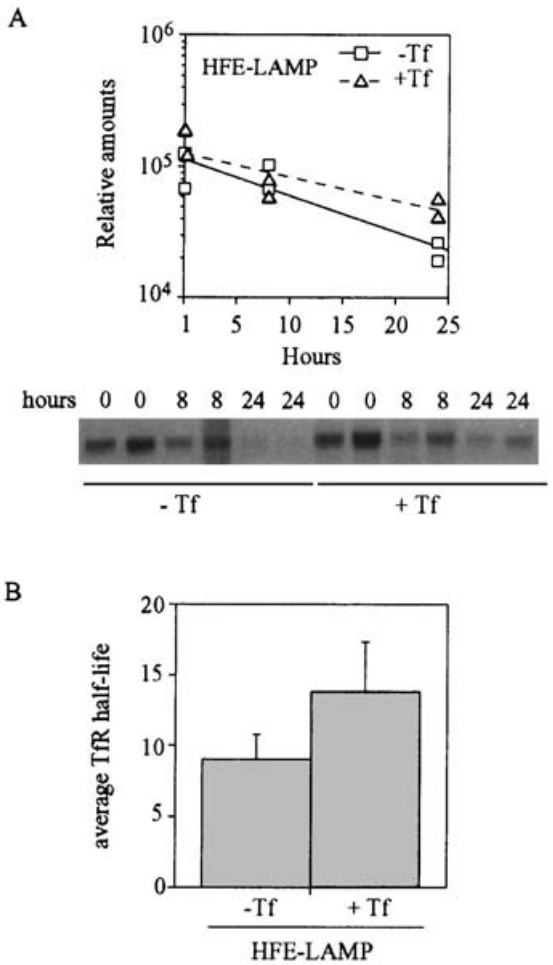

Figure 4 Tf competes with HFE for binding to TfR and increases TfR half-life in HeLa cells expressing HFE–LAMP

(**A**) Approx. 6×10^5 HFE–LAMP/tTA HeLa (Dox−) cells were labelled overnight with 50 μCi of [^{35}S]methionine/cysteine. Cells were washed and either treated with 3 mg/ml diferric-Tf (Tf+; △) or given no treatment (− Tf; □) and chased up to 24 h. After immunoprecipitation with anti-TfR, the samples were subjected to SDS/PAGE on 8 % acrylamide gels under reducing conditions. Addition of Tf competes with HFE for binding to the TfR, causing an increase in the half-life of TfR from ≈ 11.1 to 17.7 h in this representative experiment as per the corresponding gel below. (**B**) The resulting half-lives from three experiments were averaged (9.1 h for − Tf and 13.6 h for +Tf). In all cases, the half-life of TfR was greater when cells were treated with Tf.

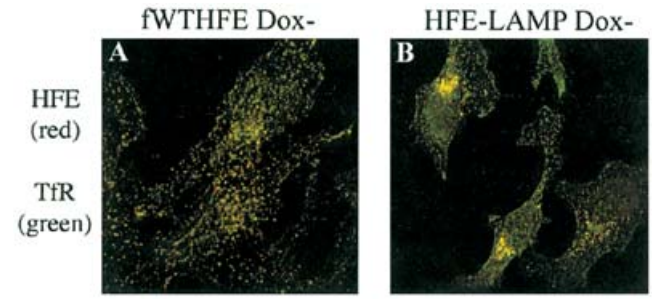

Figure 5 Immunofluorescence of HFE and TfR in HeLa cells expressing different forms of HFE

(**A**) Co-localization of HFE with TfR in punctate vesicles throughout the cell in permeabilized fWTHFE/tTA HeLa (Dox−) cells stained with sheep anti-TfR, mouse 8C10 anti-HFE, donkey anti-sheep (Alexa 488) and donkey anti-mouse (Alexa 594) antibodies. (**B**) Permeabilized HFE–LAMP/tTA HeLa (Dox−) cells stained the same as in (**A**), show co-localization of TfR and HFE in a perinuclear region.

