Defective targeting of hemojuvelin to plasma membrane is a common pathogenetic mechanism in juvenile hemochromatosis

Laura Silvestri,1 Alessia Pagani,2 Claudia Fazi,2 Gianmario Gerardi,3 Sonia Levi,1,2 Paolo Arosio,3 and Clara Camaschella1,2

1San Raffaele Scientific Institute, Dibit, Milan, Italy; 2University Vita-Salute San Raffaele, Milan, Italy; 3Dipartimento Materno Infantile e Tecnologie Biomediche University of Brescia, Italy

Introduction

The study of hereditary hemochromatosis (HH) has contributed major advances to our understanding of the systemic iron regulation.1 The key regulatory protein is hepcidin, a small antimicrobial liver peptide2 that has an inhibitory effect on the release of iron from duodenal cells and macrophages to the circulating transferrin.3 Mutations of hepcidin cause a recessive form of severe, early-onset hemochromatosis, known as juvenile hemochromatosis (JH; type 2B HH).4 The same severe phenotype is more commonly caused by mutations of HJV, the gene encoding hemojuvelin (type 2A HH).3 Studies in humans and mice provide evidence that HJV positively modulates hepcidin expression. Urinary hepcidin levels are remarkably low in patients carrying HJV mutations,5 and HJV-deficient mice have dramatically decreased liver hepcidin mRNA.6,7 The mechanism of hepcidin activation by HJV remains to be clarified. Recently, it has been shown that HJV could act as a bone morphogenetic protein (BMP) coreceptor, enhancing BMP-mediated signaling8 and likely up-regulating hepcidin expression through BMP-activated SMA and MAD proteins (SMAD).9

HJV is highly homologous to proteins belonging to the family of repulsive guidance molecules (RGMs). In the mouse, the expression of Rgma and Rgmb is limited to developing and adult central nervous system, while Rgmc, the ortholog of human HJV, is detected in the same tissues (skeletal muscle, heart, and liver) where hepcidin is expressed.3 Similar to RGM members, the HJV gene encodes a protein characterized by multiple domains, including a N-terminal signal peptide, a RGD integrin-binding motif, a partial von Willebrand factor (VWF) type D domain, and a C-terminal glycosylphosphatidylinositol (GPI) anchor domain. All RGM proteins possess a Gly-Asp-Pro-His (GDPH) sequence,10,11 which undergoes a partial autocatalytic cleavage at the Asp-Pro bond at acidic pH, compatible with that of the late secretory pathway.12 HJV is retained on the outer layer of the plasma membrane (m-HJV) through the GPI anchor motif, but can also be found as a soluble form (s-HJV) both in vitro and in vivo.13,14

Recent studies in murine muscle cells and fibroblasts have analyzed the complex biosynthesis of Rgmc during myoblast differentiation. Two classes of GPI anchor Rgmc molecules exist that are differentially processed and have distinct fates: a membrane-associated heterodimer, composed of cleaved N- and C-terminal fragments, and a full-length form, prevalent in the extracellular fluid.14

About 30 distinct missense or nonsense point mutations have been identified in the HJV gene leading to JH.3,15,16 In order to gain insights into the pathogenesis of this disorder and to characterize the functional properties of human HJV, we have studied the biosynthesis and maturation of 5 missense (G99V,3 C119F,16 F170S,17 W191C,17 and G320V) and 1 nonsense (R326X3) allelic variants associated with JH. We demonstrate that mutants with defective autoproteolytic processing (F170S, W191C, and G320V) are not correctly targeted to the plasma membrane (PM) but are mainly retained in the endoplasmic reticulum (ER), as observed for the truncated R326X variant. We also find that in our model, the HJV mutants’ ability to be released in the culture medium is independent from the membrane export. Finally, we show that iron negatively modulates the release of both wild-type (WT) and mutant s-HJV, while it has differential effects on m-HJV.


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Materials and methods

Generation of WT and HJV mutants

The whole HJV open-reading frame was amplified from human cDNA by using primers and cloning strategies described in Document S1, available on the Blood website (see the Supplemental Material link at the top of the online article). Mutant constructs were generated by site-directed mutagenesis by using oligonucleotides shown in Table S1.

