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Matriptase-2- and Proprotein Convertase-cleaved Forms of Hemojuvelin Have Different Roles in the Down-regulation of Hepcidin Expression*[S]

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Hemojuvelin (HJV) is an important regulator of iron metabolism. Membrane-anchored HJV up-regulates expression of the iron regulatory hormone, hepcidin, through the bone morphogenic protein (BMP) signaling pathway by acting as a BMP co-receptor. HJV can be cleaved by the furin family of proprotein convertases, which releases a soluble form of HJV that suppresses BMP signaling and hepcidin expression by acting as a decoy that competes with membrane HJV for BMP ligands. Recent studies indicate that matriptase-2 binds and degrades HJV, leading to a decrease in cell surface HJV. In the present work, we show that matriptase-2 cleaves HJV at Arg^{288}, which produces one major soluble form of HJV. This shed form of HJV has decreased ability to bind BMP6 and does not suppress BMP6-induced hepcidin expression. These results suggest that the matriptase-2 and proprotein convertase-cleavage products have different roles in the regulation of hepcidin expression.

Iron is an essential biological cofactor. The majority of the body's iron is bound by hemoglobin for oxygen transport by red blood cells. Although insufficient iron leads to anemia, excessive iron accumulation in the body causes oxidative damage to DNA, proteins, lipids, and other biological molecules (1). Because humans do not have a regulated mechanism for iron excretion, iron homeostasis is maintained by tightly regulating iron absorption from the intestine. Hepcidin, a liver-derived peptide hormone, acts as a negative regulator of iron absorption (2).

Hemojuvelin (HJV)[4] is a key player in the induction of hepcidin expression. Loss-of-function mutations in HJV lead to the severe iron overload disorder, juvenile hemochromatosis (3, 4). People with homozygous or compound heterozygous mutations in HJV and HJV knock-out mice have inappropriately low levels of hepcidin expression, resulting in increased iron absorption (4–6). HJV binds to the bone morphogenic proteins (BMPs): BMP2, 4, 5, and 6. Membrane HJV potentiates hepcidin expression by acting as a co-receptor for BMP ligands (7). BMP ligands up-regulate hepcidin expression by activating the BMP signaling pathway (7–9). Recent studies indicate that BMP6, in particular, is important in the regulation of iron metabolism, and its expression in the liver is positively regulated by iron levels (10–12).

HJV is a glycosylphosphatidylinositol-linked membrane protein, expressed in both skeletal muscle and liver. It can be cleaved by proprotein convertases and released as a shed form (sHJV) (13–17). sHJV generated by proprotein convertase cleavage antagonizes the function of glycosylphosphatidylinositol-linked HJV by negatively regulating BMP signaling, thereby decreasing hepcidin expression. Two mechanisms are possible for the down-regulation of BMP signaling by sHJV. First, cleavage of hemojuvelin releases HJV, such that sHJV no longer acts as a co-receptor for BMP signaling. Second, sHJV is capable of binding BMPs, thus competing with glycosylphosphatidylinositol-linked HJV (15).

Matriptase-2 is a serine protease encoded by the gene *TMPRSS6*. It is expressed mainly in the liver and is a critical regulator of iron homeostasis (18, 19). Lack of functional matriptase-2, both in humans and in mice, causes inappropriately high hepatic hepcidin expression, which results in iron-deficiency anemia (18, 19). Matriptase-2 is therefore an important suppressor of hepcidin expression. Recent studies in transfected HeLa cells indicate that matriptase-2 binds and decreases cell surface HJV by cleaving it into a ladder of smaller products (20). Because HJV and matriptase-2 are both expressed in liver, matriptase-2 has been proposed to regulate iron homeostasis by modulating the levels of cell surface HJV.

In this study we show that proprotein convertases and matriptase-2 cleave HJV in a site-specific manner and each protease generates one major cleavage product whose sizes are distinct from each other. In contrast to proprotein convertase cleavage, the sHJV product generated by matriptase-2 cleavage has reduced binding to BMP6 and does not alter BMP6-induced hepcidin expression. These data indicate that the major role of matriptase-2 is to inactivate HJV by down-regulating cell surface HJV, thereby decreasing hepcidin expression.

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[4] The abbreviations used are: HJV, hemojuvelin; BMP, bone morphogenic protein; ER, endoplasmic reticulum; FCI, furin convertase inhibitor; fHJV, FLAG-tagged HJV; PC, proprotein convertase; sHJV, shed HJV.

EXPERIMENTAL PROCEDURES

Generation of HJV and Matriptase-2 Constructs—Previously described pcDNA3-HJV (21) was used as a template to generate Arg to Ala mutations and stop codon mutations using the QuikChange XL Site-directed Mutagenesis kit (Stratagene, Santa Clara, CA) according to the manufacturer's protocol. The primers used to generate the constructs are listed in supplementary Methods. HJV-p3XFLAG-CMV-9 vector with three FLAG tags at the N terminus of HJV (7) was kindly provided by Dr. Jodie Babitt at Harvard University. The generation of the matriptase-2 expression construct is described in the supplemental Methods.

Cell Culture and Transfection—Both HepG2 and HEK293 cells were obtained from American Type Culture Collection. Stably transfected cells were maintained by the addition of 800 μg/ml G418. FuGENE 6 HD (Roche Applied Science) and Lipofectamine 2000 (Invitrogen) were used to transfect HepG2 and HEK293 cells, respectively.

Inhibitors—Cells were treated with leupeptin (Sigma) and the furin convertase inhibitor (FCI), decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Alexis, San Diego, CA). The stock solution of leupeptin (10 mM) was solubilized in water. FCI (5 mM) was dissolved in dimethyl sulfoxide. The final concentrations of the inhibitors are denoted in the figure legends.

sHJV/BMP6 Binding Assay—sHJV/PC and sHJV/M2 were filter-concentrated from the serum-free conditioned medium, collected from HEK293 cells stably expressing FLAG-tagged HJV (HEK293-fHJV), and HEK293-fHJV cells transiently transfected with pcDNA3-*TMPRSS6*, respectively. sHJV concentrations were estimated by Western blotting using baculovirus-generated sHJV as a standard. BMP6 was purchased from R & D Systems (Minneapolis, MN). sHJV·BMP6 binding assays were performed as described previously (10), except that an anti-FLAG-M2 affinity gel (Sigma) was used to pull down sHJV.

