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The Hereditary Hemochromatosis protein HFE and its chaperone β2-microglobulin localise predominantly to the endosomal-recycling compartment

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Abstract

Hereditary Hemochromatosis is an iron overload disease most frequently associated with mutations in the HFE gene. While clinical studies of the disease have received extensive attention by various groups, the localisation, trafficking and function of the HFE protein, and its chaperone β2-microglobulin (β2M), require further investigation. In this study, we present data on the cellular localisation of HFE and its clinically relevant mutants in HuTu 80 cells. We find by confocal microscopy that HFE localises to the endosomal-recycling compartment (ERC), with minimal localisation to sorting or late endosomes. Interestingly, we also demonstrate that β2M localises to the ERC where it co-localises with HFE. We find that exogenous expression of HFE results in enhanced β2M cellular levels and that β2M is necessary for cell surface expression of HFE. Finally, we have analysed the functional effects of exogenous expression of HFE and β2M on transferrin binding to the cell surface. In summary, our study sheds light on the localisation and functional effects of the HFE and its chaperone protein β2M.

Keywords: HFE; β2-Microglobulin; Hereditary Hemochromatosis; Transferrin; Endosomal-recycling compartment; Rab11

Hereditary Hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism. The clinical features of this disease are caused due to decades of progressive iron accumulation in parenchyma cells of the liver, pancreas and heart [1]. HH is a common inherited disease in people of the northern European descent, affecting approximately one in 300 individuals, with clinical outcomes ranging from mild to life threatening heart and liver diseases [1].

HFE, a key protein mutated in HH, shares homology with class I MHC molecules but appears not to be involved in antigen presentation, since HFE−/− mice do not reveal any obvious immunological abnormalities [2]. This suggests that HFE has a specialised function distinct from that of conventional MHC class I molecules. Studies have shown that HFE binds to the transferrin receptor (TfnR) to regulate transferrin-mediated iron uptake [3]; however, the precise molecular mechanism by which HFE regulates iron homeostasis remains unclear.

HFE needs to associate with a chaperone protein termed β2-microglobulin (β2M) for its stability, normal processing and cell surface expression [4,5]. β2M is expressed in most tissues but its expression is relatively high in the kidney, spleen and liver [6]. This protein is involved in the production and processing of antigens and plays a role in the stabilisation of MHC class I and MHC class I-related proteins at the cell surface [7].

Mutations in the HFE gene are known to result in HH [4,5]. Substitution of cysteine with tyrosine at position 282 in HFE eliminates a disulphide bond in HFE leading to impaired interaction with β2M and aberrant intracellular trafficking of HFE [4,5]. Although the C282Y mutation is

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known to cause HH, not all individuals homozygous for the C282Y mutation develop iron overload [8]. A second clinically relevant mutation in the HFE protein, H63D, does not prevent association of HFE with β2M, and therefore, HFE H63D is capable of reaching the cell surface where it is functional [4]. When inherited alone, the H63D mutation appears to have little effect, however, when inherited with the C282Y mutation it contributes to the HH phenotype [9]. A third clinically relevant mutation, S65C, leads to mild to moderate hepatic iron overload [10]. Interestingly, patients carrying this mutation do not manifest clinical HH nor iron associated extensive liver fibrosis [10]. Although so far no mutation in β2M has been associated with HH, reports show that HFE−/− and β2M−/− double knock out mice accumulate higher iron levels in tissues compared to the HFE−/− knock out alone, suggesting that lack of β2M contributes to the severity of the HH phenotype [8,11].

To address some ambiguities in the localisation of HFE and β2M in the literature we examined by confocal microscopy the subcellular distribution of these proteins in comparison with a range of intracellular compartment marker proteins, and with respect to each other, in HuTu 80 cells. Furthermore, we have examined the functional effects HFE and β2M have on iron binding to the cell surface of these cell types.