Generation of anti-HJV

The DNA fragment encoding from amino acid 226 to 402 of human HJV was cloned into a pGEX (Amersham Biosciences Europe GmbH, Freiburg, Germany) vector in fusion with GST. The peptide comprises the C-terminal 176 amino acids of the protein deleted of the GPI anchor motif. The proteins were expressed in Escherichia coli, purified by affinity chromatography, and used to elicit antibodies in mice.

Cell culture

Cell culture media and reagents were from Invitrogen (Carlsbad, CA) and from Sigma-Aldrich (St Louis, MO). HeLa and HepG2 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine, 200 U/mL penicillin, 200 mg/mL streptomycin, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 95% humidifier air and 5% CO₂.

Western blot analysis

HeLa cells were seeded in 100-mm diameter dishes until 70% to 80% of confluence was reached. Cells were transfected with a complex consisting of 10 µg plasmid DNA and 30 µL of the liposomal transfection reagent Metafectene (Biontex Laboratories, Munich, Germany) in 4 mL OptiMem (Invitrogen) according to the manufacturer’s instructions. HepG2 cells were transfected with 24 µg plasmid DNA and 60 µL Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction. After 18 hours the medium was replaced; cells were collected after 24 hours and lysed in RIPA buffer for Western blot analysis.

Proteins were quantified by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA); samples (50 µg) were subjected to 10% or 15% SDS-PAGE, and were transferred to Hybond C membrane (Amersham Biosciences Europe) by standard Western blotting technique. Blots were incubated with the relevant primary antisera at dilutions recommended by the manufacturer. Anti-HJV was used at a proportion of 1:500. After washing in TBS (0.5 M Tris-HCl [pH 7.4] and 0.15 M NaCl) containing 0.1% Tween-20, blots were incubated with relevant HRP-conjugated secondary antisera and developed using a chemiluminescence detection kit (ECL; Amersham Biosciences Europe).

WT and R326X HJV precursor proteins were synthesized by in vitro transcription and translation (TNT-coupled reticulocyte lysate system; Promega, Madison, WI) and used to analyze the biogenesis of WT and mutant HJV. After transfection (18 hours), cells were washed with PBS (without calcium and magnesium), detached from the dishes, centrifuged at 1000g for 20 minutes, followed by 42 000g for 20 minutes. Transfected HeLa cells were then centrifuged at 13 000g for 20 minutes, followed by 42 000g for 1 hour. The pellet was washed with 0.35 M sucrose plus 5 mM HEPES (pH 7.4), homogenized by douncing 40 times in a Dounce homogenizer (Kontes Glass, Vineland, NJ), and centrifuged at 500g for 20 minutes, followed by 42 000g for 20 minutes. The supernatants were analyzed for each sample. The Student t test was used for statistical calculation.

PI-PLC cleavage of membrane-bound HJV

A total of 10⁶ HeLa cells, transiently transfected with HJV-expressing constructs or empty vector, were incubated in PBS or DMEM plus 0.3 U/mL phosphatidylinositol-specific phospholipase C (PI-PLC) at 37°C in a 5% CO₂ incubator. After 2 hours, the supernatants were collected, and proteins were precipitated with TCA or cold acetone and loaded on a 10% SDS-PAGE.

EM analyses

HeLa cells were transiently transfected with Lipofectamine using pcDNA3.1 expressing WT and mutant HJV. After transfection (18 hours), cells were fixed, labeled with a polyclonal goat anti-cMYC using the gold-enhanced protocol, embedded in Epon-812, and cut as described previously. Immuno-electron microscopy (EM) images were acquired from thin sections following steps were carried out at 4°C. The pelleted cells were resuspended in 0.35 M sucrose plus 5 mM HEPES (pH 7.4), homogenized by douncing 40 times in a Dounce homogenizer (Kontes Glass, Vineland, NJ), and centrifuged at 500g for 20 minutes, followed by 42 000g for 20 minutes. The membrane pellet was washed with 0.35 M sucrose plus 5 mM HEPES (pH 7.4) and then centrifuged at 13 000g for 20 minutes. Transfected HeLa membrane fractions were incubated in aliquots of 50 µL for 18 hours at 4°C and at 37°C to block and activate a proteolytic process, respectively. The samples were then centrifuged at 13 000g for 20 minutes at 4°C, the membrane pellets and supernatants were analyzed by SDS-PAGE under reducing conditions, and HIV was detected by immunostaining with anti-HIV.

