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# Tranferrins Receptor Association and Endosomal Localization of Soluble HFE Are not Sufficient for Regulation of Cellular Iron Homeostasis

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**Abstract** Iron uptake and storage are tightly regulated to guarantee sufficient iron for essential cellular processes and to prevent the production of damaging free radicals. A non-classical class I MHC molecule, the hemochromatosis factor HFE, has been shown to regulate iron metabolism, potentially via its direct interaction with the transferrin receptor (TfR). In this study, we demonstrate that a soluble $\beta_2$microglobulin-HFE monochain (sHFE) folds with $\beta_2$microglobulin ($\beta_2$m) and associates with the TfR, indicating that the transmembrane and cytoplasmic domains are not necessary for assembly and trafficking through the ER-Golgi network. We also demonstrate human TfR-specific uptake and accumulation of extracellular sHFE by treated cells. The sHFE localized to the endosomal compartment albeit we observed variation in the time taken for endosomal trafficking between different cell types. The sHFE monochain was effective in reducing Tf uptake into cells, however this did not correlate to any changes in TfR or ferritin synthesis, in contrast to the HFE-induced increase and decrease of TfR and ferritin, respectively. These findings of incongruent sHFE activity suggest that either variation in affinity binding of sHFE to TfR prevents efficient modulation of iron-regulated proteins or that HFE has multiple functions some of which may be independent of TfR but dependent on interactions within the endosomal compartment for effective modulation of iron metabolism. J. Cell. Biochem. 91: 1130–1145, 2004. 

**Key words:** Hfe; TfR; hemochromatosis; iron metabolism; trafficking

Hemochromatosis factor (HFE) is a MHC-class I like glycoprotein that assembles with $\beta_2$microglobulin ($\beta_2$m) to form a heterodimeric complex. A mutation in the *HFE* gene results in the autosomal recessive disease hereditary hemochromatosis [Feder et al., 1996]. Several mutations have now been identified, however the majority of cases are due to a cysteine to tyrosine mutation at position 282 of the protein [Feder et al., 1996]. The change abrogates the disulfide bond in the $\alpha$3 domain and prohibits $\beta_2$m binding and assembly of the HFE protein [Feder et al., 1997; Waheed et al., 1997]. The disease is characterized by accumulation of excess iron in tissues, resulting in tissue damage and eventual organ failure, indicating that HFE functions by modulating iron homeostasis. Although the etiology of hereditary hemochromatosis is now known, the role of HFE and the mechanism of its function in hereditary hemochromatosis are poorly understood.

To date, HFE has been localized to various cell types. Immunohistochemical studies have illustrated HFE on the apical plasma membranes of syncytiotrophoblasts [Parkkila et al., 1997a], Kupffer cells and endothelium of the liver [Bastin et al., 1998], in crypt enterocytes of human duodenum [Parkkila et al., 1997b; Bastin et al., 1998; Waheed et al., 1999], in epithelial cell of gastromucosa, on the cell surface of macrophages and monocytes [Parkkila et al., 2000], and in EBV-transformed B cells [Chitambar and Wereley, 2001]. In a variety of transfected cells, HFE was localized to a perinuclear compartment [Gross et al., 1998; Riedel et al., 1999; Ikuta et al., 2000]. This was then

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demonstrated to be the endosomal compartment since within this perinuclear compartment HFE co-localized with TfR or Tf [Gross et al., 1998; Griffiths et al., 2000; Ramalingam et al., 2000].

In placental cells [Parkkila et al., 1997a], HFE-transfected cell lines [Feder et al., 1998; Gross et al., 1998; Salter-Cid et al., 1999], and HFE vaccinia-infected cells [Ben-Arieh et al., 2001] HFE is associated with the transferrin receptor (TfR). The crystal structure indicates that HFE binds TfR at the $\alpha_1$ and $\alpha_2$ face of the protein and that this interaction induces many changes in TfR conformation [Bennett et al., 2000].

Observations of HFE binding to TfR were quickly followed by data demonstrating that HFE modulates expression of proteins involved in iron metabolism. The presence of excess HFE increased expression of TfR and decreased ferritin, the iron storage protein [Gross et al., 1998; Corsi et al., 1999; Roy et al., 1999; Ramalingam et al., 2000; Arredondo et al., 2001; Feeney and Worwood, 2001; Vahdati-Ben Arieh et al., 2003]. In addition, HFE increased iron regulatory protein (IRP) activity [Corsi et al., 1999; Riedel et al., 1999; Roy et al., 2002], the likely mechanism by which it regulates TfR and ferritin levels. HFE has also been reported to affect iron (Fe) or Fe-Tf uptake in a variety of cells including HeLa [Feder et al., 1998; Riedel et al., 1999; Salter-Cid et al., 1999], Caco-2 [Arredondo et al., 2001], HEK293 [Feeney and Worwood, 2001], and HLF [Ikuta et al., 2000]. The process by which HFE affects cellular iron uptake remains unclear. HFE has been found to decrease the binding affinity of Tf to its receptor TfR [Feder et al., 1998; Lebron et al., 1999], however this is unlikely to be of physiological significance since the estimated tenfold reduction in Tf–TfR binding affinity is likely to be overcome by the high concentrations of serum Tf. Alternatively, it has been suggested that HFE affects TfR internalization and/or recycling. Various studies have attempted to address the question of HFE regulation of TfR recycling but the data is varied [Salter-Cid et al., 1999; Ikuta et al., 2000; Waheed et al., 2002]. Thus, the mechanisms by which HFE exerts its biological functions remain unresolved.

Soluble ligands have been demonstrated to play a regulatory role in physiological processes [Montero-Julian, 2001; Oppenheim, 2001] and some are in development as therapeutic tools [Gardnerova et al., 2000; Cappello et al., 2002; Leclerc, 2002]. In particular, Tf and TfR based therapies have been investigated as a drug delivery system capable of crossing the blood brain barrier [Li and Qian, 2002]. Likewise a soluble HFE protein may be useful as a therapeutic agent either in treatment of HFE-related human hemochromatosis or as a drug delivery tool. In this study, we constructed a soluble $\beta_2$m.HFE monochain and investigated its biochemical properties and its role in modulation of iron metabolism. Furthermore, we utilized the sHFE monochain to compare and contrast the assembly, trafficking, TfR association, and cellular localization of HFE, MuHFE (C282Y), and sHFE, in an attempt to ascertain some of the structural components necessary for HFE function and to gain a better understanding of HFE biology.

## EXPERIMENTAL

### Cell Lines and Antibodies

The following cell lines, HEK293, HeLa, Jurkat, VAD12.79, B16, and FO1 were grown in Dulbecco's modified Eagle's media (DMEM, Invitrogen Corporation, Groningen, The Netherlands) supplemented with 10% fetal calf serum and 10 mM glutamine (Biological Industries, Bet Ha'emek, Israel). Transfected cell lines (HEK293.HFE, HEK293.MuHFE, HEK293.h$\beta_2$m.sHFE, HEK293.h$\beta_2$m.sMuHFE, HEK293.h$\beta_2$m.sHFE.gfp, HEK293.h$\beta_2$m.sMuHFE.gfp, and VAD 12.79/hTfR) were selected with 1.2 mg/ml of G418 and maintained in 0.5 mg/ml G418 in supplemented DMEM.

The following antibodies were utilized: 2F5 (mouse anti-human HFE specifically recognizes human HFE-$\beta_2$m heterodimer); 8C10 (mouse anti-human HFE recognizes human HFE-$\beta_2$m heterodimer or TfR associated HFE-$\beta_2$m); HFE CT (rabbit anti-human HFE antisera; [Ben-Arieh et al., 2001]; B2.62.2 (mouse anti-human $\beta_2$m; kind gift from Prof. Lemonnier, Institute Pasteur, Paris, France; [Perarnau et al., 1988]; 9E10 (anti-myc; ATCC Rockville, MD); V1-10 (anti-human TfR, a kind gift of Dr. Z. Eshhar, The Weizmann Institute of Science, Rehovot, Israel); H68.4 (anti-TfR cross-reactive with both mouse and human TfR, Zymed Laboratories, Inc., San Francisco, CA); Cy5-conjugated goat anti-mouse Ig, Cy3-conjugated anti-rabbit Ig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and rabbit anti-Cab45 (a kind gift from Dr. Gerardo

Lederkremer, Tel Aviv University, Israel; [Scherer et al., 1996]).

