Low Intracellular Iron Increases the Stability of Matriptase-2*

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Matriptase-2 (MT2) is a type II transmembrane serine protease that is predominantly expressed in hepatocytes. It suppresses the expression of hepatic hepcidin, an iron regulatory hormone, by cleaving membrane hemojuvelin into an inactive form. Hemojuvelin is a bone morphogenetic protein (BMP) coreceptor. Here, we report that MT2 is up-regulated under iron deprivation. In HepG2 cells stably expressing the coding sequence of the MT2 gene, Tmprss6, incubation with apo-transferrin or the membrane-impermeable iron chelator, deferoxamine mesylate salt, was able to increase MT2 levels. This increase did not result from the inhibition of MT2 shedding from the cells. Rather, studies using a membrane-permeable iron chelator, salicylaldehyde isonicotinoyl hydrazone, revealed that depletion of cellular iron was able to decrease the degradation of MT2 independently of internalization. We found that lack of the putative endocytosis motif in its cytoplasmic domain largely abolished the sensitivity of MT2 to iron depletion. Neither acute nor chronic iron deficiency was able to alter the association of Tmprss6 mRNA with polyribosomes in the liver of rats indicating a lack of translational regulation by low iron levels. Studies in mice showed that Tmprss6 mRNA was not regulated by iron nor the BMP-mediated signaling with no evident correlation with either Bmp6 mRNA or Id1 mRNA, a target of BMP signaling. These results suggest that regulation of MT2 occurs at the level of protein degradation rather than by changes in the rate of internalization and translational or transcriptional mechanisms and that the cytoplasmic domain of MT2 is necessary for its regulation.

Iron is an essential nutrient for life, but it is also toxic when in excess. Because humans cannot control the excretion of excess iron from the body, systemic iron homeostasis is maintained by coordinate regulation of the iron absorption in the duodenum, iron recycling from senescent erythrocytes in macrophages, and mobilization of stored iron in the liver (1, 2). Hepcidin, a key iron regulatory hormone, plays an essential role in this process. Hepcidin is a 25-amino acid peptide secreted predominantly by hepatocytes. It inhibits iron efflux into the circulation by binding to and targeting ferroportin on plasma membrane for degradation (3). Ferroportin is the only known iron exporter. It is expressed on duodenal enterocytes, macrophages, and hepatocytes. Lack of hepcidin causes juvenile hemochromatosis, a particularly severe form of iron overload disorder (4). In contrast, inappropriately high levels of hepcidin cause iron-deficiency anemia (1, 2, 5). A high level of iron in the body up-regulates hepcidin expression thus providing a negative feedback to maintain iron homeostasis.

Recent studies document the pivotal roles of hemojuvelin (HJV)7 in the up-regulation of hepcidin expression (6–12) and matriptase-2 (MT2, encoded by the gene Tmprss6 in humans and Tmprss6 in mice and rats) in the suppression of hepcidin expression. Mutations in Tmprss6 result in increased hepcidin expression, which leads to iron-refractory iron-deficiency anemia (13). Similar phenotypes are also reported in mouse models either with knockdown of both Tmprss6 alleles or with a truncated Tmprss6 that lacks the catalytic domain (mask mice), indicating that iron-refractory iron-deficiency anemia is caused by lack-of-function mutations in Tmprss6 (14, 15). MT2 is a serine protease (16). Tmprss6 is predominantly expressed in hepatocytes (17). This type II transmembrane protease is composed of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain, which contains a membrane-proximal stem region, a predicted activation site, and a catalytic domain. MT2 lack the catalytic domain of Tmprss6 (16). Tmprss6 is predominantly expressed in hepatocytes (17). This type II transmembrane protease is composed of a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain, which contains a membrane-proximal stem region, a predicted activation site, and a catalytic domain. MT2 lack the catalytic domain of Tmprss6 (16).

