Interactions of the ectodomain of HFE with the transferrin receptor are critical for iron homeostasis in cells

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Abstract Expression of wild type HFE reduces the ferritin levels of cells in culture. In this report we demonstrate that the predominant hereditary hemochromatosis mutation, C282Y[2] HFE, does not reduce ferritin expression. However, the second mutation, H63D HFE, reduces ferritin expression to a level indistinguishable from cells expressing wild type HFE. Further, two HFE cytoplasmic domain mutations engineered to disrupt potential signal transduction, S335M and Y342C, were functionally indistinguishable from wild type HFE in this assay, as was soluble HFE. These results implicate a role for the interaction of HFE with the transferrin receptor in lowering cellular ferritin levels.

Key words: Hemochromatosis; HFE; Iron metabolism; Ferritin; Transferrin receptor

1. Introduction

Hereditary hemochromatosis is a common, autosomal recessive disease of iron metabolism [2]. Lack of functional HFE in the cells of the duodenal crypts leads to chronic absorption of iron [1,3–6]. The excess iron accumulates in the cells of parenchymal tissues leading to oxidative damage and multi-organ dysfunction [2]. The pathology of hereditary hemochromatosis indicates that HFE plays a significant role in regulated iron absorption and the maintenance of iron homeostasis, but the exact mechanism by which HFE facilitates iron homeostasis is not yet understood.

The predominant mutation in HFE that leads to hereditary hemochromatosis is a missense mutation which leads to the conversion of a critical cysteine to tyrosine at amino acid 282 [1]. HFE is a non-classical major histocompatibility complex class I type molecule that requires β-2-microglobulin binding for proper folding and efficient cell surface expression [4,7–10]. The C282Y mutation prevents the formation of a disulfide bond that stabilizes the β-2-microglobulin binding pocket in HFE [9]. The inability of this mutant to fold correctly and associate with β-2-microglobulin [8] results in reduced amounts of HFE and inefficient trafficking to the cell surface [7]. Lack of association with the transferrin receptor explains why it does not localize to the endosome as does wild type HFE [10–12].

A second HFE mutation has been identified in a significant number of individuals with hereditary hemochromatosis. This missense mutation converts amino acid 63 from histidine to aspartic acid, disrupting the formation of a salt bridge with the aspartic acid at position 95 [9]. The frequency of this mutation is significantly increased in individuals with hereditary hemochromatosis that are heterozygous for the C282Y mutation. This mutation is often associated with an intermediate iron loading phenotype [1,13]. The interaction of H63D HFE with transferrin receptor is not significantly different from that of wild type HFE. It has approximately the same affinity for the transferrin receptor [14] and co-immunoprecipitates with the transferrin receptor [8]. Because the association of HFE with the transferrin receptor is the main link between HFE and iron homeostasis, how this mutation contributes to iron overload is unclear.

Despite the efforts to understand the role of HFE in iron homeostasis, the precise mechanism behind its function remains unknown. Several groups have independently observed an iron deficient phenotype in cell lines transfected with HFE that is evidenced by increased transferrin receptor expression, decreased ferritin expression and an increase in IRP activity [11,15,16]. The labile iron pool is hypothesized to be reduced because HFE reduces iron uptake from transferrin [15–19].

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

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[2] The numbering system for HFE used in this manuscript is in accordance with that published by Feder et al. [Feder et al. (1996) Nat. Genet. 13, 399–408]. The 22 amino acid signal sequence that is cleaved upon processing is included in this numbering system.

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cell surface expression and association with the transferrin receptor to induce the low iron phenotype.

2. Materials and methods

2.1. Site-directed mutagenesis of HFE

The isolation of the H63D cDNA and generation of the C282Y mutation have been previously described [7]. A standard polymerase chain reaction (PCR) mutagenesis approach was utilized to generate the S335M and Y342C mutations. Two overlapping fragments with the appropriate base changes were produced in a first round PCR reaction. The product of the first reaction was then combined with the primers previously described [7] to yield the HFE mutant with the FLAG epitope.

