Research Article

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The hemochromatosis proteins HFE, TfR2, and HJV form a membrane-associated protein complex for hepcidin regulation

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Background & Aims: The hereditary hemochromatosis-associated membrane proteins HFE, TfR2, and HJV are required for adequate hepatic expression of the iron hormone hepcidin. While the genetic interactions are clear, it remains elusive how bone morphogenetic protein co-receptor HJV functions together with HFE and TfR2 to activate hepcidin transcription via the BMP–SMAD signaling pathway. Here, we investigate whether HFE, TfR2, and HJV physically interact on the surface of hepatocytes.

Methods: We explore protein–protein interactions by glycerol gradient sedimentation assays and co-immunoprecipitation analyses in transfected HuH7 hepatoma-derived cells.

Results: Our data demonstrate that HFE and TfR2 bind HJV in a non-competitive manner. Co-immunoprecipitation analyses provide direct experimental evidence that HFE, TfR2, and HJV form a multi-protein membrane complex. Our experiments show that like TfR2, HJV competes with TfR1 for binding to HFE, indicating that the expression of TfR2 and HJV may be critical for iron sensing. We identify residues 120–139 of the TfR2 extra-cellular domain as the critical amino acids required for the binding of both HFE and HJV. Interestingly, RGMA, a central nervous system homolog, can substitute for HJV in the complex and promote hepcidin transcription, implicating RGMA in the local control of hepcidin in the CNS.

Conclusions: Taken together, our findings provide a biochemical basis for hepcidin control by HFE, TfR2, and HJV.

Keywords: BMP/SMAD pathway; Hereditary hemochromatosis; Iron sensing.
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Abbreviations: HH, hereditary hemochromatosis; TfR, transferrin receptor; HJV, hemojuvelin; MHC, major histocompatibility complex; β2M, beta 2 microglobulin; Fe_2-Tf, transferrin-bound iron; GPI, glycophosphatidylinositol; BMP, bone morphogenetic protein; SMAD, small mother against decapentaplegic; qRT-PCR, quantitative real-time polymerase chain reaction; LDLR, low density lipoprotein receptor; IRIDA, iron refractory iron deficiency anemia; TMPRSS6, transmembrane protease serine 6; RGMA, repulsive guidance molecule A.

Introduction

Regulation of systemic iron metabolism is critical to secure adequate cellular iron supplies while preventing toxic iron accumulation. Iron homeostasis is controlled by hepcidin, a peptide hormone predominantly produced by the liver [1,2], which regulates intestinal iron absorption, macrophage-mediated iron recycling and iron mobilization from hepatic stores [3]. Hepcidin exerts its function by binding to the iron exporter ferroportin expressed on the surface of iron-releasing cells, triggering its degradation and hence reducing plasma iron levels [4].

Iron balance is disrupted in hereditary hemochromatosis (HH), one of the most frequent inherited disorders of the Western world. HH is an autosomal recessive disease, hallmarked by excessive iron absorption from the diet. Iron accumulates within parenchymal cells over time, eventually leading to organ failure. The genes encoding *HFE* [5], hemojuvelin (*HJV*) [6], transferrin receptor 2 (*TfR2*) [7] or hepcidin (*HAMP*) [8] are mutated in different subtypes of the disease. These mutations result in inappropriately low hepcidin levels, reflecting activation of hepcidin expression by HFE, TfR2, and HJV [9–11].

HFE encodes a ubiquitously expressed major histocompatibility complex class 1-like molecule (MHC-1), which associates with β2-microglobulin (β2M) [5]. Despite its similarity to classical MHC class I proteins, HFE is unable to present short peptides and does not perform any known immune function [12]. HFE binds to the transferrin receptor 1 (TfR1) [13], that supplies transferrin-bound iron (Fe_2-Tf) to most cell types [14]. Fe_2-Tf competes with HFE for binding to TfR1 due to overlapping binding sites on TfR1 [15]. HFE also interacts with TfR2 via domains that differ from those involved in HFE/TfR1 interaction [16,17]. TfR2 is a relative of TfR1 that is predominantly expressed in hepatocytes and erythroid precursors [18]. TfR2 binds to Fe_2-Tf with low affinity [19], and unlike TfR1, it can simultaneously interact with both HFE and Fe_2-Tf [20]. Furthermore, the TfR2 protein is stabilized in response to increasing iron levels [21,22]. These observations support a model in which TfR2 acts as a sensor for iron saturation of transferrin in the serum. According to this model [23], HFE association with TfR1 is inversely proportional to Tf-iron saturation. If serum Fe_2-Tf levels increase, HFE is displaced from TfR1 to allow its interaction with TfR2 and to activate hepcidin transcription. The molecular mechanism by which HFE and TfR2 regulate hepcidin expression remains elusive.