* This work was supported, in whole or in part, by National Institutes of Health Grants DK080765 and DK102791 (to A. S. Z.), DK054488 (to C. A. E.), K99DK104066 (to N. Z.), DK066600 (to R. S. E.), P50AI11199 (to H. T.), and R24AA12885 (to H. T.).
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4432 JOURNAL OF BIOLOGICAL CHEMISTRY

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domain, and a C-terminal catalytic domain (18). The cytoplasmic domain of MT2 contains an endocytosis motif that mediates the internalization of cell surface MT2 in a dynamin-dependent manner (19). The only identified iron-related substrate for MT2 is HIV (20).

In contrast to MT2, HIV is a glycosylphosphatidylinositol-linked membrane protein (21). It is mainly expressed in hepatocytes, skeletal muscle, and heart (22, 23). HIV acts as a co-receptor for BMP6 in hepatocytes to robustly induce hepcidin expression through the BMP-signaling pathway (24). Homozygous or compound heterozygous mutations in the HIV gene, HFE2, in humans markedly reduce hepatic hepcidin expression and result in juvenile hemochromatosis with the clinical manifestations indistinguishable from lack of hepcidin (4, 22). Similarly, a pronounced decrease in hepcidin expression and severe iron overload are also present in mice with a global disruption or liver-specific disruption of both Hjv alleles (6, 7, 22, 25). MT2 binds HIV through its stem region and cleaves it into a nonessential amino acids (complete medium). HepG2 cells stably transfected with pcDNA3 empty vector (HepG2-H11002) or pcDNA3-TMPRSS6 (HepG2-MT2-H11003) were maintained in the complete medium with 800 μm SIH for 18 h. Cell surface proteins were biotinylated at 4 °C as described above, followed by incubation at 37 °C for 0, 10, and 20 min in the same medium. Cell surface proteins were then subjected to Pronase digestion by incubating cells for 60 min at 4 °C in DMEM containing 30 μg/ml Pronase (Roche Applied Science) and 20 mM HEPES, pH 7.4. The Pronase activity was quenched by adding ice-cold PBS containing 10 mg/ml BSA. After four washes with PBS, cells were solubilized in NET/Triton X-100 1 × protease inhibitor mixture (Roche Applied Science). Biotinylated proteins were isolated using neutroavidin-agarose beads (Thermo Fisher Scientific). Bound proteins were eluted with 0.25 mg/ml Sulfo-NHS-Biotin (Thermo Fisher Scientific) at 4 °C for 30 min. After the reaction was terminated, cells were immediately solubilized in NET (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4)/Triton X-100 buffer subject to SDS-PAGE, followed by immunodetection of MT2, neogenin, TfR1, Na+/K+-ATPase, ferritin, and β-actin. Alternatively, bound proteins were first eluted with NET/Triton X-100, 1% β-mercaptoethanol, 0.5% SDS and then subjected to digestion with Endo-H or PNGase F (New England Biolabs) as described previously (28), followed by SDS-PAGE and immunodetection of MT2 and neogenin.

MT2 Internalization—The internalization of biotinylated cell surface MT2 in HepG2-MT2 cells was performed as described previously (19, 30) with some modifications. Briefly, HepG2-MT2 cells on polystyrene-coated 60-mm plates were incubated in complete medium (MEM, 10% FCS) with or without 100 μm SIH for 18 h. Cell surface proteins were biotinylated at 4 °C as described above, followed by incubation at 37 °C for 0, 10, and 20 min in the same medium. Cell surface proteins were then subjected to Pronase digestion by incubating cells for 60 min at 4 °C in DMEM containing 30 μg/ml Pronase (Roche Applied Science) and 20 mM HEPES, pH 7.4. The Pronase activity was quenched by adding ice-cold PBS containing 10 mg/ml BSA. After four washes with PBS, cells were solubilized in NET/Triton X-100 1 × protease inhibitor mixture (Roche Applied Science). Biotinylated proteins were isolated as described above for immunodetection.