Results

Maturation and processing of WT and mutant HJV

To analyze the biogenesis of WT and mutant HJV, we cloned the full-length human HJV coding sequence in a mammalian expression vector (pcDNA3.1), incorporating the cMYC tag at the
N-terminus, immediately downstream of the signal peptide (Figure 1A). Next, we introduced some pathogenic mutations altering conserved residues: G99V modifies the RGD domain, C119F removes a Cys predicted to be involved in a disulphide bridge, F170S modifies a Phe next to the catalytic consensus sequence GDPH, W191C introduces an extra Cys in the partial VWF type D domain, G320V at the C-terminus is the most common mutation in patients,3 and R326X removes the 100 C-terminal residues, including the GPI anchor moiety and a glycosylation site.

In addition, the recombinant C-terminal sequence of HJV, deleted of the GPI anchor motif, was expressed in E. coli and purified by affinity chromatography, and then used to elicit antibodies that recognize the C-terminus of the protein (Figure 1A).

We first addressed the question whether the mutations affect the HJV posttranslational modifications. HJV contains 3 potential N-linked glycosylation sites and is predicted to be a GPI anchor protein based on a consensus sequence proximal to the transmembrane domain. Different levels of glycosylation of the transfected HJV modify the protein electrophoretic mobility. The in vitro-transcribed HJV migrates in SDS-PAGE with the expected MW of 46 kDa, whereas cell-associated HJV migrates with an apparent MW of approximately 50 kDa (Figure 1B), suggesting the addition of carbohydrate moieties. G99V, C119F, F170S, W191C, and G320V migrate, as does the WT protein, around 50 kDa (Figure S1A). The R326X mutation, lacking the last 100 amino acids and missing 2 C-terminal cysteines, migrates slower than the in vitro-synthesized corresponding mutant (Figure S1B; bottom panel).

To analyze in detail the glycosylation status of the mutants, the cellular extracts were digested with either Endo H or PNGase F and analyzed by Western blot. Endo H is specific for the Asn-linked N-linked glycosylation sites and is predicted to be a GPI anchor protein based on a consensus sequence proximal to the transmembrane domain. Different levels of glycosylation of the transfected HJV modify the protein electrophoretic mobility. The in vitro-transcribed HJV migrates in SDS-PAGE with the expected MW of 46 kDa, whereas cell-associated HJV migrates with an apparent MW of approximately 50 kDa (Figure 1B), suggesting the addition of carbohydrate moieties. G99V, C119F, F170S, W191C, and G320V migrate, as does the WT protein, around 50 kDa (Figure S1A). The R326X mutation, lacking the last 100 amino acids and missing 2 C-terminal cysteines, migrates slower than the in vitro-synthesized corresponding mutant (Figure S1B; bottom panel).

To analyze in detail the glycosylation status of the mutants, the cellular extracts were digested with either Endo H or PNGase F and analyzed by Western blot. Endo H is specific for the Asn-linked high-mannose oligosaccharides produced in the early ER, while PNGase F also deglycosylates fully mature proteins, with complex oligosaccharides modified in the trans face of the Golgi apparatus. We find that Endo H converts the expressed WT and mutants to partially deglycosylated forms (Figure 1C); however, the pattern of F170S, W191C, and G320V is different from that of WT, suggesting they have an altered glycosylation process. After PNGase F treatment, the mobility of missense HJV mutants was analogous to that of the WT protein (Figure 1D). Moreover, Endo H and PNGase F modify R326X mobility to a similar extent, suggesting that this is not complex-glycosylated as the WT HJV (Figure 1E).

Mature HJV contains 14 cysteine residues, some of which are probably involved in intra- or interchain disulfide bonds and modify electrophoretic mobility. Thus, HeLa cells homogenates were analyzed in SDS-PAGE. Under reducing conditions, HJV migrates at approximately 50 kDa, while under nonreducing conditions, it migrates faster, at about 46 kDa, indicating a more compact conformation, likely caused by intramolecular disulfide bridges (Figure S1B). All missense mutations and R326X behave as the WT HJV, except C119F, which shows a broader band under nonreducing conditions.