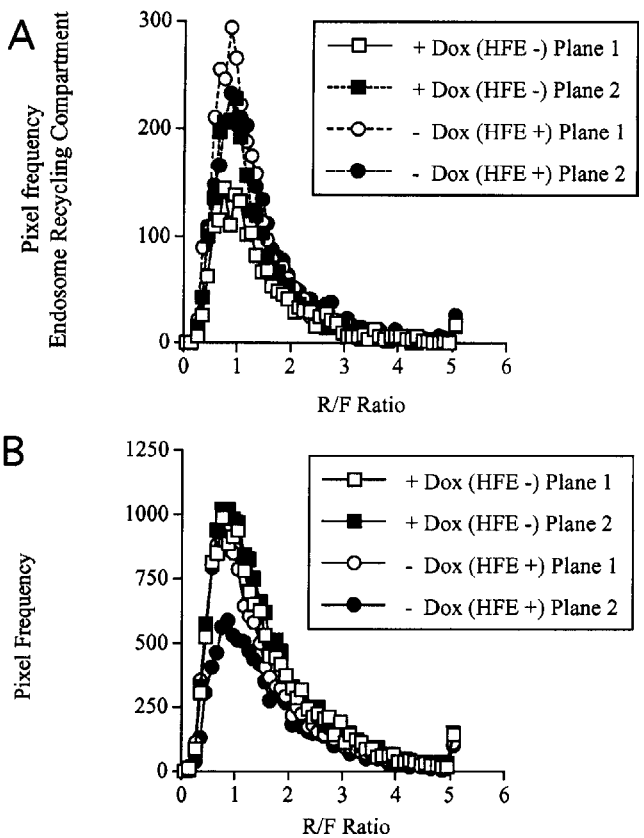

Figure 6 Measurement of endosome pH

Endosomal pH was quantified in two separate confocal planes of fWTHFE/tTA HeLa cells that do not (Dox+) or do (Dox−) express HFE. (**A**) The R/F ratios of Tf in the recycling endosomes are shown. There is no difference between the distributions from cells expressing or not expressing HFE. There were fewer endosomes in 'plane 1' of the Dox-treated cells, accounting for the lower peak value. The R/F ratio distribution in this plane was similar to the distributions in the other planes. (**B**) The R/F ratios of each pixel within the optical plane are shown. There is no difference between the distributions from cells expressing HFE or not, demonstrating that the pH of TfR-containing endosomes is not altered by HFE expression. There were fewer endosomes in 'plane 2' of the cells not treated with Dox, accounting for the lower peak value. The R/F ratio distribution in this plane was similar to the distributions in the other planes. In all cases, only pixels above the background levels for both fluorophores were analysed. These measurements were performed in duplicate. The data presented are from a representative of three experiments.

the R/F ratios from two separate confocal planes from HFE-expressing and non-expressing cells were determined pixel by pixel. As was the case for recycling endosomes, there was no significant difference in the R/F ratios between cells expressing or not expressing HFE (Figure 6B). The failure to observe any change in the R/F ratio strongly indicates that HFE expression has no effect on acidification of any of the Tf-containing endosomes. The R/F ratio in cells incubated with 40 mM methylamine, which alkalinizes endosomes, was significantly decreased, consistent with an increase in endosomal pH and thereby confirming the sensitivity of the assay (results not shown).

The effect of HFE on iron dissociation from Tf within endosomes

HFE could be acting within the endosomal compartments to inhibit the release of iron from Tf. Previous studies by Murphy,