HJV is a glycophosphatidylinositol (GPI)-anchored protein which acts as a bone-morphogenetic protein (BMP) co-receptor, driving hepcidin transcription via the BMP–SMAD signaling cascade [24]. Disease-associated mutations in HJV cause a juvenile form of HH with a severe phenotype of iron overload, indicating that the HJV/BMP pathway plays a critical role in maintaining basal hepcidin levels. BMP6, which is activated by iron, has been proposed to be the endogenous ligand for HJV [25,26]. *BMP6*−/− mice display severe iron overload due to hepcidin deficiency, resembling hereditary hemochromatosis. Recent studies have shown that, despite increased *Bmp6* mRNA levels in response to iron overload, the BMP/SMAD signaling pathway is impaired in *Hfe* and *Tfr2* knockout mice [27–29] and in HFE-HH patients [30,31]. Furthermore, HFE-induced hepcidin transcription has been shown to require functional HJV [32]. The molecular basis of these genetic interactions also remains elusive.

Here, we uncover that HH-associated membrane proteins form a multi-protein complex on the plasma membrane of hepatic cells, and we dissect biochemical parameters underlying the format of this critical regulatory complex.

Materials and methods

Plasmid construction

The HFE open reading frame was amplified from human cDNA using the primers HFE-*Bam*HI-F and HFE-*Not*I-R and cloned into *Bam*HI/*Not*I sites of the vector pcDNA3 (Invitrogen), to generate pcDNA3-HFE. To introduce a Flag or a cMYC tag downstream of the signal peptide (aa 1–23), pcDNA3-HFE was mutagenized by using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen) to insert a *Kpn*I site at position 23 (pcDNA-HFE$^{23\text{-}Kpn\text{I}}$). The primers used were HFE-*Kpn*I-Mut-F and HFE-*Kpn*I-Mut-R. Synthetic double-stranded Flag or cMyc tags were then cloned into the *Kpn*I site to generate pcDNA3-Flag-HFE (Flag-HFE) or pcDNA3-cMyc-HFE (Myc-HFE) plasmids, respectively. Oligonucleotides for tag synthesis were Flag-F-*Kpn*I, Flag-R-*Kpn*I, cMyc-F-*Kpn*I, and cMyc-R-*Kpn*I.

To create the pEYFP-HFE plasmid, we amplified the *HFE* open reading frame (ORF) from human cDNA (with the primers HFE-F-*Xho*1 and HFE-R-*Bam*HI) and we cloned the fragment into the *Xho*I/*Bam*HI sites of the pEYFP-N1 vector.

The p3XFlag-HJV plasmid (Flag-HJV), expressing the HJV open reading frame with the Flag epitope tag fused to the N-terminus (after the signal peptide at position 35), was created as previously described [24]. The pcDNA3-HJV construct was a kind gift of Dr. Clara Camaschella (Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan, Italy) [33].

The pcDNA3-TfR2-HA (TfR2-HA) plasmid, expressing the *TfR2* cDNA fused to a C-terminal HA tag, was generated by cloning the PCR-amplified *TfR2* ORF into *Hin*dIII/*Eco*RI sites of the pcDNA3 vector. The *TfR2* cDNA was PCR-amplified from HuH7 cell-derived cDNA template using the primers TfR2-*Hin*dIII-F and TfR2-HA-*Eco*RI-R.

The chimera constructs TfR2-cd-TfR1-ecto-Flag and TfR2$_{104–250}$-HA were kindly provided by Dr. Caroline Enns (University of Oregon) [17]. We replaced the Flag tag in the TfR2-cd-TfR1-ecto-Flag construct with the HA tag using the primers TfR2-cd-F and TfR1-ecto-HA-R and then cloned the chimera into the *Bam*HI/*Eco*RI sites of the pcDNA3 vector. The chimera TfR2$_{104–250}$-HA was used as a template to further truncate the TfR2 extracellular domain and to generate the new truncated chimera constructs TfR2$_{104–236}$-HA, TfR2$_{104–150}$-HA, TfR2$_{104–139}$-HA, TfR2$_{104–119}$-HA, using the primers TfR1-*Hin*dIII-F, TfR2-236-HA-*Eco*RI, TfR2-150-HA-*Eco*RI, TfR2-139-HA-*Eco*RI, TfR2-119-HA-*Eco*RI, respectively. All these chimera constructs contain the TfR1 cytosolic and transmembrane domains and carry the HA epitope tag at the C-terminus.

To generate the pcDNA3-β2M construct, the *β2M* ORF was amplified from HuH7 cDNA by using the primers β2M-*Bam*HI-F and β2M-*Not*I-R.

Flag-HJV and TfR2-HA mutant constructs (TfR2^{M172K}, TfR2^{Q690P}, HJV^{G99V}, HJV^{G320V}, HJV^{D172E}) were generated by using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen).