Immunoprecipitation of MT2 and ZIP14—HepG2-MT2 and -fZIP14 cells were incubated with or without 10 μm MG-132 in
Low Iron Increases Matriptase-2

complete medium for 6 h. MT2 and fZIP14 were immunoprecipitated using rabbit anti-MT2-coated protein A-Sepharose 4B conjugate (Invitrogen) and anti-FLAG M2-agarose affinity gel (Sigma), respectively. The eluates were subjected to SDS-PAGE and immunodetection of ubiquitin, MT2, and ZIP14.

Immunodetection—Cell lysates, conditioned medium, streptavidin eluates, immunoprecipitation eluates, or the rat liver extracts were separated by SDS-PAGE under reducing conditions, followed by transfer onto a nitrocellulose membrane. Membranes were probed with rabbit anti-neogenin 21567 antibody (1:10,000; Zymed Laboratories Inc.), mouse anti-Na+/K+-ATPase α1 (1:500; Santa Cruz Biotechnology), rabbit anti-ferritin (1:10,000; Dako), rabbit anti-ubiquitin (1:1000; Santa Cruz Biotechnology), mouse anti-FLAG (1:10,000; Sigma), or mouse anti-β-actin antibody (1:10,000; Chemicon International), followed by immunodetection using a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Chemicon International) and chemiluminescence (SuperSignal, Pierce). Alternatively, MT2 was detected using an Alexa Fluor 680 goat anti-rabbit secondary antibody (1:10,000; Invitrogen) and visualized using an Odyssey Infrared Imaging System (LiCor), and the intensities of MT2 bands were quantified. Rabbit anti-MT2 (23144) IgG cross-reacts with rat MT2 (17). Generation of Rats with Acute and Chronic Iron Deprivation and Polysome Fractionation—Rats with acute and chronic iron deprivation were generated in the animal facility at the Department of Nutritional Sciences, University of Wisconsin (17, 31). Briefly, weanling male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were randomly assigned to diet groups with free access to either a control diet (50 mg of iron/kg diet, group control) or they were pair-fed an iron-deficient diet (less than 2 mg of iron/kg diet, group ID). The pair-fed group receives the same amount of diet as consumed by the group fed the iron-deficient diet to equalize energy intake. All animals had free access to water. After feeding the diets for 3 or 14 days, animals were anesthetized with carbon dioxide, and livers were rapidly removed and snap-frozen in liquid nitrogen and then stored at −80 °C for qRT-PCR, polysomal fractionation, and Western blot analysis. There are either three or five animals/group as indicated. All procedures for animal use met the requirements of the University of Wisconsin Research Animal Resource Center.

Polysomal fractionation and RNA isolation were performed as described previously (32, 33). Briefly, liver tissues were minced and homogenized in ice-cold polysome buffer (PB: 40 mM HEPES, pH 7.4, 100 mM KCl, 5 mM MgCl2, 2 mM citrate, and 1 mM DTT). The homogenate was centrifuged at 5000 g for 20 min. The upper two-thirds of the supernatant were collected and brought to 1% sodium deoxycholate, 1% Triton X-100 in PB. A 500-μl sample was loaded on a linear 15–60% sucrose gradient in PB and centrifuged at 180,000 × g in a Sorvall TH641 rotor for 2 h at 4 °C. The gradients were then fractionated. The absorbance at 254 nm was continuously monitored and 11 1-min fractions were collected. Fractions 1–4 contained the free protein pool, the ribonucleoprotein (RNP) particle pool, the ribosomal subunits, and the 80 S monosome. Fractions 5–11 contained the light and heavy poly-somes. Total RNA was isolated from 500 μl of each gradient fraction using RNA Stat-60 reagent (Tel-Test, Inc.). RNA from each gradient was reverse-transcribed to synthesize total cDNA. The total amount of mRNA for Tmprss6, mitochondrial aconitase, and β-actin was quantified by quantitative real time PCR (qRT-PCR) with SYBR Green. The amount of mRNA in each fraction was expressed as the percentage of the combined total mRNA in all fractions.