2.2. Cell lines

The fWTHFE/tTA HeLa cell line expressing FLAG epitope-tagged HFE (fHFE) under the tetracycline responsive promoter has been previously described [11]. Cells were grown in Dulbecco's modified Eagle's essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 400 μg/ml G418 (Geneticin, Calbiochem), 200 ng/ml puromycin, and with (HFE−) or without (HFE+) 2 μg/ml tetracycline. The HEK 293 cell lines were co-transfected with the plasmids for the individual HFE mutations described above, and a plasmid containing the coding sequence for human β-2-microglobulin [7]. Isolated HFE-expressing clones were grown in DMEM supplemented with 10% FBS and 600 μg/ml G418.

2.3. Cell treatments

Cells were plated into 35 mm dishes in DMEM with 10% FBS and 50 nM human transferrin. After 3 days in culture, the growth medium was changed and desferoxamine was added to a final concentration of 50 μM, $Fe(NTA)_4$ was added to a final concentration of 100 μM, or soluble HFE (a kind gift of Pamela Bjorkman, California Institute of Technology, CA, USA) was added to a final concentration of 0.5 or 1 μM. Cells were grown for at least 16 h in the treated medium before they were lyzed.

2.4. Immunodetection of HFE and ferritin

Cells were lyzed with NET-Triton buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.4). Samples (∼300 μg) were diluted with Laemmli buffer [20] and subjected to electrophoresis on denaturing 12% SDS–polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose. Immunoblot analysis of HFE was performed as described previously using rabbit anti-HFE antibody EX1 (1:500 dilution) [7] and sheep anti-rabbit antibody conjugated to horseradish peroxidase (1:10 000 dilution, Roche Molecular Biochemicals). Immunoblot analysis of ferritin was performed as described previously [11] using rabbit anti-human ferritin antibody (1:1000 dilution, Dako), and sheep anti-rabbit antibody conjugated to horseradish peroxidase (1:10 000 dilution, Roche Molecular Biochemicals). Western blots were developed using chemiluminescence (Super Signal, Pierce) per the manufacturer's directions.

3. Results

Wild type and mutant forms of HFE were stably expressed in HEK 293 cells. Immunoblot analysis identified stable expression of the ∼40–45 kDa HFE product in all cell types except that of the parental HEK 293 cell line in which no detectable HFE was identified (Fig. 1).

Expression of HFE induces an iron deficient phenotype as indicated by reduced ferritin levels in HeLa cells [11,15–17]. To confirm that HFE had the same effect in HEK 293 cells, analysis of ferritin levels was performed for parental HEK 293 cells and those expressing wild type HFE (Fig. 2). Immunoblot analysis revealed that HFE expression reduced ferritin levels under standard tissue culture conditions. HFE did not prevent the cells from responding to changes in iron status, as ferritin expression decreased in the presence of the iron chelator, desferoxamine, and increased with the addition of Fe-NTA. Identical results were obtained in HEK 293 cells transfected without a FLAG epitope-tagged wild type HFE, indicating the FLAG epitope is not responsible for the low iron phenotype (data not shown). These results recapitulate the phenotype that was observed previously in fWTHFE/tTA HeLa cells [11,17].

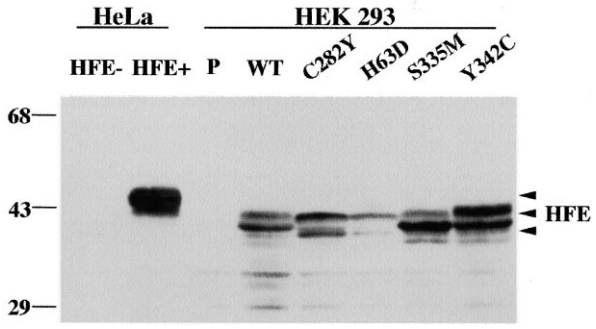

Fig. 1. Characterization of stable HFE expression in WT-, C282Y-, H63D-, S335M-, Y342C HFE/HEK 293 cell lines. Parental HEK 293 cells (lane 3), like fWTHFE/tTA HeLa cells with tetracycline added to turn off HFE (lane 1), did not express detectable levels of HFE as assayed by immunoblot. Wild type (lane 4) and mutant HFEs (lanes 5–8) were stably expressed in HEK 293 cells. The molecular weight of HFE in these cells was slightly less than that of HFE in fWTHFE/tTA HeLa cells (lane 2), probably due to differences in the extent of glycosylation in the two cell lines [11]. These results are representative of more than three independent experiments with similar results.