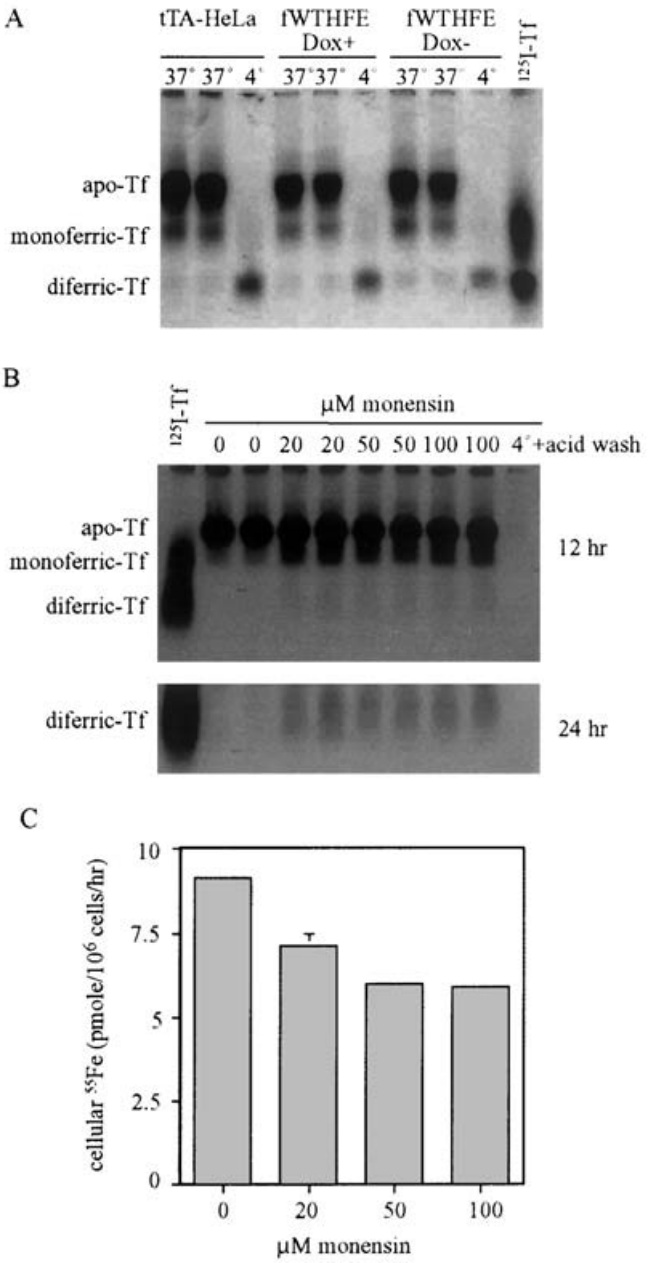

Figure 7 Effect of fWTHFE expression on Tf-associated iron dissociation within endosomes

(**A**) Urea/PAGE analysis. fWTHFE/tTA HeLa cells (Dox+ and Dox−) and tTA HeLa cells were incubated in presence of 100 nM ^{125}I-Tf at 37 °C for 30 min to allow ^{125}I-Tf to bind to cell-surface TfR, endocytose and cycle. This was followed by an acid wash to remove the fraction of cell surface-associated ^{125}I-Tf. Cell lysates were separated on urea/PAGE. The gels were fixed, dried and exposed to X-ray film. The samples were run in duplicate. In the meantime, we also ran two parallel controls of which one was cell-surface bound ^{125}I-Tf at 4 °C without acid wash and the other one was ^{125}I-Tf alone. (**B**) Effects of monensin treatment on Tf-associated iron dissociation within endosomes. tTA HeLa cells were pretreated with different concentrations of monensin (0, 20, 50 and 100 μM) for 30 min at 37 °C, followed by addition of ^{125}I-Tf to the final concentration of 100 nM with the same concentrations of monensin. Cells were incubated for another 30 min at 37 °C, subjected to acid wash, run on urea/PAGE and exposed to X-ray film as above. All the samples were run in duplicate. Two more control samples were also included: (i) ^{125}I-Tf alone without any manipulation and (ii) tTA HeLa cells incubated with ^{125}I-Tf at 4 °C, followed by acid wash to remove the surface-bound ^{125}I-Tf to confirm that the samples incubated at 37 °C represented the true status of Tf within the endosome. The positions of apo-Tf, monoferric-Tf and diferric-Tf are marked on the left. As the diferric-Tf bands are weak for all the samples, a portion of an image from a longer exposure (24 h) is presented in the lower panel. (**C**) Effect of monensin treatment on Tf-^{55}Fe uptake. To further evaluate the effects of monensin on endosomal acidification, we examined its effects on the rate of Tf-^{55}Fe uptake. The same cells were used and the experimental protocol was exactly the same as for (**B**) except that Tf-^{55}Fe was used instead of ^{125}I-Tf. After a 30 min incubation at 37 °C, cells were subjected to acid wash and the intracellular ^{55}Fe was measured. The rate of Tf-^{55}Fe uptake is expressed as pmol of ^{55}Fe/10^6 cells per h.