Hjv−/− Mice—The liver tissues from Hjv−/− mice with or without a liver-specific introduction of Hjv cDNA were the same as in our previous studies (34). Briefly, Hjv−/− mice on 129/SvEvTac (129/S) background were obtained from Dr. Nancy Andrews (Duke University). Eight-week-old male mice were injected with ~5 × 1011 AAV8-Hjv particles per mouse via the tail vein. The expression of Hjv in AAV8 vector is under the control of a strong liver-specific promoter. Two weeks later, mice were anesthetized for analysis. Each group consisted of five animals.

Isolation of Liver Cell Populations from Iron-loaded Wild-type Mice—The whole liver tissues and the isolated hepatocytes, Kupffer cells (KCs), sinusoidal endothelial cells (SECs), and hepatic stellate cells (HSCs) from control and iron-loaded wild-type mice were the same as in our previous studies (35). Briefly, wild-type (8-week-old) 129/S male mice (Taconic) were randomly assigned to two different groups with free access to either a high iron rodent diet with 2% carbonyl iron (TD.08496; Harlan Laboratories) or a control rodent diet with 48 ppm iron (TD.80394; Harlan Laboratories). After 3 weeks of feeding, livers were perfused for isolation of hepatocytes, KC, SEC, and HSC by the NonParenchymal Liver Cell Core of the Southern California Research Center for ALPD and Cirrhosis (P50AA11199, R24AA12885) as described previously (35). The purity of each cell type was greater than 95%. The whole liver tissues were collected from a parallel set of animals. All procedures for animal use met the requirements of the Department of Comparative Medicine at Oregon Health & Science University.

qRT-PCR Analysis—qRT-PCR was used to analyze the following: Tmprss6, hepcidin, ID1, SMAD7, and β-actin mRNA levels in HepG2 cells after incubation with BMP6 at 0, 5, 25, and 50 ng/ml for 18 h; Tmprss6 and β-actin mRNA levels in HepG2-Control and HepG2-MT2 cells after incubation with apo-Tf, holo-Tf, DFO, or SiH for 18 h; hepcidin, Tmprss6, mitochondrial aconitase, and β-actin mRNA levels in whole rat liver tissues and the liver polysomal fractions; and hepcidin, Bmp6, Id1, Smad7, Tmprss6, Hjv, and β-actin mRNA levels in whole liver tissues of mice or isolated liver cell populations. The procedures for total RNA isolation and cDNA preparation were described previously (17). qRT-PCR analysis was performed as reported previously (28). Primer sequences for human Tmprss6, human hepcidin, human β-actin, rat Tmprss6, rat hepcidin, rat β-actin, mouse Tmprss6, mouse hepcidin, mouse Hjv, mouse Bmp6, mouse Id1, mouse Smad7, and mouse β-actin are the same as reported previously (17, 23, 28, 34–36). The sequences for other primers are as follows: 5′-ACGATCGCATTCTTGCTCGCT-3′ (human ID1, forward) and 5′-AGAAGCCACAGGCAAGAATT-3′ (human ID1, reverse); 5′-CAATGACCCACGAGTTTATGCA-3′ (human SMAD7, forward) and 5′-GTTGAAGATGACCTCTAGCCA-3′ (human SMAD7, re-
verse); and 5′-GATCGAGCCACTATCGA-3′ (rat mitochondrial aconitase, forward) and 5′-TGGATCAAAGTCCGATC-G-3′ (rat mitochondrial aconitase, reverse). All primers were verified for linearity of amplification. The results for each gene of interest are expressed as the amount of mRNA relative to β-actin.