### DNA Expression Constructs

Human HFE and MuHFE (C282Y) cDNA cloned into pcDNA 3.1 were a kind gift from Dr. J. Feder, Progenitor, Inc., CA [Feder et al., 1997]. The h$\beta_2$m.sHFE cDNA monochain was constructed using gene splicing by overlap extension (SOE) [Horton et al., 1989]. Figure 1A is a schematic representation of the SOE method, briefly the first step involved amplification of the human $\beta_2$m cDNA and human HFE using standard reverse transcriptase (RT)-PCR methods and the following primers: the $\beta_2$m-forward primer (ggcacgaagatctgagatgtctcgctccgtgg); the $\beta_2$m-reverse primer encoded for the carboxy-terminus of *h$\beta_2$m* gene with additional sequence encoding for a glycine/serine ($G_4S$) peptide linker (gccgccaccggatccacctccgcccgagccgccacctcccatgtctcgatcccacttaac); HFE-forward primer contained sequences encoding for the glycine/serine peptide linker followed by sequences corresponding to the start of the mature form of the HFE protein (ggctcgggcggaggtggatccggtggcggcggttcccgcttgctgcgttcacac); the HFE-reverse primer contained sequences corresponding to the carboxy terminus of the $\alpha_3$ region of the HFE protein (gccagacggctcgagctcccagatcacaatgaggg).

Once the two parental cDNA segments were generated, the h$\beta_2$m.sHFE was spliced by overlap extension, depending on complimentary hybridization of the glycine/serine sequence. The PCR product was cloned upstream and in frame with 3′His.Myc epitope tags within pcDNA3.1/Myc-His vector (Invitrogen Corporation, Carlsbad, CA). The h$\beta_2$m.sMuHFE cDNA was generated in a similar manner. The h$\beta_2$m.sHFE.gfp and h$\beta_2$m.sMuHFE.gfp expression vectors were generated by introducing the green fluorescence protein (eGFP) cDNA downstream of the monochain cDNA (Fig. 1A). The correct sequences of the cloned PCR products were confirmed by automated sequencing (BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

### Stable Transfection

Cells were stably transfected with mammalian expression vectors encoding for human HFE, MuHFE, $\beta_2$m.sHFE, $\beta_2$m.sMuHFE, $\beta_2$m.sHFE.gfp, or $\beta_2$m.sMuHFE.gfp co-expressing the neomycin resistance gene. The DNA was transfected using the calcium phosphate method previously described [Fromm et al., 1998]. Transfected clones were selected with G418 resistance; expanded; and then analyzed for expression of the gene of interest either by FACS analysis or Western blot analysis.

### Protein Purification

The $\beta_2$m.sHFE protein was purified from conditioned media from the transfected cell line. Conditioned media was incubated with Ni–NTA His Bind resin (Novagen, Madison, WI) at 4°C for 18 h. The resin was washed with 20 mM Tris buffer (20 mM Imidazole, 500 mM NaCl; pH 7.5), loaded on a column and the protein eluted with 20 mM Tris buffer containing 500 mM Imidazole and 250 mM NaCl (pH 7.5). The eluted protein was dialyzed against PBS and the protein yield and purity determined by standard Bradford protein assay and SDS–PAGE analysis.

### Immunocytochemistry

Cells were seeded on cover slips coated with poly L-Lysine (Sigma Chemical Co., St. Louis, MO) in 24-well plates. Some sub-confluent cultures were incubated with 0.1% BSA–DMEM for 30 min followed by the addition of hTf-Rhodamin (5 μg/ml, Molecular Probes, Eugene, OR) for 15 min. The cells were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 20 min. Following extensive rinsing with 50 mM glycine and permeabilization with triton (0.3% in PBS) the cells were blocked for 20 min with a 1:10 dilution of normal goat serum. The cells were then incubated with the primary antibody (8C10, 9E10, V1-10, Cab45, or FITC-conjugated anti-human CD71) for 1 h followed by incubation with secondary Ab (Cy5-conjugated goat anti-mouse Ig or Cy3-conjugated anti-mouse Ig) for 1 h. The cells were washed extensively following incubations with universal buffer (10 mM Tris, 150 mM NaCl pH 7.5) and PBS. The glass coverslips were mounted on slides with MOWOIL 4–88 reagent (Calbiochem, Darmstadt, Germany). The cells were analyzed by a confocal laser scan microscope (410 Zeiss, Oberkochen, Germany), with the following configuration: 25 mW Argon–Krypton laser (488 nm lines), a 40 × NA/1.2 C-apochromat water-immersion lens or a 100Xna/1.3 plan neofluar oil immersion lens.