**HJV undergoes an autoproteolytic cleavage**

RGMs are subjected to a proteolytic processing, through the cleavage at the GDPH consensus sequence,12 similarly to other proteins, as mucin and BMPER.21,22 Consistently, under reducing conditions, HJV shows a smaller band of approximately 33 kDa, in addition to the major approximately 50-kDa species (Figure 1B). The 33-kDa band is not detectable with anti-cMYC antibody, nor it visible under nonreducing conditions (data not shown), suggesting that it consists in a C-terminal fragment held to the cleaved product by disulfide bonds. The low amount of the expected 16-kDa N-terminal fragment, generated by the cleavage,12,14 was not detectable, perhaps for the low sensitivity in blotting of the anti-cMYC antibody.

To understand whether the mutations alter the proteolytic processing, total lysates from transfected HeLa cells were analyzed under reducing conditions. As shown in Figure 2A, only G99V and C119F produce the 33-kDa fragment, while in F170S, W191C, and G320V variants, only the full-length, 50-kDa form is present. Similar results were obtained in HepG2 cells (Figure S2).

**HJV mutants are highly reduced at the plasma membrane**

We used a surface-labeling method16 to investigate the amount of WT and HJV mutants localized on HeLa and HepG2 cell surfaces 36 hours after transfection. Plasma membrane (PM) of HeLa cells exposes almost identical amounts of WT and G99V HJV...
Also, the autoproteolytically active C119F variant reaches the PM, but less efficiently than WT (19.5% vs. 9.9%). In contrast, m-HJV is reduced in the other mutants (F170S, 4.5% vs. 2.9%; W191C, 7.5% vs. 2.9%; and G320V, 8.9% vs. 2.9%), indicating an impairment of the proper HJV trafficking and sorting to the cell surface (Figure 2B). The same assay in HepG2 cells confirmed an m-HJV reduction of F170S, W191C, and G320V, whereas C119F is more competent to localize to the cell surface (Figure 2C). Similar results were obtained by flow cytometry of nonpermeabilized HeLa and HepG2 cells (Figure S3A–B). These data confirm that only the mutants that undergo autoproteolytic processing have the ability to reach the PM.

To further analyze the characteristics of m-HJV, transfected HeLa cells were treated with PI-PLC, and the supernatant was analyzed. Analysis of the supernatants under reducing conditions show that WT, G99V, and C119F releases mainly the approximately 33-kDa form (Figure 2D; top panel). However, when the supernatants are analyzed under mildly reducing conditions, an additional slow-mobility band is detectable (Figure 2D; bottom panel), suggesting the existence of disulfide bonds that join the approximately 33-kDa and the approximately 16-kDa fragments together, as already described. After PI-PLC treatments and under reducing conditions, trace amounts of the full-length approximately 50-kDa HJV is revealed by anti-cMYC, using only a highly sensitive detection method (ECL Advance; Amersham Biosciences Europe) confirming the low quantity of the uncleaved protein on the PM of WT and mutant transfected cells (data not shown). Altogether, these results indicate that the major type of m-HJV is the heterodimeric form derived from the autoproteolytic cleavage.

HJV mutants are selectively retained into the endoplasmic reticulum

To study the HJV mutants unable to reach the PM, EM and morphometric analysis on HeLa cells were performed 18 hours after transfection. As shown in Figure 3A and 3B, WT HJV is localized mainly on the PM, in part to the Golgi (G) and in low proportion into the endoplasmic reticulum (ER), suggesting a proper maturation pathway. On the contrary, C119F, W191C,
G320V, and R326X are predominantly clustered into the ER. The F170S cleavage variant shows labeling of Golgi and ER, and a minor one at PM, suggesting Golgi and ER retentions. A significant proportion of the G99V protein is found within the ER; nevertheless, this variant can still get to the cell surface quite efficiently in comparison with the others. Thus, the reduced expression on PM is probably due to the inappropriate protein folding or processing for all mutants, except G99V, where there may be a delay in the cell-surface localization.