Aisen and co-workers [3,5] showed that at reduced pH, TfR facilitates the release of iron from Tf. The association of HFE with TfR could prevent this conformational change in TfR. The amount of iron remaining bound to Tf within the endosome was measured by separation of apo-, monoferric- and diferric-Tf species on 6 M urea/PAGE gels as described by Makey and Seal [21]. These gels can separate the different forms of Tf molecules (diferric-, N-terminal monoferric-, C-terminal monoferric- and apo-Tf) into distinct bands on the basis of their different Stokes radii [21]. Taking advantage of this technique, we incubated non-transfected tTA HeLa cells and fWTHFE/tTA HeLa cells with or without inclusion of Dox in the presence of 100 nM ^{125}I-Tf for 30 min either at 37 °C to allow equilibration of Tf within the cell or at 4 °C to only allow binding to cell-surface TfR as a control. This Tf concentration was used because a 30 % decrease in Tf-mediated ^{55}Fe uptake was seen but no difference in ^{125}I-Tf uptake was measured under these conditions [14]. Surface Tf was removed by chilling cells and washing with neutral pH buffer, to remove unbound Tf, followed by acidic buffer, to remove all surface-bound Tf. Cells were then lysed and subjected to 6 M urea/PAGE. In all the cell lines internalized Tf migrated predominantly as the apo-Tf band, with a small amount of monoferric-Tf and a very faint diferric-Tf band (Figure 7A). We were unable to distinguish between the two monoferric-Tf bands as described by Makey and Seal [21]. PhosphoImager analysis of the bands showed no significant difference with respect to the ratio of each Tf species from extracts of cells either expressing HFE or not. These results indicate that fWTHFE expression does not have any effect on iron dissociation from Tf within the endosome.

To demonstrate that only intracellular Tf was measured in this assay several controls were included. At 4 °C, the non-permissive temperature for endocytosis of the TfR, the Tf that was bound to the cell surface migrated predominantly as diferric-Tf (Figure 7A, 4 °C). These results are consistent with results published previously that TfR has a high affinity for diferric-Tf, and a lower affinity for monoferric-Tf and no detectable binding to apo-Tf [27–29]. Washing the cells with acid removes all of the surface-bound Tf (Figure 7B). These results indicate that acid wash is able to remove all cell-associated ^{125}I-Tf and also suggested that data obtained at 37 °C represent only the internalized ^{125}I-Tf (Figures 7A and 7B). To exclude the possibility that acid washes could cause a loss of iron from Tf in the endosome, cells were treated with monensin, a weak base, to inhibit endosomal acidification [30]. Monensin treatment is able to decrease the rates of Tf-^{55}Fe uptake by $\approx$22, 33 and 34 % at 20, 50 and 100 μM monensin, respectively, in comparison with the untreated cells. The amount of inhibition of Tf-mediated iron uptake at these concentrations of monensin was the same as that of HFE expression in cells [14]. Under the same conditions, we performed the urea/PAGE gel analysis. Treatment of cells with monensin significantly increased the amounts of both monoferric- and diferric-Tf detected within the cell, indicating that we were able to observe changes in levels of iron-saturated Tf within the same range as we would expect for Dox− fWTHFE/tTA HeLa cells expressing wild-type HFE (Figure 7B and quantified in Figure 7C). These results indicate that HFE does not inhibit the release of iron from Tf in endosomal compartments.