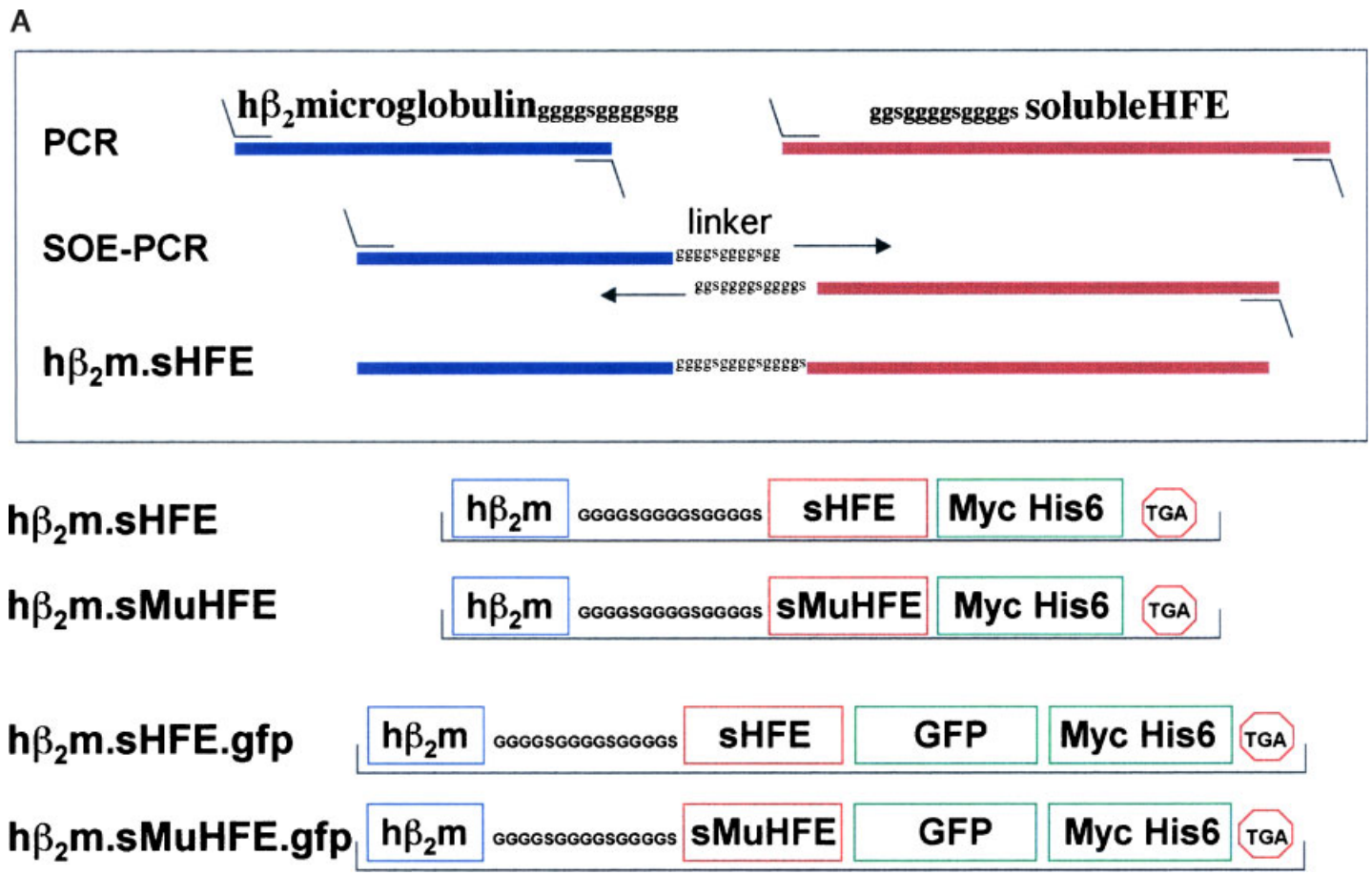



**Fig. 1.** Characterization of hemochromatosis factor (HFE), mutant HFE, and soluble HFE in transfected cells. **A**: Schematic diagram representing generation of human $\beta_2$m.sHFE monochain by splice overlap extension (SOE) PCR (''Materials and Methods''). Likewise, $\beta_2$m.sMuHFE, $\beta_2$m.sHFE.gfp, and $\beta_2$m.sMuHFE.gfp monochains were generated. The cDNA encoding for the gfp protein was cloned in frame upstream of the Myc epitope. **B**: Western blot analysis of HEK293 cells expressing either HFE (2.HFE), MuHFE (C282Y; 2.MuHFE), $\beta_2$m.sHFE (2.sHFE), $\beta_2$m.sMuHFE (2.sMuHFE), $\beta_2$m.sHFE.gfp (2.sHFE.gfp), or $\beta_2$m.sMuHFE.gfp (2.MuHFEgfp). Cell lysates (10 μg protein) were separated on a 12% acrylamide gel and the protein detected either with HFE-CT (αHFE cytoplasmic tail) or with 9E10 (αMyc) antibodies. **C**: Western blot analysis of conditioned media (120 μl) collected from the above cell lines. Secreted protein in the media was detected with 9E10 (αMyc) antibody. **D**: Cell lysates from metabolically labeled HEK293 cells expressing $\beta_2$m.sHFE (sH), $\beta_2$m.sMuHFE (sMH), $\beta_2$m.sHFE.gfp (sHg), or $\beta_2$m.sMuHFE.gfp (sMHg) were immunoprecipitated with 9E10 (αMyc) or 2F5 (αHFE-$\beta_2$m heterodimer) antibodies. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

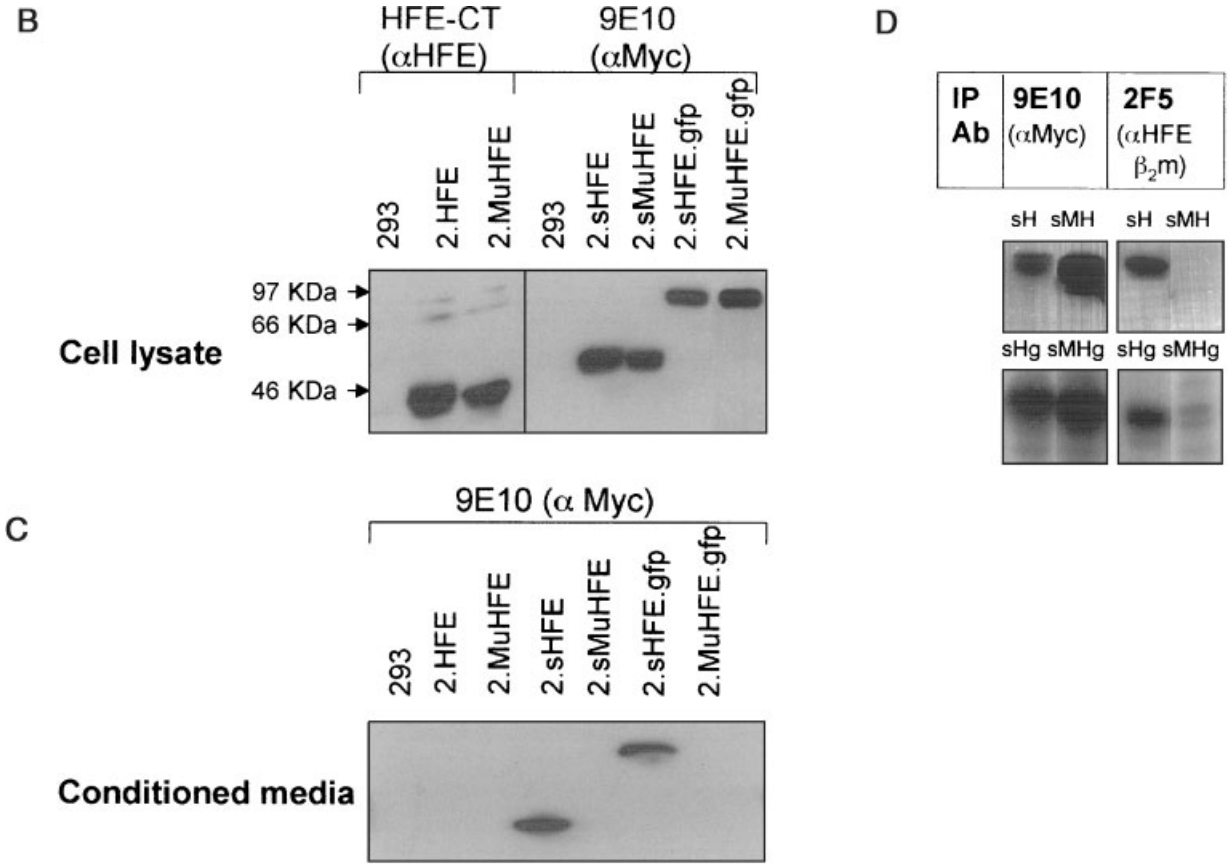



**Fig. 1.** (*Continued*)

### Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis

Standard procedures were followed. Cells were starved in methionine-free DMEM supplemented with 5% FCS for 1–2 h. Radiolabeled methionine (Express $^{35}S^{35}S$, Perkin–Elmer Life Sciences, Inc., Boston MA) was added to the cells (100 μCi/ 90 mm plate) for 40–60 min. Cells were lysed in lysis buffer (0.5% NP40, 50 mM Tris, 150 mM NaCl pH 7.5 with complete protease inhibitors [Boehringer Mannheim, GmbH, Germany]) at 4°C for 30 min. For pulse-chase experiments, supplemented DMEM was added with excess methionine (5 μM) for the required time prior to lysis. Lysates of equivalent total protein (0.2–1 mg) were incubated with antibody bound protein G/A for a minimum of 2 h at 4°C, the immunoprecipitated fractions were treated with endoglycosidase H (New England Biolabs, Inc., Beverly, MA) where indicated. Samples were separated on SDS–PAGE gels, which were either dried or the polypeptides were electrophoretically transferred to Hybond C membrane (Amersham Pharmacia Biotech UK Ltd., Buckingham, England) for Western analysis. Dried gels were exposed to X-OMAT AR X-ray films (Eastman Kodak Company, Rochester, NY).

For Western blot analysis, membranes were blocked with 5% skim milk in PBS, then probed with the relevant antibodies (either H68.4, 9E10, or HFE-CT) for 1 h followed by detection with the appropriate horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence substrates (ECL Amersham Pharmacia Biotech UK Ltd., Buckingham, England).

### Transferrin Uptake Assay

Cells were washed in PBS, resuspended at $5 \times 10^5$ in media and incubated with different concentrations of FITC-conjugated differric Tf (100–0.05 μg/ml; Molecular Probes, Eugene, OR) alone or with the addition of 0.5 μM purified sHFE monochain protein. Tf binding and

uptake were then measured by determining the cell fluorescence by FACS analysis.