Materials and methods

Plasmid construction. pEGFP-N1/HFE was constructed using a sense primer *WT-F* (5′-ATAGAATCCAATGGGCCCGCGAGCC-3′) and an antisense primer *WT-R* (5′-CTGTGGATCCAACCACTCACGTTCAG-3′) to amplify HFE from a human kidney cDNA library (Clontech). The PCR product was EcoRI/BamHI inserted into pEGFP-N1 (BD, Biosciences). The pEGFP-N1/HFE C282Y [*C282Y-F* (5′-GAAGAGCAGAGA TATACGT**AT**CAGGTGGAGCACCCAG-3′), *C282Y-R* (5′-CTGGGT GCTCCACCTG**AT**ACGTATATCTCTCTGCTCTTC-3′)], pEGFP-N1/HFE H63D [*H63D-F* (5′-GTTCGTGTTCTATGAT**G**ATGAGAGAGT CGCCGTGTG-3′), *H63D-R* (5′-CACACGGCGACTCTCAT**C**ATCAT AGAACACGAAC-3′)], and pEGFP-N1/HFE S65C [*S65C-F* (5′-GTTC TATGATCATGAG**TG**CCGCCGTGTGGAGCCC-3′), *S65C-R* (5′-GG GCTCCACACGGCG**GC**ACTCATGATCATAGAAC-3′)] constructs were generated by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) with pEGFP-N1/HFE as template. The Hemagglutinin (HA)-fused HFE WT vector was a gift from Kostas Pantopoulos. The pSPORT6/β2M construct was generated by insertion of the NotI/SalI insert from a β2M image clone (Geneservice Limited) into pSPORT6 (Clontech). The Rab11-FIP3 I738E construct has been described elsewhere [12].

Cell biology and immunofluorescence microscopy. HuTu 80 cells (duodenal epithelial adenocarcinoma—obtained from ATCC) were cultured and transfected, and immunofluorescence microscopy was performed, as described in [12]. Primary antibodies were mouse monoclonal anti-β2M (Abcam), anti-HA (Convance), anti-LBPA (gift from Jean Gruenberg), anti-TfnR (Zymed) and anti-EEA1 (Transduction Laboratories). Rabbit antibodies used were anti-β2M (Abcam), anti-Rab11a (Zymed) and anti-Rab11-FIP3 (described elsewhere [13]). Secondary antibodies were goat anti-mouse conjugated to TRITC (Jackson ImmunoResearch) or Alexa Fluor 488 (Molecular Probes), and donkey anti-rabbit conjugated to TRITC or Alexa Fluor 488. For transferrin (Tfn)-binding experiments, cells were serum starved for 2 h and then incubated for 1 h, at 4 °C, with 5 μg/ml of Alexa Fluor 594 labelled iron-saturated holotransferrin (Invitrogen). Images were recorded using a Zeiss LSM 510 META confocal microscope fitted with a 63×/1.4 plan apochormat lens.

Results

Wild-type (WT) HFE demonstrates a punctate vesicular staining pattern that is concentrated in the pericentrosomal region

In an attempt to gain a better understanding of the cellular function of HFE we investigated by confocal microscopy the subcellular localisation of HFE in HuTu 80 cells, a duodenal cell line. We found that in the majority (74.2 ± 5.3%) of cells expressing GFP-HFE, HFE displayed a punctate staining pattern, which was concentrated in a perinuclear location while the remaining cells revealed a reticular staining pattern (Fig. 1A). Upon immunostaining GFP-HFE expressing cells for γ-tubulin, a centrosomal marker protein, we found that the concentrated perinuclear GFP-HFE staining surrounds the centrosome (data not shown). To compare the staining pattern observed for WT GFP-HFE with that of the clinically relevant mutants we transfected HuTu 80 cells with the plasmids encoding HFE with various point mutations. We found that in cells expressing GFP-HFE C282Y the mutated protein displayed a reticular staining pattern in most cells (98.2 ± 1.1%) (Fig. 1A). In contrast, approximately half (50.2 ± 8.1%) of the GFP-HFE H63D expressing cells have pericentrosomally concentrated punctate staining, with the remaining cells displaying a reticular pattern (Fig. 1A). In cells expressing GFP-HFE S65C the majority of cells (71.1 ± 5%) displayed a reticular staining pattern, with the remaining cells displaying a punctate staining pattern concentrated pericentrosomally (Fig. 1A). Similar results on the subcellular distribution of HFE WT, HFE C282Y, HFE H63D and HFE S65C were observed in HeLa cells (data not shown).

HFE and β2-microglobulin predominantly localise to the endosomal-recycling compartment

As outlined above, HuTu 80 cells expressing GFP-HFE displayed a consistent punctate staining pattern that was concentrated in the pericentrosomal region. As HFE has previously been reported to traffic through the endosomal pathway [14], we stained GFP-HFE expressing cells for various endosomal compartment marker proteins to determine the precise subcellular localisation of the HFE protein. We first examined the localisation of HFE with respect to TfnR, a classical sorting/recycling endosomal marker protein [15]. We found that in cells expressing GFP-HFE and labelled for TfnR the two proteins co-localised in a pericentrosomal region, indicating that HFE indeed localised to endosomal membranes (Fig. 1B). Since in many cell types the ERC is a pericentrosomal organelle [15], we examined the localisation of HFE with respect to Rab11a, a small GTPase known to label the ERC [15], and found that HFE strongly