Immunoblot Analyses—Immunoblot analyses on cell lysates, conditioned medium, and binding eluate were performed as described previously (22) using an affinity-purified rabbit anti-HJV 18745 antibody generated against residues 1–401 of human HJV (0.22 μg/ml) (21), a mouse anti-β-actin antibody (1:10,000; Chemicon International, Billerica, MA), a rabbit anti-matriptase-2 antibody (1 μg/ml), a goat anti-BMP6 antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), and the corresponding horseradish peroxidase-conjugated (1:5,000) or Alexa Fluor 680-conjugated secondary antibodies (1:10,000) (Chemicon International). Immunoblots were visualized by chemiluminescence (Pierce supersignal; Thermo Fisher Scientific) or using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). The generation of the rabbit anti-matriptase-2 antibody is described in supplemental Methods.

siRNA Knockdown of Matriptase-2—SMARTpool siRNA specific for human *TMPRSS6* (Dharmacon) was used to knock down the matriptase-2 in HepG2 cells that stably express exogenous *TMPRSS6*, as described previously (13). RNAiMAX reagent (Invitrogen) was used for the transfection. The negative control siRNA was described previously (13).

RT-PCR Analysis—Quantitative RT-PCR was performed as described previously (23) using the primers listed in the supplemental Methods.

Immunofluorescence Microscopy—HepG2 cells were plated on number 1.5 glass cover slips (Warner Instruments, Hamden, CT) and transfected with the indicated HJV plasmids using FuGENE 6 HD (Roche Applied Science). Cells were stained using rabbit anti-HJV (1.73 μg/ml), and mouse anti-KDEL 10C3 (5 μg/ml; Stressgen, Victoria, BC), Alexa Fluor 568 goat anti-rabbit IgG (H+L) (4 μg/ml; Molecular Probes), and Alexa Fluor 488 goat anti-mouse IgG (H+L) (4 μg/ml; Molecular Probes). Cells were imaged using an 63 $\times$ oil objective on a LSM 710 microscope (Carl Zeiss).

Flow Cytometry—HEK293 cells stably expressing WT, R257A, or R288A HJV were grown in 100-mm dishes (a 100-mm dish is sufficient for six samples). Cells were detached by incubating in cell dissociation buffer (Invitrogen). For surface HJV detection, cells were incubated in affinity-purified rabbit anti-HJV 18745 antibody (4 μg/ml) in FACS-specific buffer (Hanks' without calcium or magnesium, 10 mM HEPES, pH 7.4, 1% FBS) for 1.5 h at 4 °C, washed, and then incubated in Alexa Fluor 488 goat anti-rabbit IgG (3.3 μg/ml) (Invitrogen) for 30 min at 4 °C. For detection of total HJV, cells were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde, washed, permeabilized in PBS with 0.1% Triton X-100, and treated as described for nonpermeabilized cells. Three samples were analyzed for cell surface and total HJV expression along with no antibody and nonspecific IgG controls for each cell line. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson) at the Oregon Stem Cell Center Flow Cytometry Core, Oregon Health and Science University.

RESULTS

Cleavage of HJV by Matriptase-2 Generates One Major Form of Soluble HJV in HEK293 Cells—Recent studies showed that membrane HJV can be cleaved by both the furin family of proprotein convertase and matriptase-2 and that the cleaved forms are shed from cells (17, 20, 24, 25). We therefore wanted to characterize the differences between matriptase-2 and proprotein convertase cleavage of HJV. The size of sHJV generated by matriptase-2 cleavage was compared with that produced by proprotein convertase cleavage. HEK293 cells stably expressing HJV (HEK293-HJV) were transiently transfected with either empty vector or a plasmid containing matriptase-2 cDNA (pcDNA3-*TMPRSS6*). Similar to a previous report (25), the sHJV that was shed by the empty vector-transfected HEK293-HJV cells migrated at ~40 kDa in SDS-PAGE, and its secretion was blocked in the presence of decanoyl-Arg-Val-Lys-Arg-chloromethylketone, a FCI that inhibits proprotein convertases (Fig. 1). The inhibition of the 40-kDa sHJV by FCI suggests that it is the product of proprotein convertase cleavage. In matriptase-2-transfected HEK293-HJV cells (HEK293-HJV/M2), however, we detected the major form of sHJV in the conditioned medium migrating at ~36 kDa (Fig. 1).

The secretion of the 36-kDa sHJV was not inhibited by FCI, but it was blocked by leupeptin, a serine-cysteine protease

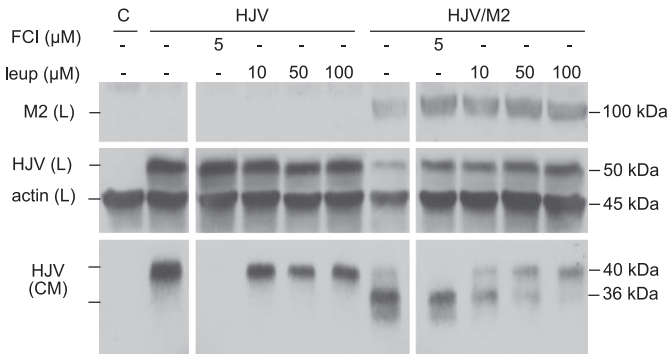

FIGURE 1. **Cleavage of HJV by matriptase-2 generates one major isoform of soluble HJV in HEK293 cells.** HEK293 cells stably expressing HJV were subcultured into 12-well plates in DMEM/10% FBS on day 1. On day 2, cells were transfected with either pcDNA3 empty vector (HJV) or pcDNA3-TMPRSS6 (HJV/M2) using Lipofectamine 2000. At about 24 h after transfection (day 3), culture medium was changed to 1 ml of DMEM/2% FBS per well with or without (5 μM), or leupeptin (*leup*; 10, 50, or 100 μM). After 24 h of incubation (day 4), conditioned medium (*CM*) was collected, and cell lysate was prepared. The total lysate and 12% conditioned medium were subjected to SDS-PAGE, followed by immunodetection of matriptase-2 (*M2*), HJV, and β-actin in the lysate (*L*) and HJV in conditioned medium. HEK293 cells stably transfected with pcDNA3 empty vector (*C*) were included as a negative control for HJV. The samples were run, transferred, and probed with antibodies on a single blot for the two *panels*. The upper portion of the gel was probed with anti-matriptase-2 and the middle with anti-HJV followed by anti-actin. The molecular masses of the sHJV are denoted to the *right* of the immunoblot and were calculated by interpolation between the 45-kDa and 31-kDa markers. Similarly, the 50-kDa size of cell-associated HJV was calculated by interpolation between the 45-kDa and 66-kDa markers and the size of matriptase-2 by interpolation between the 116-kDa and 97-kDa markers. Immunoblots were imaged using chemiluminescence. The images in each *row* come from the same blot. The experiments were performed three times with consistent results.