To generate the p3XFlag-RGMA plasmid, we amplified by PCR the *RGMA* coding sequence downstream of the signal peptide (after aa 47) from a construct expressing the *RGMA* ORF (kindly provided by Prof. Michael Butros, DKFZ, Heidelberg, Germany) using the primers RGMA-*Not*I-F and RGMA-*Xba*I-R. The fragment was cloned into the *Not*I/*Xba*I sites of the p3XFlagCMV vector (Sigma), downstream of the pre-pro-trypsin signal peptide and Flag tag.

To create the pCDNA-Flag-HJV-ΔEx4 vector, we first produced a pCDNA3-GPI plasmid which allows to clone the protein of interest with the GPI-anchor sequence at the C-terminus. The last 93 nucleotides of exon 2 and the whole exon 3 (with the pre-pro-trypsin signal peptide and the Flag tag at the N-terminus) were amplified from the Flag-HJV plasmid and successively cloned into the *Eco*RI/*Xho*I sites of pCDNA3-GPI. The primers used are Pre-Pro-F-*Eco*RI and Ex3-R-*Xho*I.

The C-terminal GFP-tagged LDL-Receptor (LDLR-GFP) was a kind gift of Dr. Runz (Molecular Metabolic Disease Unit, Institute of Human Genetics, Heidelberg) [34].

All oligonucleotide sequences are shown in Supplementary Table I.

All cDNAs were sequenced to verify the fidelity of the constructs (GATC Biotech).

Cell culture and transfection

The human hepatocarcinoma cell line HuH7 was cultured in DMEM (Dulbecco's modified Eagle's medium) with GlutaMAX (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 1% penicillin, 1% streptomycin, and 1 mM sodium pyruvate. Cells were maintained in a 5% CO_2 atmosphere at 37 °C. For transient transfection experiments, HuH7 cells were seeded at 16×10^3 cells/cm^2 16 h prior to the experiment. Plasmid transfections were performed with TransIT-LT1 Transfection Reagent (Mirus), according to manufacturer's guidelines. 0,2 μg of DNA/cm^2 and TransIT at a ratio of 3 μl per 1 μg of DNA was used. Twenty-four hours following transfection, cells were harvested and used for subsequent analyses.

Fe_2-Tf treatment of HuH7 cells

HuH7 cells were seeded onto 10 cm plates (1.2×10^6 cells/plate) and 8 h after transfection of the expression plasmids, the culture medium was exchanged to fetal bovine serum-free medium. Sixteen hours later, cells were treated with Fe_2-Tf (25 μM; Sigma T4132) for 3 h and then collected for co-immunoprecipitation experiments.

Preparation of cell lysates

Cells were washed twice in ice cold Dulbecco's phosphate-buffered saline (PBS). Cell pellets were lysed in ice-cold NET buffer (1% Triton X-100 (v/v), 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4) supplemented with 1× Complete Mini Protease Inhibitor Mixture (Complete Mini, Roche Applied Science). Following agitation at 4 °C for 30 min, lysates were centrifuged at 10,000 rpm for 15 min at 4 °C and supernatants were collected. Membrane fractions were prepared by resuspending the cell pellets in 1 ml of ice-cold hypotonic lysis buffer (Saccharose 250 mM, KCl 50 mM, $MgCl_2$ 5 mM, Tris–HCl 20 mM ph 7.4), as described previously [35]. After centrifugation at 0,6 g for 10 min at 4°, the pellet containing the membrane fractions was washed once in the hypotonic lysis buffer and then resuspended in ice-cold NET buffer. The protein concentration was measured using the BCA (bicinchoninic acid) Protein Assay (Pierce).

Immunoprecipitation assay and Western blot analysis

Immunoprecipitation assays were performed by incubating 500 μg of protein lysates with agarose-conjugated mouse anti-FLAG M2 antibody (Sigma Aldrich A2220) or anti-GFP antibody (GFP-Trap, Chromotek) for 2 h at 4°, according to manufacturer's instructions. After four washing steps in NET buffer, samples were eluted with 2× Laemmli buffer [36] and denatured by incubation at 95 °C for 5 min. Cell lysates (10% of the starting material used for immunoprecipitation) and immunoprecipitates were subjected to 10 or 12% SDS–PAGE, and proteins were transferred to a nitrocellulose membrane (Whatman) for protein immunodetection using mouse anti-TfR1 (Zymed Laboratories Inc., 1:1000), rabbit anti-HA (Sigma Aldrich H6908, 1:2500), rabbit anti-Flag (Sigma Aldrich F7425, 1:5000), rabbit anti-Myc (Sigma Aldrich C3956, 1:1000), rabbit anti-GFP (Abcam, 1:5000), and mouse anti-α-tubulin (Sigma Aldrich T5168). Blots were then incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (Sigma Aldrich) and then subjected to chemiluminescence (Amersham Biosciences, ECL Plus). The resulting bands were digitalized and quantified using the NIH Image J software (www.rsb.info.nih.gov/nih-image/). The levels of co-precipitated protein in the immunocomplex were calculated as