DISCUSSION

We and others have previously shown that TfR and HFE are capable of association soon after their synthesis in the endoplasmic reticulum and co-localize in endosomes. In this study, we demonstrate that these two proteins can remain associated as they pass through acidic vesicles inside the cell. Immunoprecipitation experiments show that a chimaeric form of HFE used throughout our studies (HFE–LAMP) can co-immunoprecipitate the TfR when expressed in HeLa cells. The chimaera is also capable of reducing Ft levels in the cells to the same extent as the FLAG-epitope-tagged wild-type form of HFE.

Alterations in the half-life of TfR in the HFE–LAMP/tTA HeLa cell line in the absence and presence of Dox were measured to determine whether TfR and HFE interacted within the endocytic compartments of the cell. The typical half-life for a normally recycling TfR has been reported to be anywhere from 14 to 24 h [31–35]. In this cell line, the half-life was measured to be 14.7 h. When HFE–LAMP was induced by withdrawal of Dox, the half-life of TfR decreased to 7.9 h. Thus, interaction of TfR and HFE–LAMP causes a greater degradation rate for the TfR cycle that we have engineered than what a normally recycling TfR would incur. We conclude that the decrease in the TfR half-life is the result of its trafficking to the lysosome. Furthermore, when an excess of diferric-Tf is added to cells expressing HFE–LAMP, a longer half-life of TfR is observed. We conclude that competition of Tf and HFE for binding to the TfR allows the Tf–TfR complex to cycle through the endosome and circumvent the lysosome. This result also confirms that the HFE–LAMP–TfR complex reaches the cell surface prior to endocytosis and sorting to the lysosome. Since lysosomal targeting is due to the motif on the HFE–LAMP protein, these results indicate that TfR is targeted to the lysosome through its interaction with HFE–LAMP.

Immunofluorescence was used to determine the co-localization of the TfR and HFE–LAMP in HeLa cells. Typically, immunofluorescence of TfR and HFE in HeLa cells reveals complete co-localization in a punctate pattern throughout the cell [14,25]. In cells expressing HFE–LAMP, we also see co-localization of HFE–LAMP and TfR. This localization, however, is redistributed to a perinuclear compartment distinct from the wild-type cells, confirming that TfR trafficking is altered due to its interaction with the HFE–LAMP.

A surprising result in our study was the observation of an increased half-life of TfR in fWTHFE/tTA HeLa cells. We found that the half-life of TfR in this cell line is nearly doubled from 25 to 44 h when fWTHFE is expressed. We have shown previously that the fWTHFE does not affect TfR recycling rates, or its cellular distribution [14]; therefore, HFE must stabilize TfR in some manner. Such a stabilization could be key in determining the function of the HFE–TfR complex in iron homoeostasis and requires further experimentation.

Since HFE interacts with the TfR in endosomal compartments and specifically reduces iron uptake from Tf, we tested two hypotheses to explain the mechanism of HFE-mediated decreases in iron uptake. We found that HFE does not raise the intracellular pH of the endosome, nor does HFE inhibit the release of iron from Tf. Thus this study excludes the possibility that, in HeLa cells, HFE down-regulates Tf-associated Fe uptake through inhibition of endosomal acidification or disruption of Fe dissociation from Tf within the endosome. Whereas competition of HFE and Tf for binding to the TfR at the cell surface could decrease Tf-mediated iron uptake at very low diferric-Tf concentrations, it does not explain differences in Tf-mediated iron uptake at normal diferric-Tf concentrations. We found that even sub-physiological concentrations of diferric-Tf (100 nM) allow for saturation of the TfR in the presence of HFE [14]. If HFE expression does not change TfR cycling kinetics or Tf uptake, but does decrease Tf-mediated iron uptake, it must do so from the endosome. The data presented here argue that HFE does not mediate this decrease through changes in iron release from Tf. We speculate, therefore, that HFE or the HFE–TfR complex may act directly on the endosomal iron transporter that is responsible for the exit of iron from the endosome into the cytoplasm of the cell. Further studies will focus on this possibility.

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