inhibitor (Fig. 1). These results are consistent with generation of this cleavage product by matriptase-2, a serine protease (26). Notably, a faint band of lower molecular mass than the major matriptase-2 cleavage product was detected, which could be the result of heterogeneous cleavage. It is not the result of heterogeneous *N*-linked glycosylation of HJV (22). Interestingly, we found that the blockage of matriptase-2-cleaved sHJV by leupeptin was correlated with the appearance of the proprotein convertase-cleaved sHJV in the conditioned medium (Fig. 1). These results suggest both that the cleavage site for matriptase-2 is different from the cleavage site for proprotein convertases and that in the presence of excess matriptase-2, cleavage by a proprotein convertase is overridden by the processing by matriptase-2.

Endogenously Expressed Matriptase-2 in HepG2 Cells Is Able to Cleave HJV—We wanted to test whether the cleavage products identified in the medium of HepG2 cells are produced by proprotein convertases and matriptase-2. HepG2 cells were used because they are human hepatoma cell line and the liver is the major site of matriptase-2 expression in the body. Two different species of sHJV were previously detected in the conditioned medium from HepG2 cells that stably express HJV (HepG2-HJV) (13). Furin is a proprotein convertase that has been implicated in HJV cleavage and is ubiquitously expressed in tissues, including the liver (27). We first examined the relative abundance of matriptase-2 mRNA *versus* furin mRNA in HepG2 cells and compared it with that in HEK293 cells, rat liver, rat muscle, rat hepatocytes, and human liver. The level of matriptase-2 mRNA was slightly higher than that of furin in HepG2 cells (Fig. 2*A*). Their ratio was similar to those detected in rat liver, rat hepatocytes, and human livers, but was much higher than in HEK293 cells and rat muscle. These results indicate that HepG2 cells endogenously express both furin and matriptase-2 and that HEK393 cells do not express appreciable amounts of matriptase-2 mRNA.

The protease inhibitors, FCI and leupeptin, were also used to distinguish between the two protease activities. FCI blocked the release of the larger sHJV form, migrating at 40 kDa, whereas leupeptin blocked the 36-kDa form of sHJV (Fig. 2*B*). Cell surface biotinylation studies indicated that the cleavage of HJV was blocked by leupeptin after HJV had trafficked to the cell surface (supplemental Fig. 1). In the presence of both FCI and leupeptin, both forms of sHJV were no longer detectable in the medium, suggesting the lack of involvement of other classes of proteases.

To verify further that the smaller form of sHJV is indeed derived from matriptase-2 cleavage, we generated a HepG2 cell line that was stably transfected with matriptase-2 to increase the processing of HJV (HepG2-M2) (Fig. 2*C*). Transient introduction of HJV into HepG2-M2 cells increased the relative amount of the 36 kDa band and decreased the 40 kDa band. Formation of the 36 kDa band was blocked by leupeptin, but not FCI (Fig. 2*C*; lower panel). Knockdown of matiptase-2 by siRNA also reduces the lower molecular mass cleavage product (Fig. 2*D*). Interestingly, when HJV is expressed, more matriptase-2 protein is detected; however, the underlying mechanism is not known (Fig. 2*C*). Together, these results support that endogenously and exogenously expressed matriptase-2 in HepG2 cells is able to cleave HJV that is released from cells and migrates as a 36 kDa band.

Proprotein Convertase-mediated Cleavage of HJV Is Blocked in the R332A HJV Mutant—Treatment of cells with FCI blocks shedding of the 40 kDa (upper band) of HJV from HepG2 cells (Fig. 2) and the single, 40 kDa band of HJV from HEK293 cells (Fig. 1). To confirm that the 40 kDa band is generated by cleavage at the canonical proprotein convertase cleavage site cluster, mutations were made in the critical Arg residues in this motif (Fig. 3*A*). The proprotein convertase cleavage cluster in HJV has three overlapping R*XX*R motifs. Mutation of Arg^{329} should disrupt the first two motifs whereas mutation of Arg^{332} should disrupt the second two motifs. The cleavage and secretion of these HJVs were assessed in HEK293 cells (Fig. 3*B*). Although all of the HJVs are detected in the cell lysates, there is reduced secretion of R329A HJV and no detectable secretion of R332A HJV. When these constructs were transfected into HepG2 cells, the R329A mutation reduced shedding of the 40-kDa form into the medium (Fig. 3*C*). The R332A mutation also selectively and substantially reduces shedding. Mass spectrometry analysis confirmed that the second and third sites are both cleaved by proprotein convertases (supplemental Fig. 2). Thus, the single endogenous HJV cleavage product in HEK293 cells is produced by cleavage in the canonical proprotein convertases cleavage site cluster.