## RESULTS

### Expression of HFE, Mutant HFE, and Soluble HFE in HEK293 Cells

HEK293 cells (abbreviated to 2 when referring to transfected cell lines) transfected with cDNA encoding either HFE (2.HFE), mutant HFE (2.MuHFE; Cys282Tyr), soluble $\beta_2$m.HFE monochain (2.sHFE), soluble $\beta_2$m.HFE.gfp monochain (2.sHFE.gfp), or soluble mutant $\beta_2$m.HFE.gfp monochain (2.sMuHFE.gfp) were analyzed for protein expression by Western blot analysis of cell lysates (Fig. 1B). HFE (45kD), mutant HFE (45kD), sHFE monochain (55kD), sHFE.gfp monochain (85kD), and sMuHFE.gfp monochain (85kD) were all expressed in the HEK293 cells. However, only sHFE and sHFE.gfp proteins and not sMuHFE and sMuHFE.gfp were secreted into the culture media (Fig. 1C). These data demonstrate that only the sHFE and the sHFE.gfp monochains assemble sufficiently to allow exit out of the ER-Golgi network and secretion. In contrast, the soluble form of the MuHFE is not transported via the secretory pathway. To further investigate sHFE monochain assembly with $\beta_2$m, the transfected cell lines were metabolically labeled and immunoprecipitated with antibodies that either recognize the myc epitope (9E10), that is all forms of soluble HFE, or the 2F5 antibody which specifically recognizes HFE-$\beta_2$m conformed heterodimers. The soluble proteins were all immunoprecipitated with the anti-myc antibody (9E10), however only sHFE and sHFE.gfp proteins and not the sMuHFE counterparts were recognized by the 2F5 antibody (Fig. 1D). These data show that the sHFE monochains are able to fold properly with the covalently bound $\beta_2$m while the sMuHFE monochains do not. The covalent linkage of $\beta_2$m to sHFE via a spacer arm as well as the Myc epitope, His tag and the gfp protein do not affect the 2F5 epitope.

### Stability and TfR Association of HFE and Soluble HFE

Next we analyzed the kinetics of intracellular assembly and stability of HFE and sHFE complexes (Fig. 2). Radiolabeled HFE-$\beta_2$m heterodimers were immunoprecipitated from 2.HFE cells at all time points tested (Fig. 2A). The mature form of the protein increased following 2 h of chase and was constant from 4 to 22 h of chase, indicating that HFE complexes are highly stable. The decrease in intracellular radiolabeled sHFE with chase time correlated with the increase in secreted

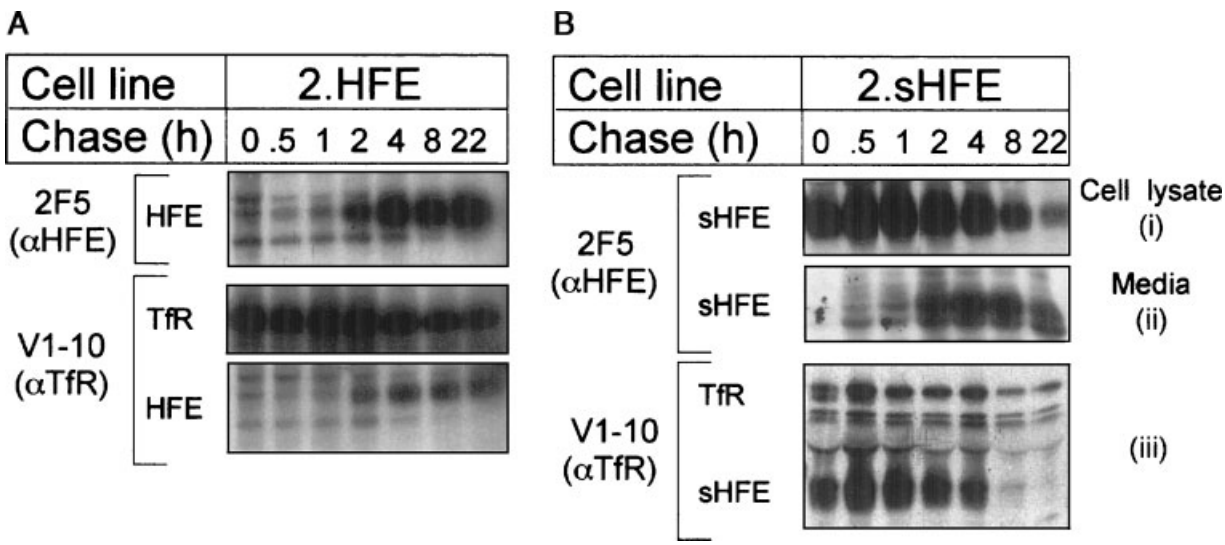



**Fig. 2.** Intracellular assembly and stability of HFE and sHFE complexes. Cell lysates from metabolically labeled and chased HEK293 expressing either HFE (2.HFE; **A**) or sHFE monochain (2.sHFE; **B**) were immunoprecipitated with 2F5 (αHFE-$\beta_2$m; (A) **top panel**, (B) [i]) or V1-10 (αTfR; (A) **bottom panel**, (B) [iii]) and treated with endoH. Immunoprecipitates were separated on SDS–PAGE. Conditioned media from metabolically labeled and chased 2.sHFE cells (1 ml) were also immunoprecipitated with 2F5 (B [ii]).

sHFE found in the culture media (Fig. 2B [i] and [ii]). The secretion of sHFE was first visible by 30 min and the majority of labeled sHFE protein was secreted into the culture media by 8 h. The secreted sHFE was detected after 22 h of chase indicating that the conformed protein was stable in the culture media, furthermore we have determined that the sHFE monochain is stable for prolonged time at 4°C (data not shown).

To address the question of TfR association, TfR was immunoprecipitated with V1-10 and the associated HFE or sHFE were analyzed. Figure 2 demonstrates that HFE (A, bottom panel) and sHFE (B [iii]) co-immunoprecipitated with TfR confirming association of HFE, $\beta_2$m, and TfR in a complex. The co-immunoprecipitation of sHFE monochain with TfR demonstrates that the conformation of the TfR binding site is not significantly affected by the lack of the TM and cytoplasmic region of HFE or by the existence of the Myc epitope and His tag. In addition, we observed co-immunoprecipitation of the sHFE.gfp monochain with TfR (data not shown) also indicating that the gfp hybrid protein does not affect the TfR binding site, whereas neither MuHFE or sMuHFE were detected in association with TfR (data not shown). These data indicate that only conformed HFE is able to bind to TfR and that this binding is independent of the transmembrane and cytoplasmic domains. However, while the half-life of TfR bound HFE is approximately 22 h (Fig. 2A); [Vahdati-Ben Arieh et al., 2003] the half-life of TfR bound sHFE was approximately 4 h (Fig. 2B) indicating that most of the sHFE is secreted to the media and does not recycle with TfR. These data are supported by confocal analysis of sHFE localization (Fig. 3).

Figure 3A demonstrates co-localization of HFE with Tf, a marker of recycling endosomes. Although we observed some co-localization of sHFE with Tf, the majority of sHFE appeared to be localized in a compartment other than recycling endosomes. This observation was consistent for sHFE detected with an antibody and the auto-fluorescing sHFE.gfp hybrid. The sMuHFE.gfp protein demonstrated diffuse staining not specific to the endosomes.

To further localize the sHFE protein, we double stained cells with an antibody directed against a Golgi marker, Cab45, and 8C10 antibody to detect $\beta_2$m-associated HFE or sHFE. Although there was some co-localization of membrane HFE with Cab45 as expected due to newly synthesized protein, this was a minor fraction of the total HFE protein. In contrast, both sHFE and sHFE.gfp co-localized mainly with Cab 45, suggesting that the bulk of sHFE protein is in the Golgi.

### Extracellular Binding of sHFE

The data described so far, demonstrate that both HFE and sHFE associate with TfR in the ER, however most of the sHFE did not recycle with TfR in endosomal compartments in the transfected cells. Thus, it was not clear whether sHFE retains the ability to reassociate with TfR at the cell surface. To test this issue both human and mouse cell lines were utilized as target cells for radiolabeled sHFE. Since our previous data (unpublished observations) demonstrated lack of high-affinity binding of hHFE to mouse TfR, we postulated that the sHFE should bind to human cells but not to mouse cells. Moreover, we have generated a mouse cell line that expresses human TfR (VAD12.79/hTfR) to control for the specificity of the binding reaction. Human cell lines (FO1, HeLa), mouse cell lines (VAD12.79, B16), and the mouse cell line expressing human TfR (VAD12.79/hTfR) were incubated with radiolabeled [$^{35}$S]h$\beta_2$m.sHFE for 1 or 20 h. Cell lysates were immunoprecipitated to detect the presence of radiolabeled sHFE in association with TfR. A representative experiment is shown in Figure 4. Following 1 h of treatment, all cell lines demonstrated the presence of labeled sHFE, however the signal associated with mouse cells was weaker than that associated with human cells. In the human cell lines FO1 and HeLa the amount of labeled sHFE increased dramatically at 20 h. In contrast, the mouse cell lines expressing only the mouse TfR showed a dramatic decrease in the amount of immunoprecipitable sHFE. Interestingly, this decrease did not occur in the mouse cell line expressing human TfR (12.79 hTfR) implying that binding and possibly internalization and accumulation depend on the expression of human TfR. Similar data were observed with immunoprecipitation with the 2F5 Ab (data not shown). The recognition of HFE complexes by 2F5 suggests that TfR and HFE dissociate in the intracellular compartments following internalization. These results demonstrate that sHFE

is able to bind preferentially human TfR, and accumulate in the cells.