RESULTS

Iron Deprivation Increases MT2 Levels in HepG2 Cells—Previous studies indicated that acute iron deficiency in weanling rats results in a rapid decrease in transferrin (Tf) saturation, an increased MT2 protein in the liver, but an unchanged hepatic Tmprss6 mRNA levels (17). We wanted to determine whether the increase of MT2 results from the decreased Tf saturation or the reduced intracellular iron load. Tf is the major iron transport protein in plasma, and its degree of saturation with iron is indicative of iron in the body. HepG2 cells stably expressing transient Tmprss6 coding sequence (HepG2-MT2) were used for the studies. HepG2 cells are a human hepatoma cell line that endogenously expresses readily detectable Tmprss6 mRNA by qRT-PCR but undetectable protein levels by Western blot using available antibodies (36). HepG2-MT2 cells were incubated with either 25 μM iron-free Tf (apo-Tf) or 25 μM diferric Tf (holo-Tf) for 18 h. Holo-Tf served as a physiological iron source to increase iron loading in the cells. Apo-Tf was used to chelate the non-Tf-bound iron in the medium. Previous studies indicate that apo-Tf facilitates iron release from hepatic cells (37, 38) and reduces iron uptake into the immature erythroid cells of mice and the cultured K562 cells (39). Incubation with apo-Tf led to increased cell death in both total MT2 (input) and biotinylated cell surface MT2 (Fig. 1A). The Na⁺,K⁺-AT-Pase, a cell surface protein, was used as a loading control for cell surface biotinylation. As a negative control, no MT2 was detected in HepG2 cells stably transfected with pcDNA3 empty vector (HepG2-Ctrl). The increase of cell surface MT2 was ~2.3-fold (Fig. 1B), which is similar to the extent of increase of MT2 in the liver of rats fed an iron-deficient diet for 1 day (17).

By also determined whether the level of neogenin was changed with iron loading of cells because MT2 also binds to neogenin and neogenin facilitates MT2 cleavage of HJV from the plasma membrane. Depletion of neogenin leads to an accumulation of MT2 within the cells (28). Neogenin levels remained unchanged with apo- and holo-Tf treatments (Fig. 1A) indicating that neogenin was not responsible for the increased MT2 levels when cells were treated with apo-Tf.

Tmprss6 mRNA was measured to determine whether it was regulated at the message level. As expected in HepG2-MT2 cells that express MT2 under the control of the CMV promoter, neither apo-Tf nor holo-Tf altered Tmprss6 mRNA (Fig. 1C). The endogenous Tmprss6 mRNA levels in apo- and holo-Tf-treated HepG2-Ctrl cells also did not change with treatment indicating a lack of transcriptional control (Fig. 1C).

Interestingly, we detected a mild increase in Tfr1 levels (~1.5-fold) in apo-Tf treated cells when compared with the untreated cells (Fig. 1A, lane 3 versus 2). The increase in Tfr1 is an indirect indicator of iron deprivation. This is consistent with the previous observations that apo-Tf facilitates iron release from hepatic cells (37, 38) and the hypothesis that excess apo-Tf reduces the cellular iron uptake by increasing the amount of monoferric Tf (39). Each molecule of monoferric Tf delivers less iron than holo-Tf into the cells. As expected, holo-Tf-treated cells showed an increase in ferritin, suggesting that these cells were iron-overloaded (Fig. 1A, lane 4 versus 2). These observations suggest that apo-Tf increases MT2 by lowering intracellular iron.

To determine the contribution of iron depletion to the increased MT2, we examined the effects of an impermeable iron chelator, DFO. HepG2-MT2 cells were incubated with 0, 25, 50, 75, and 100 μM DFO for 18 h. Interestingly, DFO increased MT2 levels both in cell lysate and on the cell surface in a concentration-dependent manner (Fig. 1D). The levels of cell surface MT2 reached a plateau at 50 μM DFO (Fig. 1, D and E). The two different molecular mobilities of cell surface MT2 suggest that MT2 is heterogeneous process. DFO mainly increased the upper band of the two MT2 species (Fig. 1D) but did not change the MT2 mRNA levels (data not shown). There are seven potential Asn glycosylation sites in the MT2 sequence (20). To determine whether these two forms of MT2 represent differential maturation of Asn-linked oligosaccharides, biotinylated plasma membrane proteins were subjected to the digestion with either Endo-H, which cleaves high mannos oligosaccharides added co-translationally in the endoplasmic reticulum, or PNGase-F, which cleaves both high mannose and Golgi-modified complex oligosaccharides. The upper MT2 band was partially sensitive to Endo-H digestion, and the lower bands are fully sensitive to Endo-H digestion (Fig. 1F). Both bands are sensitive to PNGase-F digestion indicating that the oligosaccharides on MT2 are responsible for the heterogeneity detected on gels. No change in the oligosaccharides on neogenin was detected (Fig. 1F), suggesting that the increase in processing of MT2 is not a general phenomenon. Together, these observations suggest that the decreased cellular iron load, rather than apo-Tf itself, increases MT2 in both the cell lysate and cell surface.