Immunofluorescence microscopy was employed to determine whether the R332A mutation in HJV blocks proprotein

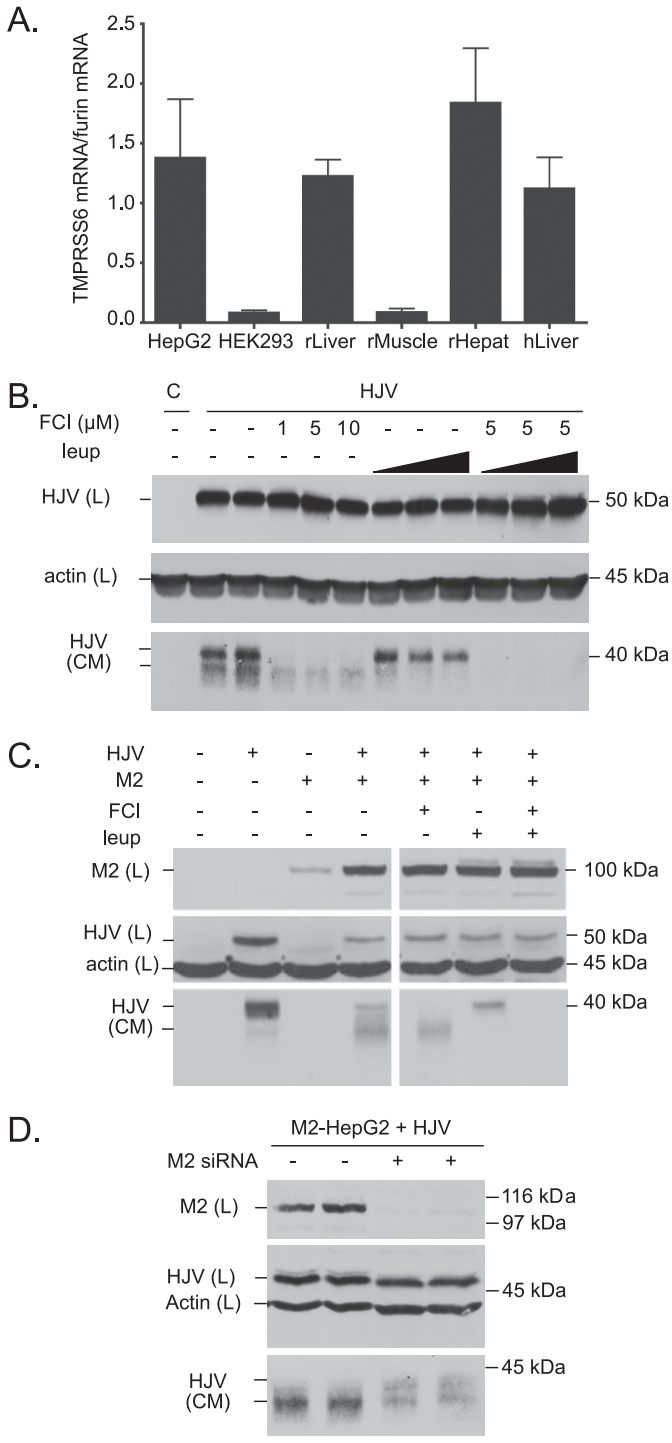

FIGURE 2. **Endogenously expressed matriptase-2 in HepG2 cells is able to cleave HJV.** *A*, quantitative RT-PCR analysis of TMPRSS6 (matriptase-2) and furin mRNA in HepG2 cells, HEK293 cells, rat liver, rat skeletal muscle (gastrocnemius), rat hepatocytes, and human liver is shown. The relative amounts of *TMPRSS6* mRNA *versus* furin mRNA in each specific cell type or tissues (mean ± S.D. (*error bars*)) are presented. Results are from three individual cDNA preparations of HepG2 and HEK293 cells, and five samples of rat liver (*rLiver*), rat muscle (*rMuscle*), rat hepatocytes (*rHepat*), and human liver (*hLiver*). *B*, secretion of the two isoforms of soluble HJV from HepG2-HJV cells is inhibited by either FCI or leupeptin (*leup*). HepG2 cells stably expressing HJV (HepG2-HJV) were subcultured into a 12-well plate in MEM/10% FBS. About 48 h after subculture, culture medium was changed to MEM/2% FBS with or without FCI (1, 5, and 10 μM) or leupeptin (10, 50, or 100 μM). Conditioned medium and cell lysates were prepared, and immunoblotting was performed as in Fig. 1. HepG2 cells stably transfected with pcDNA3 empty vector (*C*) were included as a negative control for HJV. *C*, increased expression of matriptase-2 in HepG2 cells suppressed the secretion of HJV that is cleaved by proprotein convertases. HepG2 cells stably transfected with pcDNA3 empty vector or pcDNA3-TMPRSS6 (matriptase-2) were subcultured into 12-well plates and transfected with either pcDNA3 empty vector or pcDNA3-HJV (*HJV*) as in Fig. 1. Conditioned medium (*CM*) and cell lysate were collected with or without FCI (5 μM) or leupeptin (100 μM) treatment. Immunodetection was performed as in Fig. 1. HepG2 cells stably transfected with pcDNA3 empty vector were included as a negative control for HJV. Immunoblots were imaged using chemiluminescence. These experiments were performed at least three times with consistent results. *D*, knockdown of matriptase-2 reduces HJV cleavage. Transfection of matriptase-2 or control siRNAs was conducted in 12-well plates in complete medium. After about 24 h, HJV was introduced using FuGENE 6 HD transfection reagent. Conditioned medium was collected, and cell lysates were prepared for immunodetection after about 72 h of siRNA transfection. siRNA knockdown greatly reduces the levels of matriptase-2. Matriptase-2 siRNA transfection reduces the amount of the 36-kDa HJV cleavage product and also leads to a slight increase in the 40-kDa furin cleavage product. Standard molecular mass markers are marked on the *right side* of the blot.

convertase-mediated cleavage of HJV by causing misfolding and endoplasmic reticulum (ER) retention of the protein (Fig. 3*D*). WT, R329A, and R332A HJV constructs were transiently transfected into HepG2 cells, which were subsequently detected with anti-HJV and anti-KDEL antibodies. The anti-KDEL antibody recognizes proteins with the KDEL ER-retrieval sequence and serves as a marker for the ER. Like WT HJV, the R329A and R332A HJVs have a punctate appearance (*red*) in contrast with the lacy morphology of the ER (*green*). Immunofluorescent microscopy indicates both cell surface and intracellular staining of all of these forms of HJV. In addition, little co-localization of HJV with the ER is detected. Therefore, the reduction in cleavage of R329A HJV and the block in cleavage of R332A HJV are not caused by the inability of these forms of HJV to fold and traffic past the ER.