### Localization of sHFE Following Extracellular Binding

In order to confirm internalization of sHFE in treated cells and to identify the compartment(s) in which sHFE is located, we analyzed sHFE treated cells by confocal microscopy. Figure 5A demonstrates sHFE in HeLa and HEK293 cells following treatment with sHFE-conditioned media in comparison to untreated cells. The majority of sHFE taken up by HeLa and HEK293 cells was co-localized with human Tf indicating its presence in the endosomal compartment (Fig. 5B, top panel). The time required for endosomal localization varied between cell types, HEK293 cells demonstrating a slower progression (4 h vs. 30 min for HeLa). Overall, these findings indicate that the transmembrane and cytoplasmic domains are not essential for cellular internalization and localization of HFE in the recycling endosomes.

### Soluble HFE Inhibits Holo-Tf Uptake but Does not Modulate TfR and Ferritin Synthesis

To determine the biological effects of sHFE we initially assessed the ability of sHFE to affect holo-Tf uptake by cells. HeLa cells were incubated with either FITC conjugated diferric-Tf

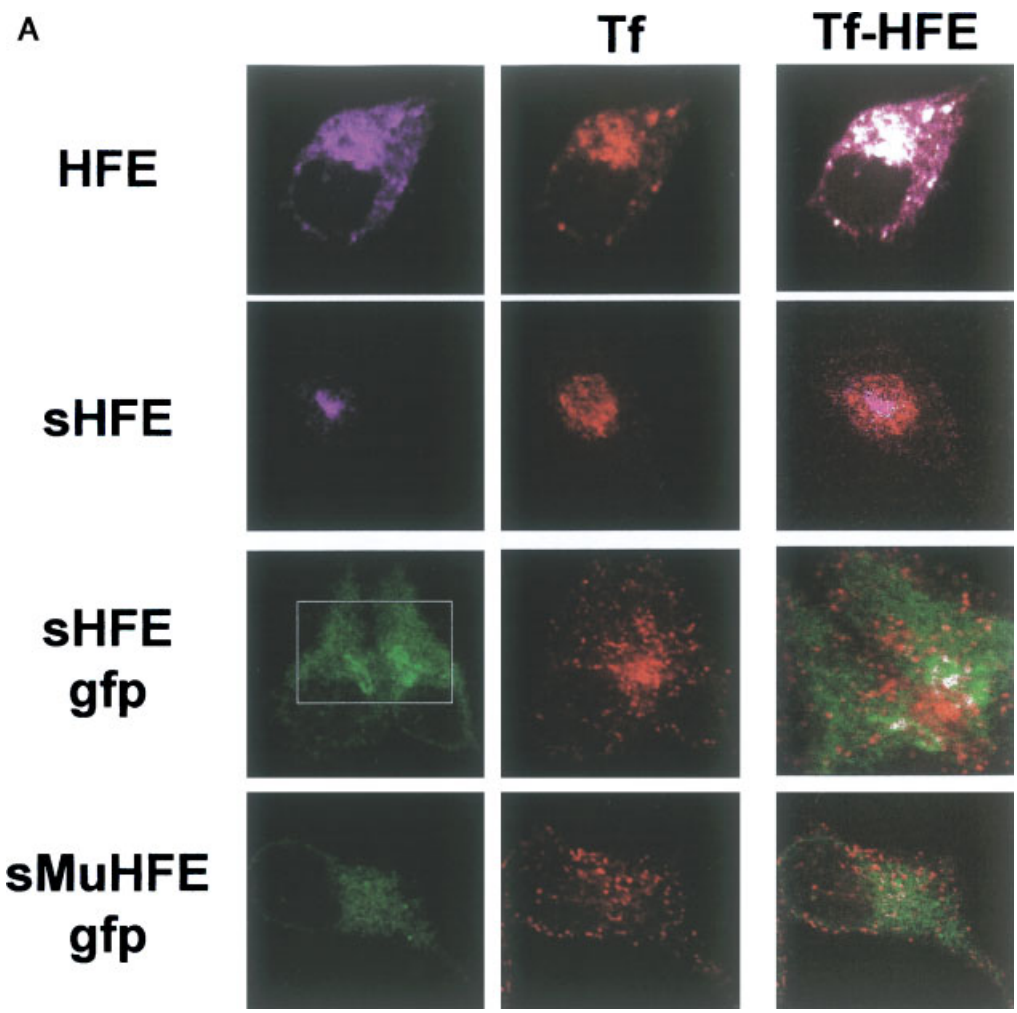



**Fig. 3.** Cellular localization of HFE, $\beta_2$m.sHFE, $\beta_2$m.sHFE.gfp, and $\beta_2$m.sMuHFE.gfp in transfected cells. Transfected cells were fixed and stained with the following antibodies: 8C10 ($\alpha$HFE-$\beta_2$m) or $\alpha$Cab45 (Golgi marker). The secondary antibodies used were Cy5-conjugated goat anti-mouse Ig or Cy3-conjugated anti-mouse Ig. **A**: Cells were treated with Rhodamine conjugated-Tf for 15 min prior to fixation and staining. Tf (red) was co-localized with HFE (purple), sHFE (purple), sHFE.gfp (green), or sMuHFE.gfp (green). **B**: Co-localization of the Golgi marker (Cab45) with HFE (purple), sHFE (purple), and sHFE.gfp (green). Co-localization of the fluorescent colors as determined by computational program is indicated by white. White boxes indicate magnification area for co-localization. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

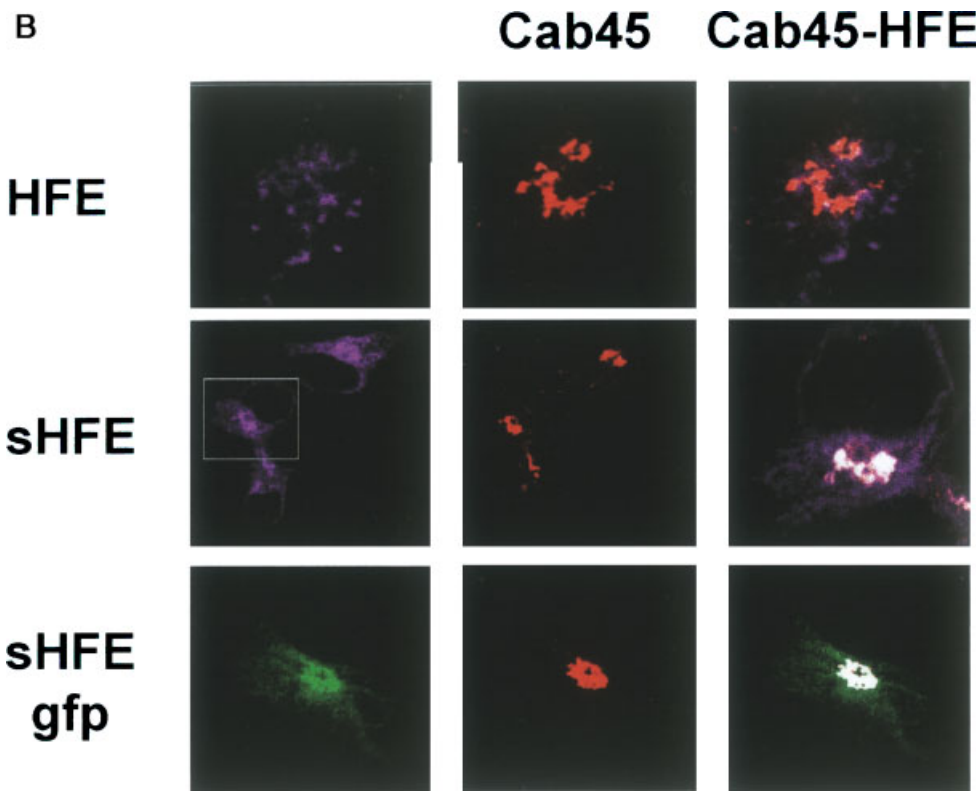



**Fig. 3.** (*Continued*)

alone or with 0.5 μM of purified sHFE protein. Figure 6 demonstrates dose-dependent uptake of Tf–FITC by HeLa cells that reached a plateau at 50 μg/ml of TF–FITC. Co-incubation of sHFE resulted in a significant (ANOVA; $P < 0.05$) decrease in Tf–FITC accumulation by cells, first observed at Tf–FITC concentration of 25 μg/ml. These results indicate that