While the reduction in cellular ferritin levels in cells expressing HFE has been independently observed by several investigators [11,15–17], its correlation with iron homeostasis for the entire organism is not fully understood. To determine whether this low iron phenotype is reflected in the disease state, we compared the iron deficient phenotype for HEK 293 cells expressing wild type HFE with those expressing the two hereditary hemochromatosis disease alleles (Fig. 3). Immunoblot analysis revealed that under standard tissue culture conditions, C282Y HFE did not reduce ferritin levels to that of wild type HFE-expressing cells. The ferritin levels detected in C282Y HFE/HEK 293 cells were not significantly different from that of the HEK 293 parental cell line (data not shown). Conversely the H63D HFE disease allele, whose contribution to the disease state may not be as severe as the C282Y HFE,

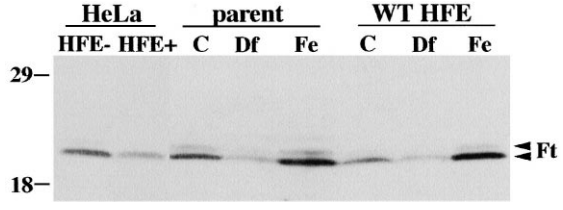

Fig. 2. Wild type HFE-expression reduced ferritin levels in HEK 293 cells. Immunoblot revealed that full length HFE expression reduced ferritin levels in HEK 293 cells grown under standard tissue culture conditions (C, lane 6) when compared to the parental cell line (C, lane 3) as it does in fWTHFE/tTA HeLa cells (lane 1 versus lane 2). Ferritin levels still responded to cellular iron status in the presence of HFE, however, since they decreased with the addition of desferoxamine, an iron chelator (Df, lane 7), and increased with the addition of $Fe(NTA)_4$, an iron salt (Fe, lane 8). These results are representative of more than three independent experiments with similar results.

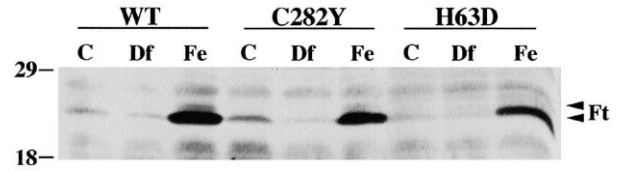

Fig. 3. C282Y HFE-expression did not reduce ferritin levels while H63D HFE-expression reduced ferritin levels to that of wild type HFE in HEK 293 cells. Expression of the C282Y HFE disease allele protein product in HEK 293 cells grown under standard tissue culture conditions (C, lane 4) did not reduce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

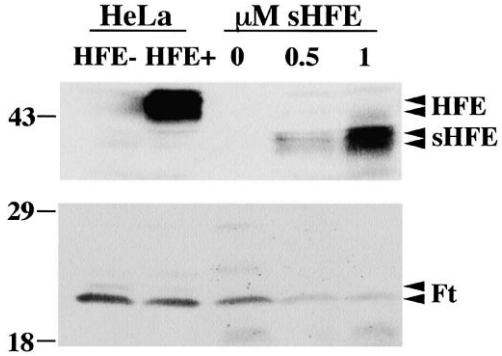

Fig. 5. Soluble HFE reduces ferritin expression in HEK 293 cells. Immunoblot reveals that addition of soluble HFE (sHFE) to the growth medium of parental HEK 293 cells reduced ferritin expression (lower panel, lane 3 versus lanes 4 and 5) as full length HFE does in fWTHFE/tTA HeLa cells (lower panel, lane 1 versus lane 2). Immunoblot analysis of soluble HFE confirmed the association of soluble HFE with these cells (upper panel, lanes 4 and 5). These results are representative of more than three independent experiments with similar results.

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

Despite extensive characterization of the interaction between HFE and the transferrin receptor, the exact mechanism by which HFE lowers ferritin levels has not been determined. One possible mechanism by which HFE could function would be through a signal transduction pathway initiated by the cytoplasmic domain of HFE. We assayed for the iron deficient phenotype in cell lines with either the Y342 or S335 in the cytoplasmic domain changed to C or M, respectively (Fig. 4). The original tyrosine and serine amino acids are potential candidates for phosphorylation. Immunoblot analysis indicated that cells expressing Y342C and S335M HFE showed reduced ferritin levels compared to those of the HEK 293 parent cells. The ferritin content of these cell lines was not significantly different from those of the HEK 293 cells expressing wild type HFE (data not shown), indicating these mutants were functional according to the limits of this assay.