R288A Mutation in HJV Specifically Blocks the Cleavage of HJV by Matriptase-2—Previous studies indicated that matriptase-2 cleaves its substrates at the carboxyl side of Arg residues (26). Based on our observations that sHJV generated by matriptase-2 cleavage is smaller than that cleaved by proprotein convertases, we reasoned that the major cleavage site by matriptase-2 lies N-terminal to the proprotein convertase cleavage site. From the molecular mass of the cleaved product and the preference of matriptase-2 to cleave after Arg, we predicted that Arg^{257} or Arg^{288} could be the sites of matriptase-2 cleavage.

HEK293 cells stably expressing HJV, R257A HJV (R257A), or R288A HJV (R288A) were transiently transfected with either pcDNA3 *TMPRSS6* or control (C), pcDNA3 empty vector, to determine which mutation blocks matriptase-2 cleavage. When matriptase-2 (M2) was introduced into HEK293-R257A and R288A HJV cells, however, the 36-kDa form of the sHJV was only detected in R257A HJV, but not in R288A HJV (Fig. 4*A*). Furthermore, leupeptin did not block the secretion of R288A HJV. As expected, introduction of matriptase-2 into HEK293-HJV cells shifted the molecular mass of sHJV from 40 to 36 kDa (Fig. 4*A*). These results suggest that Arg^{288} of HJV is likely the matriptase-2 cleavage site. Similar results were obtained when R288A HJV was introduced into HepG2-M2 cells (supplemental Fig. 3). Efforts to verify the cleavage site by mass spectrometry were unsuccessful (supplemental Fig. 2). Although we obtained 83% coverage of the

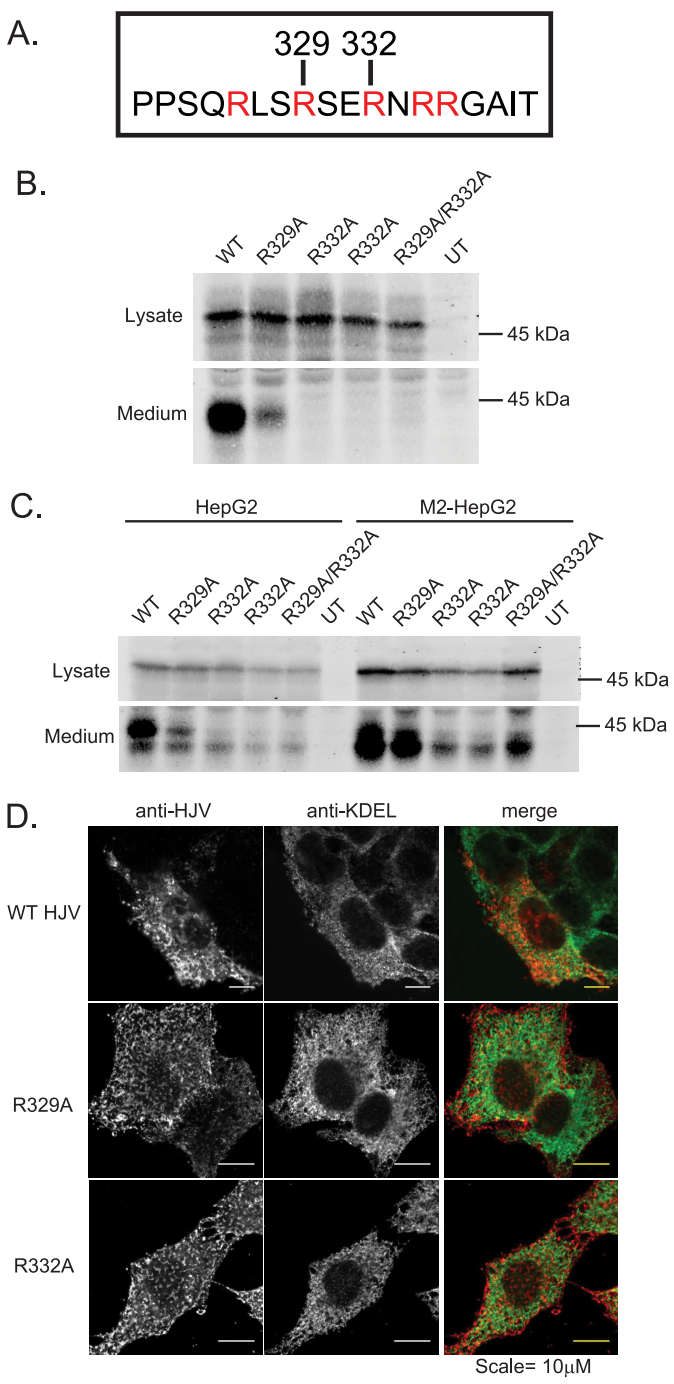

FIGURE 3. **Proprotein convertase-mediated cleavage of HJV is blocked by mutation of Arg^{332}.** *A*, schematic of the proprotein convertase cleavage motifs in human HJV is shown. The arginines that were mutated to alanines are labeled. *B*, HJV cleavage is blocked by the R332A mutation in HEK293 cells. These constructs were transiently transfected into HEK293 cells, and the presence of sHJV in the medium was assessed by immunoblot analysis. Fluorescent secondary antibodies were used to visualize HJV. Whereas the R329A mutation reduces HJV cleavage, the R332A mutation completely abolishes proprotein convertase-mediated cleavage of HJV in HEK293 cells. Two separate R332A HJV transfections are shown. This experiment was repeated with consistent results. Immunoblots were imaged using chemiluminescence. *C*, R332A mutation blocks the upper, proprotein convertase-generated cleavage product in HepG2 cells. Mutated HJV constructs were transfected into HepG2 cells or HepG2 cells stably expressing matriptase-2 (*M2-HepG2*) and HJV were analyzed in the medium and lysate as in *B*. *UT*, untransfected. This experiment was repeated with consistent results. *D*, R329A and R332A forms of HJV are not ER-retained. WT, R329A, and R332A HJV-pcDNA3 constructs were transiently transfected into HepG2 cells. Cells were stained with rabbit anti-HJV (*red*) and mouse anti-KDEL (*green*) antibodies and corresponding fluorescent secondary antibodies, and imaged by confocal microscopy. A single section is shown for each image, and the merged image is shown in the *third panel*. The *scale bar* is shown in the *bottom right corner*. The images shown are representative of several captured images for each form of HJV.