**HJV is released as a soluble form in the cell culture medium**

To assess whether HJV is released in the medium, transiently transfected HeLa cells were grown in serum-free media for 24 hours at 37°C; then, cell culture supernatants were analyzed by using anti-HJV and anti-cMYC. Anti-HJV recognizes 2 different species: a major one of 42 kDa and a minor one of approximately 33 kDa detectable only in WT, G99V, and C119F (Figure 4A). The approximately 42-kDa species has faster mobility than the cell-associated HJV and is recognized also by the anti-cMYC (Figure 4B). Since cMYC is localized at the N-terminus of the protein, the truncation of s-HJV must take place at the C-terminus.

The total amount of soluble form is comparable in WT and mutants, except C119F and G320V, which are reduced (C119F, 62.4% ± 17.9%; G320V, 62.3% ± 27.7%; P < .01 vs WT). R326X, lacking the GPI anchor moiety, is efficiently secreted, and its soluble form has the same MW of the cellular form (Figure 4C).

BFA is a fungal antibiotic which efficiently inhibits protein maturation across the post-ER pathways. To further study the posttranslational modification of HJV, WT-transfected HeLa cells were incubated with different concentrations of BFA and HJV analyzed in total lysates, PMs, and media (Figure 4D). After treatment with 100 ng/mL BFA, no band corresponding to s-HJV (42 and 33 kDa) is detectable in the medium, while the full-length cell-associated HJV is increased. In addition, the 33-kDa cleaved band is no more detectable in the cellular lysates, suggesting that at this concentration BFA blocks protein export from the trans-Golgi network (TGN; Figure 4D). These results suggest that both s-HJV and cleaved m-HJV follows a BFA-dependent maturation pathway.

**Iron modulates the release of s-HJV**

It has been previously demonstrated that the release of s-HJV is dependent upon iron quantity in the culture medium. Under low-iron concentrations, HJV was efficiently released; when iron was added (both in the form of ferric ammonium citrate [FAC] or diferric transferrin), s-HJV progressively decreased. In principle, iron could modulate HJV trafficking to the PM or influence cellular mechanisms releasing s-HJV. To address this question, transiently transfected HeLa cells were incubated for 24 hours in the presence or absence of 50 μM FAC, and the media were analyzed by Western blot.

As shown in Figure 5A, iron supplementation causes a decrease of s-HJV in WT and all mutants, indicating that the HJV variants are as iron sensitive as the WT. By using the surface-labeling method, a significant increase of m-HJV is evident after FAC incubation in WT, but not in the mutants (Figure 5B; data not shown). Thus, although the transfected cells regulate the release of the s-HJV variants in response to iron, they are unable to efficiently expose additional m-HJV molecules on the PM.

**The protein released in the medium does not originate from m-HJV**

The results of PI-PLC treatment indicate that the membrane exposes mainly the cleaved form of HJV. The larger size of s-HJV compared with m-HJV (42 kDa vs 33 kDa) and its efficient release even from the membrane-defective mutants suggest that in our system it does not originate from GPI shedding.

To demonstrate that s-HJV does not originate from a proteolytic cleavage of m-HJV, membranes isolated from transiently transfected HeLa cells were incubated at 4°C and at 37°C to block and activate a proteolytic process, respectively. We found that isolated membranes are richer in the 33-kDa band than the total lysates

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**Figure 4. HJV is secreted in the medium through a BFA-dependent pathway.** (A-B) Transfected HeLa cells were incubated for 24 hours in serum-free media. Total lysates (50 μg) and concentrated media were analyzed by using anti-HJV (A) and anti-cMYC (B). (C) R326X- or mock-transfected HeLa cells were incubated in DMEM with 2% FBS; after 2 hours, total lysates and TCA-precipitated media were analyzed by Western blot using anti-HJV. (D) BFA treatment of WT HJV-transfected HeLa cells. HeLa cells were incubated 24 hours with increasing amount of BFA. Concentrated media, PI-PLC supernatant, and total cellular lysates were loaded on 10% SDS-PAGE, and proteins were revealed by anti-HJV. The 50-kDa uncleaved m-HJV band is no more detectable in the cellular lysates, suggesting that at this concentration BFA blocks protein export from the trans-Golgi network (TGN; Figure 4D). These results suggest that both s-HJV and cleaved m-HJV follows a BFA-dependent maturation pathway.