To determine the involvement of other elements in the HFE cytoplasmic domain, the ability of soluble HFE to induce the same low iron phenotype as the full length HFE in HEK 293 cells was assayed. A soluble form of HFE lacking the cytoplasmic and transmembrane domains has been used to investigate the interaction of HFE and the transferrin receptor [9]. This form of HFE has high affinity for the transferrin receptor [8,9], and reduces the affinity of the transferrin receptor for transferrin [8,18]. Incubation of cells with soluble HFE resulted in a reduction of ferritin levels in parental HEK 293 cells in a concentration-dependent manner (Fig. 5). Superficially, the soluble HFE appears to reduce ferritin levels to a greater extent than the full length HFE (Fig. 5). We repeated the experiments three times and when the starting amounts of ferritin in both cell lines were the same, then no reproducible difference was detected between the final levels of ferritin in cells containing full length or in those treated with soluble HFE (data not shown). Like the results from the Y342C and the S335M mutants, the result that the soluble HFE lowers ferritin levels also argues against the possibility that the transmembrane or cytoplasmic domains of HFE modulate cytoplasmic components of iron metabolism.

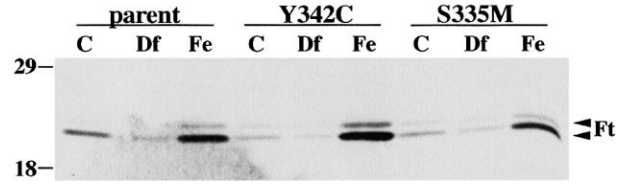

Fig. 4. Y342C and S335M HFE expression reduced ferritin levels to that of wild type HFE in HEK 293 cells. Expression of the Y342C (lanes 4–6) and S335M (lanes 7–9) HFEs in HEK 293 cells reduced ferritin expression to levels below that of the parental cell line (lanes 1–3) as assayed by immunoblot. Ferritin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

4. Discussion

A comparison of the wild type and disease-associated forms of HFE on ferritin levels in cultured cells was assessed. We observed that wild type HFE expressed in HEK 293 cells produces the same low iron phenotype that was originally identified in HFE-expressing HeLa cells [11,15–17].

C282Y HFE is severely reduced in function because it does not fold correctly [9] as manifested by; lack of association with β-2-microglobulin [7], lack of efficient trafficking to the cell surface [7,10], lack of association with the transferrin receptor [8] and the inability to maintain normal iron homeostasis in mice [6]. In this study, we show that it is also unable to recapitulate the low iron phenotype induced by wild type HFE in HEK 293 cells. The observation that this mutation in HFE does not reduce ferritin expression suggests that HFE must be efficiently expressed at the cell surface and/or tightly associated with the transferrin receptor in order to produce the low iron phenotype.

Unlike C282Y HFE, H63D HFE has been shown to traffic to the cell surface [7,10] and to associate with transferrin

receptor [8]. In this study, we show that cells transfected with the H63D HFE also have a low iron phenotype. These observations suggest that the H63D HFE would be functional in vivo and yet, the H63D allele occurs with increased frequency in individuals diagnosed with hereditary hemochromatosis [1,13]. The difference between the disease phenotype observed in humans with the H63D mutation and the wild type phenotype observed in the H63D HFE-expressing cell culture system may be the level of expression of the protein. Since the H63D HFE lacks the ability to form a salt bridge that may be important to the stability of the protein [9], the H63D HFE may be less stable than the wild type HFE. A small but significant decrease in functional HFE in vivo may lead to iron overload over the course of a lifetime. The hereditary hemochromatosis pathology resulting from the H63D mutation is milder than that of individuals homozygous for the C282Y HFE mutation.

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

Our results support the hypothesis that HFE acts through the transferrin receptor-mediated pathway of iron uptake to regulate cellular iron homeostasis. Severe mutations in HFE, such as the C282Y mutation, which prevents association with the transferrin receptor, do not manifest a low iron phenotype. These results predict that mutations in HFE that lower the affinity of HFE for the transferrin receptor, such as the W103A HFE [14], would not result in the low iron phenotype. Recent results indicate that this is indeed true [12]. Future experiments will be directed at determining the mechanism by which the HFE interaction with transferrin receptor in the extracellular domains reduces iron uptake from transferrin.

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