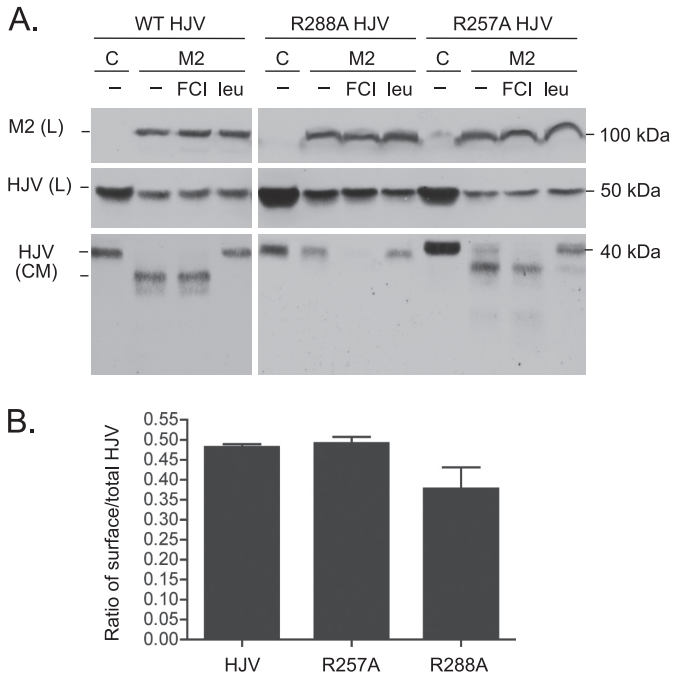

FIGURE 4. **Matriptase-2-mediated cleavage of HJV is blocked by mutation of Arg^{288}.** *A*, HEK293 cells are shown. HEK293 cells stably expressing HJV, R257A HJV, or R288A HJV were transfected with either pcDNA3 empty vector (*C*) or pcDNA3-TMPRSS6 (*M2*). Conditioned medium (*CM*) and cell lysate were collected after incubation with 5 μM FCI or 100 μM leupeptin (*leu*) for 24 h. Immunoblot analysis was performed as in Fig. 1. This experiment was done three times with consistent results. *B*, R288A HJV reaches the cells surface. Flow cytometry was performed on HEK293 cells stably expressing WT, R257A, and R288A HJV as described under "Experimental Procedures." The ratio of cell surface to total HJV was calculated, and the experiment was performed in triplicate. All three of these forms of HJV are detected on the cell surface to similar extents.

peptides of sHJV isolated from the medium, peptides in the region of matriptase-2 cleavage were not detected by mass spectrometry using arginase-, aspartyl-, or trypsin-generated fragments (supplemental Fig. 2). The lack of coverage of this area could possibly be due to posttranslational modification, heterogeneous matriptase-2 cleavage, or exoprotease digestion, which precludes confirmation of the matriptase-2 cleavage sites by mass spectrometry. Notably, a faint second band lower than the major matriptase-2 cleavage product can be detected. It could be the result of heterogeneous cleavage by matriptase-2.

Matriptase-2 is expressed at the cell surface (26). Misfolding of the R288A HJV and lack of trafficking to the cell surface could be a possible explanation for the lack of cleavage by matriptase-2. To test this possibility, flow cytometry was performed to determine the ratio of cell surface to total HJV in HEK293 cells that stably express WT, R257A, or R288A HJV. All three forms of HJV were detected on the cell surface (Fig. 4*B*). The average amount of HJV on the cell surface relative to total HJV was 49% for WT HJV, 48% for R257A HJV, and 38%

for R288A HJV. The differences between the different forms of HJV are not statistically significant by ANOVA. These results show that the R288A HJV reaches the cell surface. Therefore, the lack of R288A HJV cleavage by matriptase-2 does not result from sequestration of this mutated protein in the ER.

Truncation of HJV after Arg^{257} or Arg^{288} Results in ER Retention of HJV—To confirm that Arg^{288} is the site of HJV cleavage by matriptase-2, we created truncations that could be compared with the size of shed HJV generated by matriptase-2 cleavage. These truncated forms of HJV were created by placing stop codons (X) after the candidate Arg residues at positions 258 and 289. When these truncated forms of HJV were expressed in HEK293 cells, they were detected in the cell lysate but did not appear to be secreted (Fig. 5*A*). In contrast, HJV truncated at the proprotein convertase cleavage motif cluster was secreted (supplemental Fig. 4). The glycosylation differences of the P258X HJV in the lysate and the shed HJV produced by matriptase-2 cleavage in the medium precluded comparison of the sizes of these two forms of HJV.

Immunofluorescence microscopy was performed to compare the subcellular localization of the P258X and Q289X HJV to the anti-KDEL ER marker. Both forms of HJV extensively co-localized with the ER (Fig. 5*B*). Additionally, in contrast to WT HJV, very little punctate staining was evident. These results indicate that P258X and Q289X HJVs are not shed because they are retained in the ER, possibly due to misfolding. Furthermore, these data suggest that the region between the Arg^{288} and the proprotein convertase cleavage cluster is important for folding and secretion of HJV. Together, these results demonstrate that endogenous matriptase-2 cleaves HJV in a site-specific manner, which would result in a reduction in membrane HJV available for BMP signaling.

sHJV Generated by Matriptase-2 Has Reduced Binding to BMP6 and Does Not Suppress BMP6-induced Hepcidin Expression—sHJV generated by proprotein convertase cleavage (sHJV/PC) binds to BMP6 and inhibits BMP6-induced hepcidin expression (10). To determine the potential role of sHJV generated by matriptase-2 cleavage (sHJV/M2) in the regulation of hepcidin expression, we tested its ability to bind BMP6. Concentrated sHJV/M2 with a FLAG tag at the N terminus was incubated with BMP6, followed by pulldown of HJV. sHJV/PC with a FLAG tag at the N terminus was included as a positive control. The anti-FLAG beads pulled down both sHJV/PC and BMP6 in the positive control. In contrast, less BMP6 was pulled down with sHJV/M2 (Fig. 6*A*). These observations indicate that sHJV/M2 has reduced binding to BMP6.