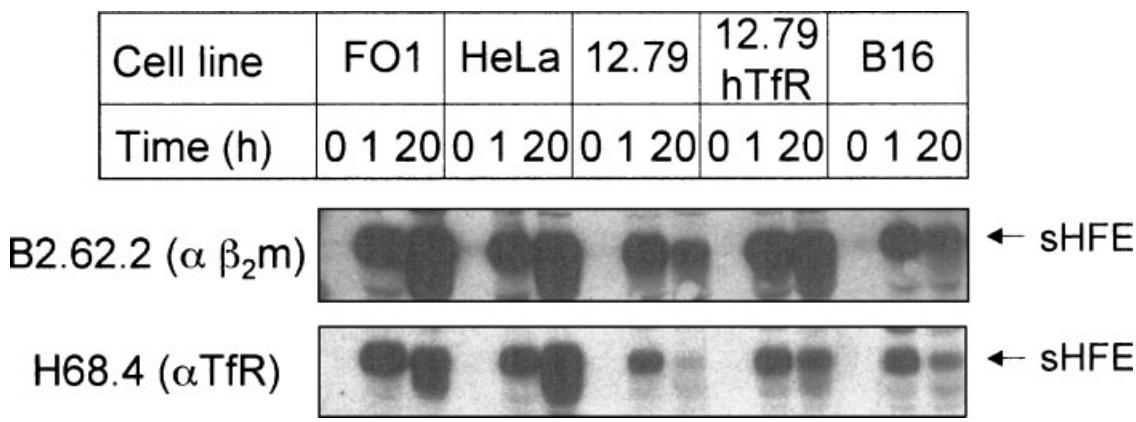



**Fig. 4.** Extracellular uptake of $\beta_2$m.sHFE preferentially by cells expressing the human TfR receptor. Human cell lines FO1 and HeLa and mouse cell lines VAD12.79, VAD12.79/hTfR (expressing human TfR), and B16 were incubated with conditioned media containing [$^{35}$S]-labeled $\beta_2$m.sHFE for 0, 1, or 20 h. The cells were then lysed and immunoprecipitated with B2.62.2 ($\alpha\beta_2$m) or H68.4 (αTfR) antibodies. The **top panel** represents labeled cell-associated sHFE and the **bottom panel** represents sHFE co-immunoprecipitated with TfR (human or mouse).

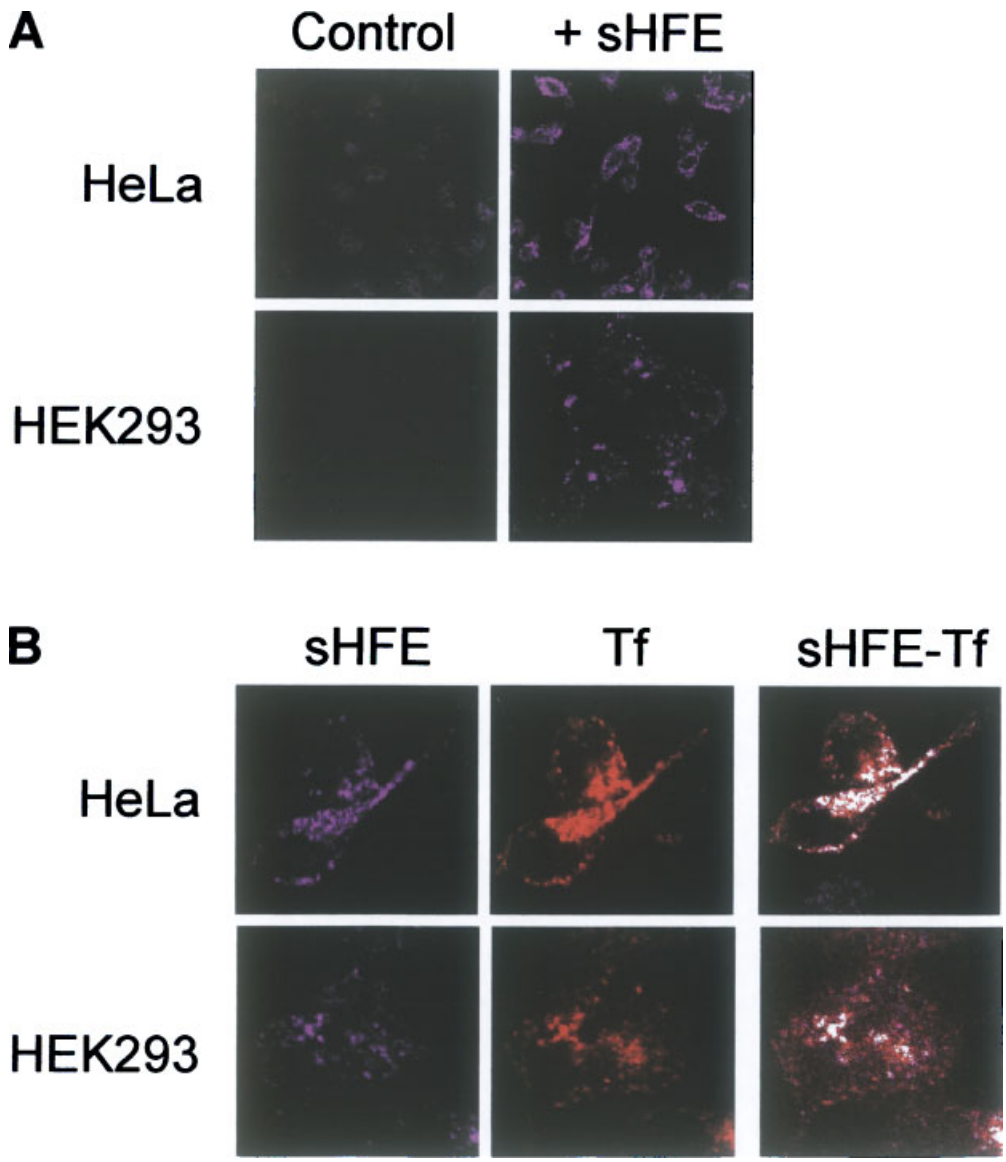



**Fig. 5.** Visualization of $\beta_2$m.sHFE uptake by cells and its localization in the recycling endosomes. **A**: Human cell lines HeLa and HEK293 were incubated either with control media or $\beta_2$m.sHFE-conditioned media (20 h). Cells were fixed and $\beta_2$m.sHFE was detected with 9E10 (anti-Myc Ab) and Cy5-conjugated goat anti-mouse Ig (purple). **B**: To localize the $\beta_2$m.sHFE cells were treated as above, then treated with fresh sHFE-containing media (2 h HeLa or 24 h HEK293) and Rhodamine conjugated-Tf (30 min; red) prior to fixation and staining. Cells were stained with the 9E10 (αMyc; purple). Co-localization of the fluorescent colors as determined by computational program is indicated by white. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the sHFE monochain is effective in regulating Tf uptake, perhaps by competition for binding with TfR or regulation of TfR recycling.

