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Liver hemojuvelin protein levels in mice deficient in matriptase-2 (Tmprss6)

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ABSTRACT

Mutations of the *TMPRSS6* gene, encoding the serine protease matriptase-2, lead to iron-refractory iron deficiency anemia. Matriptase-2 is a potent negative regulator of hepcidin. Based on *in vitro* data, it has recently been proposed that matriptase-2 decreases hepcidin synthesis by cleaving membrane hemojuvelin, a key protein of the hepcidin-regulatory pathway. However, *in vivo* evidence for this mechanism of action of matriptase-2 is lacking. To investigate the hemojuvelin–matriptase-2 interaction *in vivo*, an immunoblot assay for liver membrane hemojuvelin was optimized using hemojuvelin-mutant mice as a negative control. In wild-type mice, two hemojuvelin-specific bands of 35 kDa and 20 kDa were detected in mouse liver membrane fraction under reducing conditions; under non-reducing conditions, a single band of approximately 50 kDa was seen. Phosphatidylinositol-specific phospholipase C treatment confirmed binding of the detected protein to the cell membrane by a glycosylphosphatidylinositol anchor, indicating that the major form of mouse liver membrane hemojuvelin is a glycosylphosphatidylinositol-bound heterodimer. Unexpectedly, comparison of liver homogenates from *Tmprss6*+/+ and *Tmprss6*−/− mice revealed significantly decreased, rather than increased, hemojuvelin heterodimer content in *Tmprss6*−/− mice. These data do not provide direct support for the concept that matriptase-2 cleaves membrane hemojuvelin and may indicate that, *in vivo*, the role of matriptase-2 in the regulation of hepcidin gene expression is more complex.

Introduction

Iron homeostasis in man is precisely regulated at the level of iron absorption in the small intestine. The key iron metabolism regulatory compound is hepcidin, a hepatocyte-derived peptide which controls iron export from macrophages and enterocytes [1,2]. Hepcidin expression is in turn determined by several signaling pathways, which modulate hepcidin transcription in response to iron stores, inflammation and the rate of erythropoiesis [3]. The main component of the iron-dependent regulation is hemojuvelin (Hjv), a hepatocyte membrane protein encoded by the *HFE2* gene. Hjv interacts with bone morphogenetic protein ligands [4], and this interaction at the hepatocyte membrane initiates a cascade of intracellular phosphorylations which ultimately lead to an increase in hepcidin gene transcription. The importance of Hjv in hepcidin regulation is evident from the fact that mutations in the *HFE2* gene cause severe juvenile hemochromatosis, which has similar phenotype as juvenile hemochromatosis caused by mutations in the hepcidin gene itself [5].

Recently, another membrane protein participating in the control of iron metabolism has been identified. The serine protease matriptase-2, encoded by the *TMPRSS6* gene, is a negative regulator of hepcidin expression [6–8]. *TMPRSS6* mutations cause inappropriately high expression of hepcidin, resulting in diminished iron absorption, iron sequestration in macrophages, and, ultimately, iron-refractory iron deficiency anemia. It has been demonstrated that, under *in vitro* conditions, matriptase-2 efficiently cleaves membrane hemojuvelin [9,10], and similar mechanism of hemojuvelin protein regulation is postulated to function *in vivo*. According to the current model, mutated forms of matriptase-2 would be unable to cleave membrane Hjv, resulting in the presence of high levels of Hjv on the hepatocyte membrane, continued stimulation of the bone morphogenetic protein signaling pathway, and inappropriately high hepcidin expression. The model has been recently further strengthened by reports that mice deficient in both Hjv and matriptase-2 display the same low hepcidin mRNA levels as mice deficient in Hjv [11,12].

Despite the importance of hemojuvelin in iron metabolism, there are only few experimental studies dealing with Hjv protein levels *in vivo*. The main reason for this absence of experimental *in vivo* data is the unavailability of robust antibodies that recognize endogenous murine hemojuvelin in tissue lysates [11,13]. Commercial antibodies display non-specific bands [13], whose presence makes the identification of Hjv protein on immunoblots unreliable. Therefore, the aim of the present study was to develop a reliable Hjv immunoblot assay, by

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using *Hjv*−/− mice as a negative control. As a next step, the optimized assay was employed to compare membrane Hjv protein levels between *Tmprss6*+/+ and *Tmprss6*−/− mice. Unexpectedly, it was found that the content of liver membrane-bound Hjv heterodimer, the major form of Hjv detected by the assay, was decreased, rather than increased, in mice lacking matriptase-2.