**Figure 5. Iron modulates the secretion of s-HJV both in WT and mutants.** (A) Transfected HeLa cells were incubated in serum-free media in the presence (+) or absence (−) of 50 μM FAC, after 24 hours, total lysates (50 μg) and concentrated media were analyzed with anti-HJV. Scales refer to relative molecular mass in kilodaltons. (B) Iron modulation of m-HJV was quantified by using the described binding assay. Error bars indicate SD.


![Figure 6. s-HJV does not originate from isolated membranes.](image)

(Figure 6), which is in agreement with m-HJV as a cleaved heterodimer. Trace amounts of 50-kDa and 33-kDa species in the supernatants were evident only when the film was overexposed and the band intensity after incubation at 4°C and 37°C was comparable. This suggests that the release of the 50-kDa and 33-kDa forms is unrelated to a proteolytic cleavage, but is likely unspecific and due to the large amount of cellular HJV in isolated membranes.

We conclude that in our model the s-HJV is secreted.

**Discussion**

HJV mutations associated with JH are heterogeneous and widely spread along the gene-coding sequence. Although the evidence is that HJV up-regulates hepcidin, it is still unclear how mutations impair hepcidin activation, thereby causing the inappropriate high iron absorption and the severe iron loading that characterize the disease. Available studies concern the most frequent G320V mutation and the corresponding G313V of Rgmc, the murine ortholog of HJV. Human G320V was shown to be unable to interact with its putative receptor, neogenin, in HEK293 kidney cells, while murine G313V caused a reduced enhancement of the endogenous BMP signaling that up-regulates hepcidin. A similar defect was reported for the G99V human mutant. Still, the reason for the failure of hepcidin activation remains unexplained.

To explore the biosynthesis and maturation of HJV mutants we developed an expression vector encoding 5 missense (G99V, C119F, F170S, W191C, and G320V) and 1 truncated (R326X) variants identified in patients. The analyses of transfected HeLa and HepG2 cells, expressing WT and mutant HJV, provide new information both on the processing of HJV to the membrane and the impairments caused by the mutations.

Western blotting and Endo H and PNGase F digestions confirm that HJV has complex glycosylation moieties, indicating that it transits through the ER and Golgi apparatus. Like mucin and BMPER, which undergo an autocatalytic cleavage at the GDPH motif, respectively, reduction of m-HJV impairs the activation of the signal pathway involved in hepcidin up-regulation. The pathogenetic mechanism of G99V and C119F variants is also partially retained into the ER, in the late secretory pathway. In agreement with this model, the G99V and C119F mutants, which are cleaved, find their way to the Golgi, suggesting an ER retention mechanism, probably activated by the incorrect folding of the variants. Interestingly, F170S traffics more efficiently to the Golgi, indicating that this uncleaved mutant partially escapes from the ER control, perhaps because properly folded, but is still unable to reach the PM. This result suggests that autoproteolytic cleavage is necessary for export to the membrane and is compatible with it occurring beyond the ER, in the late secretory pathway. In agreement with this model, the G99V and C119F mutants, which are cleaved, find their way to the PM, although with different efficiency. We observed some differences in the amount of m-HJV according to the cell type used. In particular, the export of C119F is more efficient in HepG2 than in HeLa cells.

EM and morphometric analysis at 18 hours post-transfection show that G99V and C119F variants are also partially retained into the ER, while at 36 hours they are efficiently exposed on membranes, suggesting a delay in the cell-surface localization.