Lack of Changes in Shed MT2 under High and Low Iron Conditions—The ecto-domain of MT2 can be shed from cells (20, 40). To test the possibility that depletion of intracellular iron blocks MT2 shedding and therefore increases cellular MT2, we compared the accumulated amount of shed MT2 in the conditioned media of HepG2-MT2myc cells after incubation in the presence of 100 μM DFO or 10 μg/ml ferric ammonium citrate (FAC) for 18 h. FAC is widely used as a non-Tf form of iron source to load cells with iron. Consistent with the previous observations (20, 40), shed MT2 is readily detectable in the conditioned media of two different clones of HepG2-MT2myc cells (Fig. 2). Neither decreased intracellular iron (DFO) nor increased iron load (FAC) altered the amount of soluble MT2 in the conditioned medium (Fig. 2). The increased levels of ferritin compared with the actin loading control indicate that the FAC-treated cells were indeed iron-loaded. These results suggest that the increased MT2, upon iron depletion, does not appear to result from the decreased MT2 shedding.

Depletion of Cellular Iron Decreases the Degradation of MT2—Because neither shedding of the MT2 ecto-domain nor changes in neogenin levels could account for the increase in MT2, we wanted to examine whether iron deprivation affects
the rate of MT2 degradation. SIH, a membrane-permeable iron chelator, was employed for the studies. SIH is a widely used chelator to rapidly decrease intracellular iron. HepG2-MT2 cells were incubated with or without 100 μM SIH for 18 h. As shown in Fig. 3A, incubation with SIH resulted in significant increases in both cell lysate and cell surface MT2, which is similar to that of DFO in Fig. 1D. As expected, depletion of intracellular iron elevated TfR1 levels (Fig. 3A, lane 3 versus 2). Here, HepG2-Ctrl cells are included as a negative control to show the specific MT2 bands. HepG2-Ctrl cells had a higher basal level of TfR1 than HepG2-MT2 cells (Fig. 3A, lane 1 versus 2). No changes in neogenin were observed. These results strengthen the association of decreased cellular iron with the increases in MT2 protein.

MT2 undergoes a rapid degradation in HepG2 cells (17). To test the hypothesis that iron depletion prevents the degradation of MT2 to increase the steady state level of MT2, we examined the rate of MT2 degradation in the presence of SIH. HepG2-MT2 cells were first preincubated with 100 μM SIH for 18 h to deplete the intracellular iron, followed by measuring the degradation of MT2 at 2, 4, and 6 h in the presence of the same concentration of SIH and 100 μg/ml cycloheximide. Cycloheximide blocks the biosynthesis of proteins. As shown in Fig. 3B, depletion of intracellular iron slowed the degradation of MT2.
when compared with the parallel controls with no addition of SIH in the medium. Quantitative analysis of the bands revealed a significant reduction starting at 2 h of incubation (Fig. 3C). Thus these results suggest that the increase of MT2 under iron deficiency results from the reduced degradation of MT2 and imply that the cytoplasmic domain of MT2 is responsible for this process.