Methods

Hjv−/− mice [14] were a generous gift from Prof. Silvia Arber, Basel, Switzerland. Generation and biochemical characterization of *Tmprss6*−/− mice have been described previously [8], the animals display increased *Hamp* mRNA levels and absent *Tmprss6* expression. Male *Tmprss6*−/− and *Tmprss6*+/+ mice, aged 8 weeks, were used for Hjv determinations. All animal experiments were approved by the Ethics Committee of the First Faculty of Medicine in Prague. Anti-Hjv antibody (AF3634), generated against the mature region of mouse Hjv (amino acids 36–393), was purchased from R&D Systems (Minneapolis, MN, USA), anti-pan-cadherin antibody (4068) from Cell Signaling (Boston, MA, USA). Secondary antibodies (705-036-147 and 713-036-137) were obtained from Jackson ImmunoResearch Europe, UK.

For Hjv protein determination in whole tissue lysates, tissue samples were homogenized (5×10 s) with an Ultra Turrax homogenizer in 4 volumes of Ripa buffer (Sigma Aldrich) with a protease inhibitor mix (Roche Diagnostics, Mannheim, Germany) and centrifuged for 15 min at 6000×*g*. 80 μg of supernatant protein was loaded on a pre-cast 4-20% Tris–Glycine minigel (Invitrogen, Carlsbad, CA, USA). For liver membrane Hjv protein determinations, liver was homogenized in 5 volumes of 0.25 mM sucrose, pH 7.6, containing 1 mM EDTA and protease inhibitors. Homogenate was centrifuged for 15 min at 6000×*g*, and membranes were obtained by ultracentrifugation of the supernatant at 80,000×*g* for 45 min. 60 μg of the membrane protein was separated by SDS electrophoresis under both reducing (2% β-mercaptoethanol) and non-reducing conditions. Proteins were blotted on a PVDF membrane, blots were blocked with 5% skimmed milk in tris-buffered saline containing 0.1% of Tween 20, and incubated overnight with a solution of the primary antibody in 5% milk. Primary antibody dilutions were 1:1000 for hemojuvelin, 1:100,000 for Gapdh and 1:4000 for pan-cadherin. After washing, blots were incubated with a 1:20,000 solution of the secondary antibody in 5% milk and proteins were visualized by chemiluminescence. Densitometry was performed on a Biorad GS-80 scanner, statistical analyses of densitometry and PCR results were performed using the Mann–Whitney nonparametric test.

For phosphatidylinositol-specific phospholipase C (Pi-PLC) cleavage of the GPI anchor, liver membrane samples (approximately 2 mg of protein) were treated with Pi-PLC (Sigma-Aldrich, 100 mU) at 37 °C for 3 hours and subsequently recentrifuged at 45,000×*g* for 45 min. 60 μg of supernatant protein was loaded on gel for immunoblots.

Liver mRNA isolation was performed as previously described [8]. For real-time PCR determination of hepcidin gene (*Hamp*) and Hjv gene (*Hfe2*) expression, a Roche LightCycler instrument in combination with the Fast Start SYBR Green protocol (Roche LightCycler, Roche Diagnostics GmBH, Germany) was used. Target mRNA content was calculated relatively to *Gapdh* mRNA content, assuming exact doubling of amplified DNA in each PCR cycle. Primer sequences (forward and reverse) were *Gapdh*, CGGTGTGAACGGATTTGC and GCAGTGATGGCATGGACTGT; *Hfe2*, CAATCCTGCGTCTTTGATGTT and GAAGCAAAGCCACAGAACAAA; *Hamp* CTGAGCAGCACCACCTATCTC and TGGCTCTAGGCTATGTTTTGC.