We also compared the effects of concentrated sHJV/M2 and sHJV/PC (Fig. 6*B*) on BMP6-induced hepcidin expression in HepG2 cells (Fig. 6*C*). In agreement with the results of BMP6 binding assay, sHJV/PC, but not sHJV/M2, suppressed the induction of hepcidin expression by BMP6 (Fig. 6*C*). Although matriptase-2 has been shown previously to reduce hepcidin expression by decreasing the amount of cell surface HJV available for signaling (20), these results suggest that sHJV/M2 lacks the additional ability to reduce BMP signaling by competing with membrane-bound HJV for binding to BMPs.

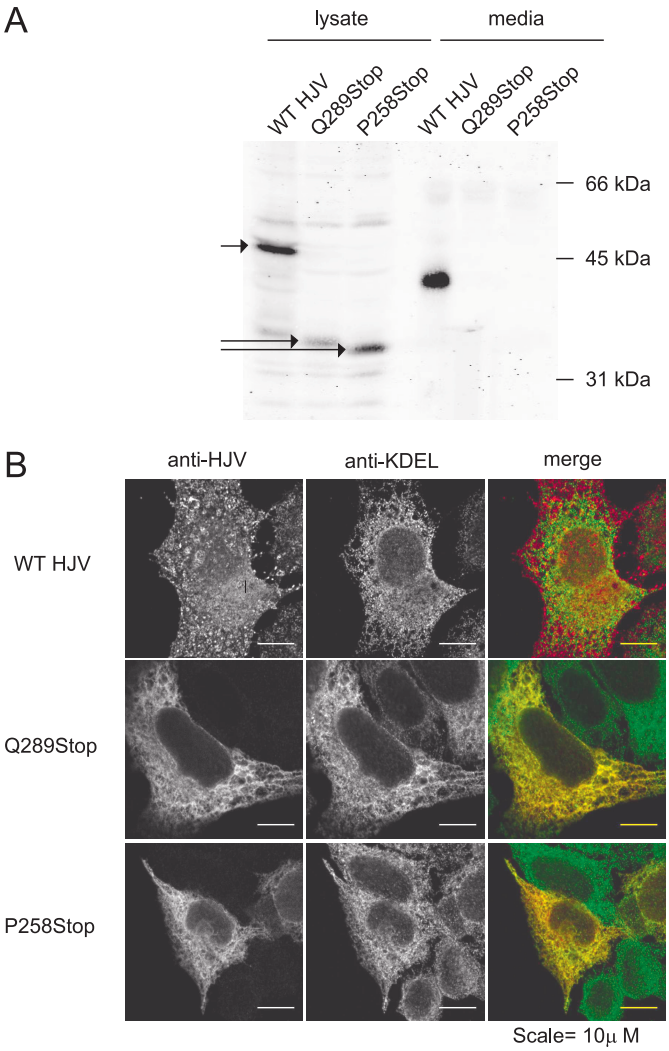

FIGURE 5. **Truncation of HJV after Arg^{257} or Arg^{288} results in ER retention of HJV.** *A*, P258X- and Q289X-HJV are not secreted. Site-directed mutagenesis was used to put a stop codon at positions 258 or 289 in HJV. These constructs were transfected into HEK293 cells, and the presence of HJV was analyzed in the medium and the lysate by immunoblotting. Fluorescent secondary antibodies were used to visualize the bands. *Arrows* mark the HJV detected in the lysate. This experiment was repeated with similar results. *B*, P258X- and Q289X-HJVs are retained in the ER. The constructs were transfected into HepG2 cells and then stained using rabbit anti-HJV (*red*) and mouse anti-KDEL (*green*) antibodies and corresponding fluorescent secondary antibodies and imaged by confocal microscopy. A single section is shown for each image, and the merged image is shown in the *third panel*. The *scale bar* is shown in the *bottom right corner*. The images shown are representative of several captured images for each form of HJV.

DISCUSSION

In the present study we show that matriptase-2 cleaves glycosylphosphatidylinositol-linked HJV in a site-specific manner and generates one major HJV cleavage product. Site-directed mutagenesis of HJV Arg^{288} to Ala abolished cleavage by matriptase-2, indicating Arg^{288} as the likely cleavage site. These results are in contrast to a previous study by Silvestri *et al.* showing that expression of matriptase-2 produces a ladder of cleaved HJV products in the conditioned medium (20).