Although the mechanism of HFE biological activity is not well understood, previously it has been demonstrated that excess HFE leads to increased TfR and decreased ferritin, suggesting a state of reduced intracellular iron pools. To further investigate the biological activity of sHFE we determined its effect on the iron pools in the transfected cells and in cells treated with sHFE. In order to rule out any clonal variation among the transfected clones, the parental HEK293 cells as well as five clones of HFE or MuHFE transfectants, and four clones of sHFE transfectants were analyzed in parallel for the expression of TfR and ferritin (Fig. 7A). The data clearly show that the level of TfR was elevated in all five clones expressing HFE, while no such effect was observed in cells expressing

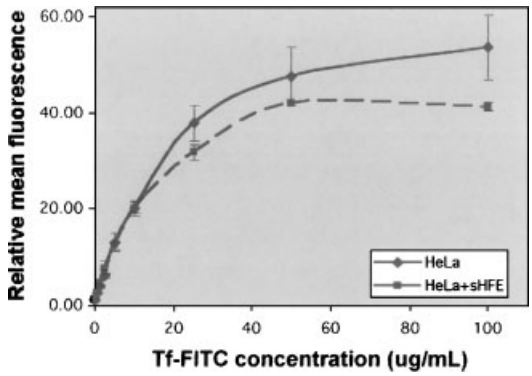
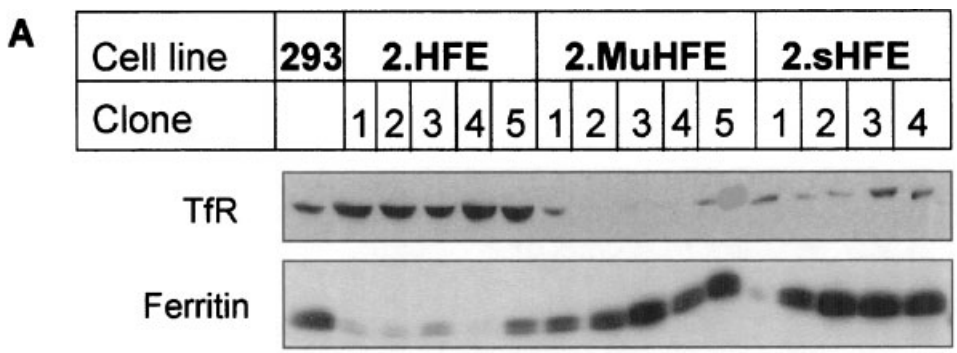



**Fig. 6.** $\beta_2$m.sHFE significantly affects Tf uptake by cells. HeLa cells ($5 \times 10^5$) were incubated with various concentrations of FITC-conjugated diferric Tf (0.05–100 μg/ml) either in the presence or absence of 0.5 μM purified $\beta_2$m.sHFE for 30 min at 37°C. Tf uptake was detected by FACS analysis and the data is presented as the increase in mean fluorescence of test samples compared to control cells (no Tf–FITC; mean ± SEM, n = 3).

MuHFE or sHFE (Fig. 7A). Furthermore, the five HFE expressing clones demonstrated reduced ferritin synthesis, while HEK293 cells expressing the mutant or the sHFE did not show any significant decrease in the levels of ferritin synthesis.

Likewise, when we treated various cell lines, including HEK293, HeLa, Jurkat, and Daudi with sHFE protein no consistent differences were detected in the level of newly synthesized TfR or ferritin between untreated and treated cells (Fig. 7B). Furthermore, concentrations of up to 1 μM of purified sHFE (Fig. 7C) neither increased TfR nor decreased ferritin in HeLa cells, in contrast to the effects of the wild type membrane HFE in HEK293 transfected cells (Fig. 7A) or previously reported HeLa cells [Corsi et al., 1999].



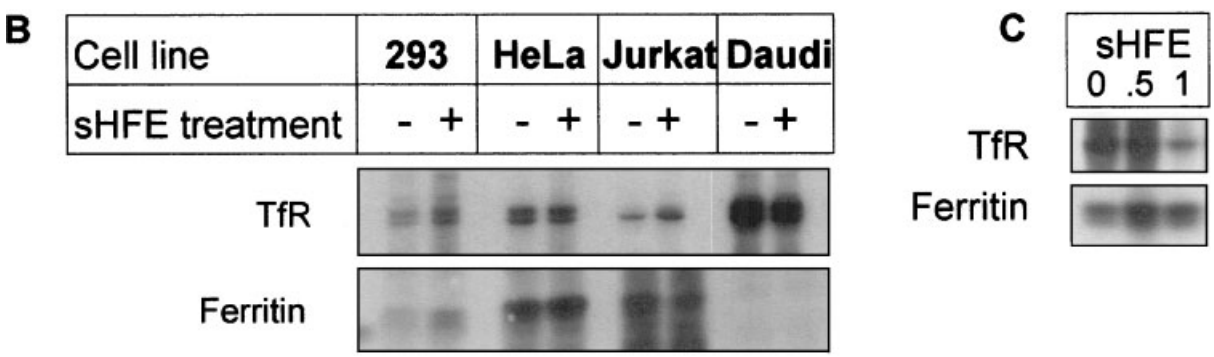




**Fig. 7.** Regulation of TfR and ferritin by HFE but not $\beta_2$m.sHFE in transfected and/or treated cells. **A**: Western blot analysis of TfR (detected with H68.4 Ab) in cell lysates (80 μg of protein; **top panel**) and ferritin immunoprecipitates of metabolically labeled cells (**bottom panel**) from HEK293 cells and individual clones expressing HFE (2.HFE clones 1–5), MuHFE (2.MuHFE clones 1–5), or $\beta_2$m.sHFE (2.sHFE clones 1–4). **B**: HEK293, HeLa, Jurkat, and Daudi cells were treated with control media (−) or $\beta_2$m.sHFE media (+) for 20 h and then were metabolically labeled, lysed, and immunoprecipitated with V1-10 (αhTfR, **top panel**) and αferritin (**bottom panel**) antibodies. **C**: HeLa cells were treated with increasing concentrations of purified $\beta_2$m.sHFE (0.5 and 1 μM) for 20 h and then the cells were metabolically labeled and immunoprecipitated with V1-10 (αhTfR, **top panel**) and αferritin (**bottom panel**) antibodies.

These results suggest that despite the intracellular association of sHFE and TfR in sHFE transfected cells or the cell surface association of sHFE to TfR in treated cells, and its subsequent internalization to endosomes, these biochemical events are insufficient to mediate changes in intracellular iron.

## DISCUSSION

In this study, we constructed a soluble HFE monochain and characterized its cellular trafficking, localization, and TfR-association in comparison to HFE in an attempt to elucidate the structure–function relationship of HFE. We determined the extracellular binding, uptake, localization, and function of sHFE monochain in a variety of cell lines in order to investigate the potential development of sHFE as a therapeutic tool. We developed a $\beta_2$m covalently linked soluble HFE monochain to ensure that $\beta_2$m was not limiting in this system since previously $\beta_2$m deficiency has been implicated in alteration of HFE function [Waheed et al., 2002] or exacerbation of HFE-induced iron overload as demonstrated in the compound HFE-$\beta_2$m knockout mice [Levy et al., 2000].

We have also constructed a soluble MuHFE monochain (containing the C282Y mutation) and found it to behave similarly to the membrane mutant HFE (C282Y). In accordance with previous data [Feder et al., 1997; Parkkila et al., 1997a; Waheed et al., 1997, 1999] sMuHFE monochain did not assemble with the covalently linked $\beta_2$m (Fig. 1D). The sMuHFE proteins did not mature and traffic to the cell surface to be secreted (Fig. 1C), instead they remained intracellular, probably destined for degradation as determined by the quality control mechanisms of the ER [Parodi, 2000; Fewell et al., 2001]. Consequently, sMuHFE proteins were dysfunctional and did not alter the cellular iron metabolism as indicated by the absence of modulation of TfR and ferritin as seen for HEK293 cells expressing MuHFE (Fig. 7 and data not shown).

As in previous reports we observed that HFE assembled with $\beta_2$m and was co-immunoprecipitated with TfR (Fig. 2A). We were able to co-immunoprecipitate both immature and mature forms of HFE with TfR (Fig. 2A) suggesting that some association occurs in the ER as is the case in HeLa cells [Gross et al., 1998]. However, most of the TfR was bound to mature HFE indicating that the majority of the TfR bound HFE is outside of the ER network. In fact, confocal microscopy data demonstrated that a high percentage of HFE was localized with TfR in the recycling endosomes as determined by co-localization with Tf (Fig. 3A). This is similar to previous observation of HFE–TfR or HFE–Tf co-localization in other transfected cell types [Gross et al., 1998; Griffiths et al., 2000; Ramalingam et al., 2000; Davies et al., 2003]. In addition to endosomal localization we also observed abundant HFE heterodimers on the cell surface as detected by FACS analysis (data not shown; [Vahdati-Ben Arieh et al., 2003], these heterodimers may either traffic from the ER-Golgi network alone or they may be present as a result of HFE–TfR dissociation either at the Golgi or in endosomal compartments as previously proposed [Lebron et al., 1998].