Results

Immunochemical detection of Hjv in whole liver homogenates resulted in several non-specific bands; however, comparison of samples from *Hjv*+/+ and *Hjv*−/− mice enabled clear identification of two hemojuvelin-specific bands at approximately 35 kDa and 20 kDa (Fig. 1A). The 35 kDa band appeared more strongly on immunoblots than the 20 kDa band and was therefore used for subsequent densitometry quantifications. Hemojuvelin-specific bands were also seen in homogenates of muscle and myocardium (Supplementary Figure 1). This tissue distribution of hemojuvelin protein is in agreement with the tissue distribution of *Hfe2* mRNA, which is present in mainly muscle, myocardium and liver [5]. In contrast to previously reported data [15], no Hjv-specific bands were detected in homogenates from kidney or testis (Supplementary Figure 1).

Hjv protein exists in soluble and membrane-bound forms [16]. Since signal transduction by the Hjv/Smad pathway depends on membrane-bound hemojuvelin, crude membrane fractions were utilized for Hjv determination in subsequent experiments. Membranes were prepared by ultracentrifugation, and membrane Hjv content was examined in livers from *Hjv*+/+, *Hjv*+/− and *Hjv*−/− mice. The results again showed the presence of the 35 and 20 kDa bands in the liver membrane fraction of *Hjv*+/+ and *Hjv*+/− mice (Fig. 2A); compared to *Hjv*+/+ mice, the intensity of the 35 kDa band was decreased in *Hjv*+/− mice (Fig. 2B). Levels of *Hfe2* mRNA were decreased in *Hjv*+/− mice by about 50% (Fig. 3A). As noted previously [17], there was no significant change in *Hamp* mRNA levels between *Hjv*+/− and *Hjv*+/+ mice (Fig. 3B).

At present, it is not exactly known which forms of Hjv are expressed at the mouse hepatocyte membrane *in vivo*. One of the possible candidates is a glycosylphosphatidylinositol (GPI) anchored heterodimer, composed of two disulfide bond-linked Hjv fragments (AA 33–165 and AA 166–393), which originate by autoproteolytic cleavage of the full-length peptide at a labile Asp-Pro bond [16,18,19]. The observed size of Hjv-specific bands obtained under reducing conditions (Fig. 4A) agrees with this concept, provided the fragments are glycosylated as predicted [16,18,19]. When liver membranes were analyzed under non-reducing conditions, the Hjv-specific bands moved as a single spot of approximately 50 kDa (Fig. 4B), which is again in agreement with the reported heterodimer composition. Hjv-specific bands were found only in membrane fraction; following ultracentrifugation of liver homogenate, no Hjv-specific bands were seen in the supernatant (Fig. 4A). To confirm that the detected protein is indeed bound to the membrane by a GPI anchor, membrane samples were treated with Pi-PLC and recentrifuged. As expected for a GPI-bound protein, after Pi-PLC treatment the Hjv-specific bands were found in the supernatant fraction, both in reduced and non-reduced samples. In reduced samples, Pi-PLC treatment decreased the intensity of the 20 kDa band; however, the signal of the major 35 kDa band significantly increased, and a new minor Hjv-specific

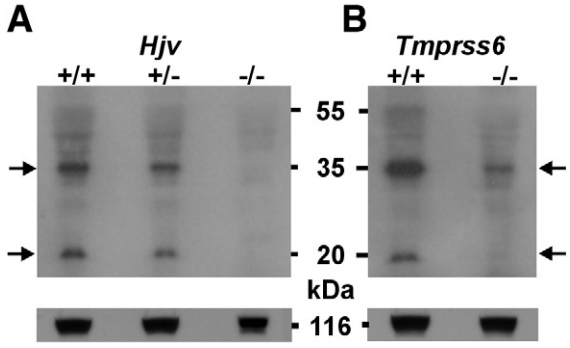

Fig. 1. Hemojuvelin protein in whole liver homogenates. A: Immunoblot of Hjv protein in whole liver homogenates from *Hjv*+/+, *Hjv*+/− and *Hjv*−/− mice. B: Immunoblot of Hjv protein in whole liver homogenates from *Tmprss6*+/+ and *Tmprss6*−/− mice. 80 μg of protein was loaded per lane, pan-cadherin (116 kDa) was used as loading control. Arrows indicate the positions of hemojuvelin-specific bands.