Altogether, our results indicate that altered maturation and reduced membrane localization characterize most of the mutants studied (Table 1), and likely are common mechanisms leading to HJV-associated JH. Other truncated variants (Q312X,25 C321X,26 and R385X17) probably share the same pathogenetic mechanism with R326X, and the cleavage is expected to be inhibited in A168D15 and D172E, which occur close to and within the GDPH motif, respectively. Reduction of m-HJV impairs the activation of the signal pathway involved in hepcidin up-regulation. The pathogenetic mechanism of G99V and C119F that are present on the PM, although show some maturation defects, remains to be fully elucidated. We hypothesize a defect of the binding to the ligand (BMP) or to co-receptors, which

**Table 1. Scheme of maturation and processing of the WT and mutant HJV**

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<th>WT</th>
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ND indicates not determined.
generates the inadequate activation signal for hepcidin produc-
tion, as demonstrated for G99V.8

Cleavage of the GPI anchor protein by membrane-associated
phospholipases or proteases represents a common mechanism to
release the soluble counterparts of GPI-anchored proteins in
extracellular fluid.27 However, the activity of phospholipases and
proteases would not be selective in discriminating the different
GPI-anchored proteins, suggesting the possibility of alternative
mechanisms.28 HeLa cells release s-HJV into the medium mainly
as an approximately 42-kDa species, as HEK293 and Hep3B cells
do.13 This species is efficiently released from all mutants, with the
exception of C119F and G320V, and without any relationship with
the amount of m-HJV. A faint band of released 33 kDa is detected
only in WT and some variants. That the soluble 42-kDa form
cannot be derived from a protease-mediated shedding of m-HJV is
supported by the following findings: (1) the discrepancy between
the amount of membrane and soluble forms observed in some
variants; (2) the evidence that PI-PLC releases mainly the 33-kDa
cleaved moiety from the membrane; and (3) the absence of the
42-kDa isoform in isolated membrane supernatant. Thus, the
42-kDa s-HJV seems to derive from an independent secretory
pathway, as reported for the GPI anchor folic acid receptor.29 The
minor band of 33 kDa found in the soluble fractions of the variants
competent for the cleavage process could represent their soluble
autocatalytic product, as reported for MUC5AC.29 Whether this
secretory mechanism is the result of HJV overexpression, or
whether it occurs in physiologic conditions and in which cell types,
remains to be established.

A partial processing of mouse G313V Rgmc has been recently
described. Lack of cleavage was shown in transfected Cos-7 cells,
although in that system some uncleaved protein reaches the PM and
may release some Rgmc.14 We believe that the discrepancy with
our results could be due to the high level of overexpression of the
murine variant, caused by adenovirus infection, or to a different
maturatin process of the variant in nonmurine cells.

To understand whether both m- and s-HJV are involved in the
pathogenesis of JH, we studied the effect of iron addition to the
transfected cells. As previously shown for WT,13 iron addition
reduces the release into the medium of both WT and mutant s-HJV.
On the contrary, WT, but none of the mutants present on the cell
surface, responds to iron treatment by increasing m-HJV. These
findings strengthen the central role of m-HJV in the disease. s-HJV
might fulfill the function of sequestering BMP to inhibit both BMP
signaling and hepcidin expression,8 which is compatible with a role
in signaling iron deficiency.13 The finding that s-HJV is strongly
reduced by iron in all mutants as well as in WT is in agreement with
this interpretation and indicates that s-HJV has a minor role, if any,
in the disease.

Our results are consistent with the proposed dual role of HJV in
iron metabolism: s- and m-HJV might reciprocally regulate
hepcidin expression in response to iron changes,13 and depend-
ning on the different cell types, m-HJV could be most important
in the hepatocytes, where the protein behaves as BMP corecep-
tor to respond to iron excess up-regulating hepcidin. In other
cells, which do not express hepcidin, such as skeletal muscle
cells,14 the significance of m-HJV remains unclear, while s-HJV
secretion could signal the iron needs.13 Whether s-HJV binds
BMP and/or antagonizes its effect on membrane receptors
remains to be established.

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Authorship

Author contributions: L.S. designed the experimental work, per-
formed research, and wrote the manuscript; A.P. performed re-
search and analyzed data; C.F. and G.G. performed research; S.L.
helped design research and analyze data; P.A. contributed to
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Correspondence: Clara Camaschella, Universit`a Vita-Salute
San Raffaele, Via Olgettina, 60, 20132 Milano, Italy; e-mail:
clam.camaschella@hsr.it.

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Defective targeting of hemojuvelin to plasma membrane is a common pathogenetic mechanism in juvenile hemochromatosis

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