The precise function of HFE remains unclear; nevertheless it's implicated in the induction of an iron-deprived state in cells over-expressing HFE (macrophages being an exception; [Montosi et al., 2000; Drakesmith et al., 2002], since these cells respond by increasing TfR and decreasing ferritin [Corsi et al., 1999; Riedel et al., 1999; Roy et al., 2000; Arredondo et al., 2001; Waheed et al., 2002]. This is precisely what we observed in HEK293 cells over expressing HFE, an increase in both TfR content and synthesis and decrease in ferritin synthesis (Fig. 7). To date several mechanisms have been proposed for the modulation of intracellular iron by HFE. These include HFE reduction of TfR affinity for Tf [Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998] and HFE regulation of TfR uptake and/or recycling [Salter-Cid et al., 1999; Ikuta et al., 2000]. Our data suggest that HFE may regulate TfR mediated cellular iron uptake by retention of TfR inside the cell. While we observed that cells over-expressing HFE had increased TfR (total protein) and that this corresponded to increased synthesis (data not shown), we could not consistently detect changes in cell surface expression of TfR (data not shown) suggesting one of the two things: (1) HFE acts to retain TfR in the ER-Golgi network or (2) HFE retains TfR in the endosomal compartments and interferes with TfR recycling. In our pulse-chase studies we did not observe any difference in TfR maturation rate between cells over-expressing HFE and the parental cells, similar observations were made by Salter-Cid et al. [1999] studying HeLa

transfectants, indicating that HFE does not affect TfR trafficking to the cell surface. Hence, it is likely that HFE retains TfR in the endosomal compartment.

Like HFE, the $\beta_2$m-sHFE monochain we constructed properly folded with the covalently-linked $\beta_2$m as detected by a conformationally dependent antibody, suggesting that the transmembrane domain and cytoplasmic tail are not essential for proper folding and assembly of the protein. The sHFE monochain trafficked through the ER-Golgi network and was secreted into the culture media, in which it was stable. In addition, sHFE associated with TfR (Fig. 2B). However, in comparison to HFE the half-life of TfR-associated sHFE was shorter (Fig. 2). This phenomenon is likely due to dissociation of sHFE either in the low pH Golgi compartment [Lebron et al., 1998] or at the cell surface. Localization of the sHFE protein in transfected cells demonstrated that the majority of the protein is in the Golgi compartment (Fig. 3B) and not in the endosomes, unlike HFE, this is probably due to secretion of the protein and its dilution into the culture media.

In order to assess the extracellular binding of sHFE to TfR, we treated several mouse and human cell lines with sHFE-containing media and immunoprecipitated sHFE and TfR-associated sHFE. In these experiments we observed sHFE binding to TfR, with an apparent specificity for human TfR compared to mouse TfR. In addition, sHFE accumulated in the cells with time, suggesting that the protein is stable in the cells, alternatively that the rate of internalization exceeds that of degradation. Furthermore, we assessed the localization of sHFE in treated cells (Fig. 5B). In both, HeLa and HEK293 cells the sHFE localized to the recycling endosomes, albeit following different incubation periods indicating cellular differences in internalization rate.

Binding and internalization of sHFE monochain was matched with functional inhibition of Tf–FITC uptake and accumulation by cells treated with the sHFE monochain. This effect may either be due to sHFE competition with Tf for binding to TfR at the cell surface [Feder et al., 1998], alternatively, sHFE may alter the recycling of TfR thereby decreasing Tf accumulation in the cell [Salter-Cid et al., 1999]. Interestingly however, despite the observed TfR binding and reduction in Tf–FITC cellular accumulation by sHFE monochain, this protein did not induce an iron-deprived state as indicated by modulation of ferritin and TfR (Fig. 7), either in sHFE expressing HEK293 cells or a variety of cells treated with the sHFE monochain. These data suggest that either sHFE binding affinity to TfR is insufficient to mediate these effects or that there are TfR independent mechanisms or interactions that the sHFE is unable to participate in.

Although, previously sHFE treated cells demonstrated reduced ferritin [Roy et al., 2000; Drakesmith et al., 2002], sHFE-expressing cells have not been reported till date. The difference between our study and previous reports may be due to the differences in the constructs. Whereas, our sHFE is a monochain with $\beta_2$m covalently bound produced by human cells, the sHFE previously used was produced from CHO cells transfected with sHFE and $\beta_2$m [Lebron et al., 1998]. The inability of $\beta_2$m to dissociate from sHFE may contribute to the difference. Consequently, we constructed another sHFE without the bound $\beta_2$m and in cells expressing this protein we observed the same results, that is no changes in TfR and ferritin compared to parental cells (data not shown). The only other difference in the constructs is the presence of the Myc and His epitopes, these, however did not affect TfR binding, trafficking, or cellular localization. Furthermore, we utilized similar or higher concentrations of sHFE to previous reports [Roy et al., 2000; Drakesmith et al., 2002]. The concentrations utilized were sufficient to induce some biological activity as demonstrated by sHFE-induced reduction of Tf uptake but not others such as regulation of intracellular iron pools as reflected by lack of modulation of ferritin and TfR expression. This partition of sHFE biological activity reveals the complexity of HFE function. The disparity in biological activity of sHFE may be due to differences in binding affinity to TfR, otherwise it might be due to the requirement of different structural features for different HFE functions.

The failure of sHFE to regulate cellular iron metabolism in transfected cells may be due to the physical absence of the transmembrane and cytoplasmic domains, if these domains are required for high-affinity interaction with TfR. This is difficult to assess since quantitative analysis of binding affinity as determined by Biocore studies utilize soluble forms of the proteins [Lebron et al., 1998]. Alternatively, the transmembrane and cytoplasmic domains

may be required for interactions with other intracellular proteins and/or signal transduction. A previous study attempted to address the issue of signal transduction with HFE cytoplasmic mutants of two amino acids that were potential candidates for phosphorylation, these mutants demonstrated the same level of ferritin reduction as that of the HFE transfectants, thus arguing against the role for these amino acids in signal transduction [Roy et al., 2000]. This, however, does not eliminate a potential function for the transmembrane and cytoplasmic domains since they may be involved in as yet unidentified functions such as interaction with other proteins in the endosomes.

We cannot absolutely preclude that the absence of sHFE regulation of iron metabolism in HEK293 transfected or treated cells is not related to insufficient amounts of this protein or differences in TfR binding affinities, however we believe that this is unlikely since sHFE bound to TfR sufficiently and at a lower concentration (0.5 μM) downregulated Tf accumulation in the cells. Alternatively, the difference between HFE and sHFE may be due to differences of binding capabilities or interactions with other proteins within the endosomal compartment. To date no other proteins have been reported to interact with HFE. However, HFE has recently been implicated in the release of iron from specific cell types [Drakesmith et al., 2002]. Once iron dissociates from Tf it must be transported from the endosomes and into the cytosol. Although this process is not completely understood, the transporter DMT-1 has been implicated in this process [Tabuchi et al., 2000]. Therefore, HFE may interact with DMT-1 to regulate Fe transport to the cytosol. If this is indeed the case, HFE function might not be directly related to TfR binding, but rather HFE might utilize TfR simply to traffic to the endosomal compartment in a "piggy-back" fashion, since HFE itself does not appear to have an endosomal localization sequence.

Overall, we have demonstrated that the sHFE monochain is able to associate with human TfR, enter the cells, and traffic with human TfR to endosomal compartments. At high concentrations, sHFE monochain is able to reduce Tf uptake by cells but not affect the intracellular iron pools as indicated by TfR and ferritin expression. The disparity in biological activity has revealed a complexity in HFE function that is likely to involve the endosomal compartment. This protein may be uniquely useful in future developments of treatments for HFE-related hereditary hemochromatosis. Alternatively, due to its specific binding to TfR and cellular internalization of sHFE it may be engineered as a drug delivery tool, in particular it may be advantageous as a drug delivery system to overcome the blood brain barrier.

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