**Cytoplasmic Domain of MT2 Is Necessary for the Increase of Cell Surface MT2 under Iron Depletion**—We wanted to examine the mechanism involved in the iron-dependent stabilization of MT2. A recent study showed that cell surface MT2 undergoes endocytosis in a dynamin-dependent manner (19). To determine the role of the MT2 cytoplasmic domain in iron regulation of MT2, we first examined the degradation pathway that is responsible for this process. Many plasma membrane proteins are targeted to lysosomes for degradation with or without ubiquitination. Others, like ZIP14 (an iron transporter), can also be degraded by proteasomes. The iron-dependent degradation of ZIP14 involves ubiquitination and extraction from plasma membrane-derived vesicles (41). To distinguish between these pathways, HepG2-MT2 cells were incubated with either the lysosomal inhibitors, bafilomycin A1 (100 nM), leupeptin (100 μM), and aprotinin (5 μM), or the proteasome inhibitors, MG-132 (10 μM) and epoxomicin (1 μM). Only the lysosomal inhibitors were able to increase the levels of cell surface MT2 with no evident effect on total cell MT2 after 6 h of incubation. SIH exhibited a similar effect as the lysosomal inhibitors (Fig. 4A). In contrast, incubation with the proteasome inhibitors did not alter the cell surface MT2 (Fig. 4A), indicating that MT2 is not degraded in the proteasome. No ubiquitin was detected in the immunoprecipitated MT2 eluate after 6 h of incubation with MG-132 (Fig. 4B, left panel), although as a control, significant increases of ubiquitin were observed in the immunoprecipitated ZIP14 eluate after MG-132 treatment (Fig. 4B, right panel). The inhibitor results suggest that the increase of cell surface MT2 under iron depletion is likely due to the decreased trafficking to lysosomes, rather than proteasomal degradation.
We next determined whether deletion of the putative endocytosis motif (MT2ΔCD9) or truncation of the most of MT2 cytoplasmic domain (MT2ΔCD46) could abolish the iron regulation of MT2 using the constructs as illustrated in Fig. 5A. The endocytosis motif of human MT2 has been mapped to the first nine amino acids in its N terminus (19). We generated stable clones of HepG2 cells that expressed either MT2ΔCD9 or MT2ΔCD46. HepG2-MT2ΔCD46 cells expressed a relatively low level of MT2 (Fig. 5B). This might be a clonal effect, because the transient transfection studies in HepG2 cells showed comparable levels of MT2 (Fig. 5F). As expected, incubation in the presence of 100 μM SIH for 6 h increased cell surface MT2 by ~1.9-fold (Fig. 5, B and C). Interestingly, deletion of the putative endocytosis motif largely abolished the iron regulation of cell surface MT2, and deletion of most of the MT2 cytoplasmic domain completely abolished the iron regulation of cell surface MT2 (Fig. 5, B and C). Consistently, both the total cellular MT2ΔCD9 and MT2ΔCD46 exhibited significantly decreased rates of degradation when compared with wild-type MT2 (Fig. 5, D and E). Similar results were observed in two more HepG2-MT2ΔCD9 or HepG2-MT2ΔCD46 clones. Together, these results indicate that the MT2 cytoplasmic domain, likely the endocytosis motif, is responsible for the regulation of cell surface MT2 by iron depletion.

Iron Depletion Affects the Post-internalization Processing of MT2—Cell surface MT2 undergoes constitutive internalization (19). To test the hypothesis that iron depletion increases the level of cell surface MT2 by suppressing its internalization, we compared the internalization rates of biotinylated cell surface MT2 in HepG2-MT2 cells with or without iron depletion by SIH. After a 10- and 20-min warm-up, MT2 remaining on the cell surface was removed by Pronase digestion (30 μg/ml) at 4 °C. This concentration of Pronase was able to eliminate more than 95% of cell surface MT2 and over 90% of Na⁺,K⁺-ATPase with a minimal effect on cell integrity and viability. As expected, SIH treatment increased the level of cell surface MT2 (Fig. 6, A, lane 5 versus 1). In agreement with the previous report (19), we detected the internalization of biotinylated cell surface MT2, as evidenced by the appearance of Pronase-resistant MT2 in the cells after incubation at 37 °C (Fig. 6A, lane 5 versus 1). In agreement with the previous report (19), we detected the internalization of biotinylated cell surface MT2, as evidenced by the appearance of Pronase-resistant MT2 in the cells after incubation at 37 °C (Fig. 6A, lane 5 versus 1). In agreement with the previous report (19), we detected the internalization of biotinylated cell surface MT2, as evidenced by the appearance of Pronase-resistant MT2 in the cells after incubation at 37 °C (Fig. 6A, lane 5 versus 1). In agreement with the previous report (19), we detected the internalization of biotinylated cell surface MT2, as evidenced by the appearance of Pronase-resistant MT2 in the cells after incubation at 37 °C. This concentration of Pronase was able to eliminate more than 95% of cell surface MT2 and over 90% of Na⁺,K⁺-ATPase with a minimal effect on cell integrity and viability. As expected, SIH treatment increased the level of cell surface MT2 (Fig. 6, A, lane 5 versus 1). In agreement with the previous report (19), we detected the internalization of biotinylated cell surface MT2, as evidenced by the appearance of Pronase-resistant MT2 in the cells after incubation at 37 °C (Fig. 6A, lane 5 versus 1).
To further determine the contribution of internalization to iron regulation of cell surface MT2, we examined the ratios of cell surface versus total cellular MT2. We reasoned that if iron depletion reduces the rate of MT2 internalization, it would lead to an accumulation of MT2 at the cell surface thus increasing the ratio of cell surface-versus-total MT2. No significant difference was observed between HepG2-MT2 cells with or without iron depletion by SIH (Fig. 6, C and D). In both cases, ~15% of total MT2 was detected on the cell surface. These observations reinforce the idea that iron depletion does not alter MT2 internalization.