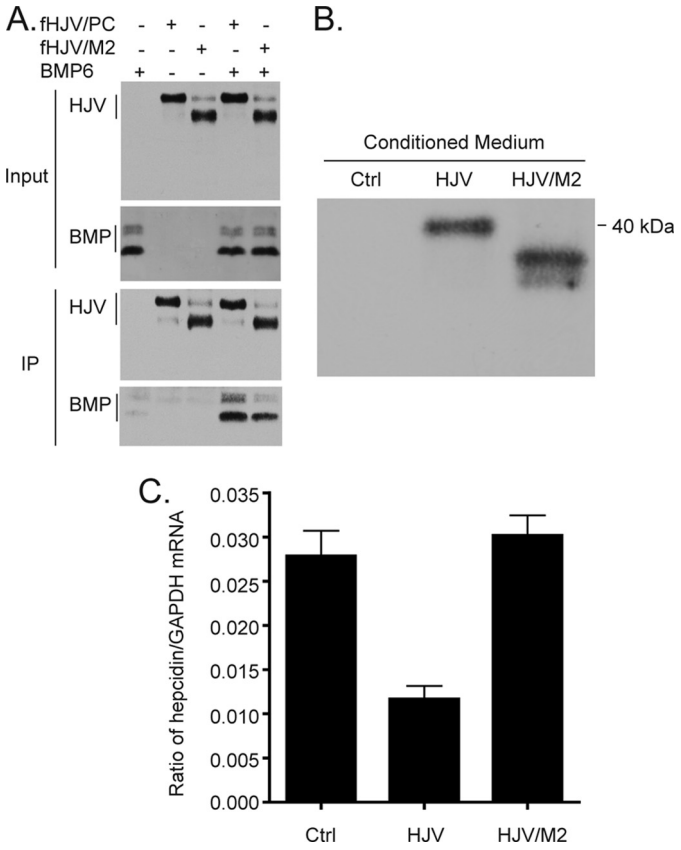

FIGURE 6. **sHJV generated by proprotein convertase cleavage suppresses BMP6-induced hepcidin expression, but sHJV produced by matriptase-2 does not.** *A*, sHJV/BMP6 binding assay is shown. BMP6 (500 ng) alone, fHJV/PC (500 ng) alone, fHJV/M2 (500 ng) alone, or BMP6 (500 ng) in combination with fHJV/PC (500 ng) or fHJV/M2 (500 ng) was incubated at 4 °C overnight in 250 μl of 50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH 7.4. sHJV was pulled down with an anti-FLAG-M2 affinity gel (Sigma). sHJV and BMP6 in 2% of input, sHJV in 10% of eluate, and BMP6 in 90% of eluate were immunodetected using the corresponding antibodies. This experiment was performed three times with consistent results. *B*, immunoblot analysis of sHJV in the concentrated conditioned medium from HEK293, HEK293-HJV, and HEK293-HJV/matriptase-2 cells is shown. 10 μl of the media described in *C* was subjected to SDS-PAGE followed by immunodetection of HJV using chemiluminescence. *C*, sHJV produced by matriptase-2 does not suppress BMP6-induced hepcidin expression in HepG2 cells. HepG2 cells were subcultured into 12-well plates. After 48 h of culture, medium was switched to MEM/1% FBS and incubated for 6 h to serum-starve the cells. The medium was then changed to MEM/1% FBS with 10% concentrated conditioned medium in volume from HEK293 (*C*), HEK293-HJV (*HJV*) and HEK293-HJV/matriptase-2 cells (*HJV/M2*), respectively, as well as 2.5 ng/ml BMP6. The estimated final concentrations of sHJV in HJV and HJV/M2 groups were about 2 μg/ml. After 18 h of incubation, total RNA was isolated, and cDNA was prepared for quantitative RT-PCR analysis of hepcidin and GAPDH mRNA. The hepcidin mRNA levels are expressed as the amount relative to that of GAPDH in each specific sample. The results are from three separate experiments and the mean values ± S.D. (*error bars*) are presented.

This discrepancy might be due to the different cell types used or to the levels of matriptase-2 expressed.

We also show that expression of HJV increases the levels of matriptase-2. Matriptase-2 binds HJV (20). Formation of a complex between HJV and matriptase-2 could stabilize matriptase-2 and reduce its degradation. The physiological implications of this observation are not known and need to be examined.

The proprotein convertase cleavage site in HJV does not overlap with that of matriptase-2. In addition to the different sizes of the two cleaved products, we found that the R332A mutation in HJV blocks proprotein convertase-mediated cleavage but not matriptase-2 cleavage. HJV has an overlapping cluster of three R*XX*R motifs, all of which are consensus sequences for proprotein convertase cleavage (27). The R329A mutation in HJV, which is predicted to disrupt the first and second cleavage motifs, reduces proprotein convertase-mediated HJV cleavage, but does not abolish it, indicating that the third site is likely utilized to some extent. This also indicates that at least one of the first two sites is utilized. The R332A HJV mutation, which is expected to abolish cleavage at the second and third site, diminishes proprotein convertase-mediated secretion. Therefore, the second and third sites are both capable of being cleaved by proprotein convertases. The third site was shown previously to contribute to proprotein convertase-mediated cleavage of HJV by mutational analysis (17) and by N-terminal protein sequencing of the proprotein convertase products of a HJV-alkaline phosphatase fusion construct (25). These data extend our understanding of the ability of proprotein convertases to cleave HJV at multiple consensus motifs.

Notably, the matriptase-2 HJV cleavage product decreases when proprotein convertase cleavage is inhibited by FCI or mutation of the R332A cleavage site. The degree to which blocking proprotein convertase-mediated cleavage of HJV reduces matriptase-2 cleavage is somewhat variable from experiment to experiment. Several possibilities could explain this effect. Proprotein convertase-mediated cleavage could promote subsequent matriptase-2 cleavage. Alternately, matriptase-2 cleavage could occur with or without prior proprotein convertase cleavage. In the absence of cleavage by proprotein convertases, matriptase-2 cleavage may occur at multiple sites and results in smaller cleavage products that are not detected by immunoblot analysis. Further experiments are needed to examine the order of HJV cleavage by both proprotein convertases and matriptase-2 with respect to HJV trafficking and regulation by iron.

The liver is the major organ that expresses both HJV and matriptase-2, and we found that HepG2 cells endogenously express matriptase-2 mRNA. Importantly, the ratio of matriptase-2 mRNA to that of one proprotein convertase, furin, in HepG2 cells is similar to the ratios in the liver, and in isolated primary hepatocytes. HepG2 cells are therefore a good model cell line to study the cleavage of HJV by matriptase-2.

Previous studies detected two major soluble HJV products in the conditioned medium of HepG2 cells that express HJV (13, 22, 28). Here, we show that they are the cleavage products of matriptase-2 and proprotein convertases. Expression of the proprotein convertase, furin, is ubiquitously detected in all tissues as well as in HepG2 cells. Other proprotein convertases are also expressed in the liver (29). Our experiments do not differentiate among the different possible proprotein convertases that could be involved in HJV processing.

The role of sHJV generated by matriptase-2 cleavage in the regulation of BMP6-induced hepcidin expression was analyzed. The sHJV generated by proprotein convertase cleavage binds to BMPs, which may sequester BMPs away from membrane-anchored HJV and BMP receptors to reduce signaling

(9, 15, 30, 31). Here, we show that in contrast to the proprotein convertase cleavage product, the smaller matriptase-2-generated sHJV has reduced binding to BMP6 and was unable to antagonize BMP6-induced hepcidin transcript levels in HepG2 cells. Previous studies have shown that cellular HJV is essential for the induction of hepcidin expression (4–7, 15) and that matriptase-2 reduces the amount of cell surface HJV and suppresses HJV-induced hepcidin expression (20). These results, in conjunction with our data, support a model in which matriptase-2 suppresses hepcidin expression by cleaving cellular HJV to produce a form of HJV with reduced affinity for BMPs. This study shows that endogenously expressed matriptase-2 cleaves HJV in a site-specific manner, strengthening the link between matriptase-2 and the regulation of hepcidin expression.

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