To gain further insight into the mechanism for iron regulation of MT2, we determined the degradation rate of biotinylated cell surface MT2 in HepG2-MT2 cells with or without iron depletion by SIH. Consistent with the observation that iron depletion decreased the degradation of total cellular MT2 (Fig. 3, B and C), the degradation rate of cell surface MT2 was also reduced by iron depletion (Fig. 6, E and F). These observations indicate that the increased total cellular and cell surface MT2 under iron depletion results from the decreased MT2 degradation in the lysosome.

Lack of Translational Regulation of Tmprss6 mRNA in Rats—The steady state levels of a protein are controlled by its rate of synthesis and degradation. We wanted to test in animal models whether iron deprivation facilitates the translation of Tmprss6 mRNA. The levels of several proteins involved in cellular iron homeostasis are regulated at the translational level by iron regulatory proteins (IRP), the key iron sensors whose ability to bind stem loop structures in mRNA are negatively correlated with cellular iron load. IRPs bind the iron-responsive elements

FIGURE 5. Cytoplasmic domain of MT2 is responsible for the increase of cell surface MT2 under iron depletion. A, sequences of the N-terminal cytoplasmic tails of MT2, MT2ΔCD9, and MT2ΔCD46. B, deletion of the MT2 cytoplasmic domain abolishes the increase of cell surface MT2 under iron depletion. HepG2-MT2, MT2ΔCD9, and MT2ΔCD46 cells were incubated with or without 100 μM SIH for 6 h, followed by biotinylation of cell surface proteins at 4 °C. The eluted cell surface proteins and about 10% of input lysate were subjected to SDS-PAGE and immunodetection of MT2, Na\(^+\)/K\(^+\) ATPase (NaKATPase), and β-actin using specific antibodies. Two images of MT2 with different exposure times are presented. Experiments were repeated three times with consistent results. C, quantitation of cell surface MT2 in B. The intensities of cell surface MT2 bands in B were quantified using an Alexa Fluor 680 goat anti-rabbit secondary antibody. The relative amounts of MT2 in the SIH-treated group versus the untreated control group for each cell line are presented. The differences between the control and SIH groups were evaluated using the two-tailed Student’s t test. D, deletion of the MT2 cytoplasmic domain slows down the degradation of cellular MT2. HepG2-MT2, MT2ΔCD9, and MT2ΔCD46 cells were plated in a 12-well plate. After culture for 48 h in MEM, 10% FCS (complete medium), fresh complete medium with 100 μg/ml cycloheximide (CHX) was changed, and cell lysates were collected after 0, 1, 2, 4, and 6 h of incubation. Cell lysates were subjected to SDS-PAGE and immunodetection of MT2 and β-actin using specific antibodies. Ctrl-HepG2 cells were included as a negative control (Ctrl) for MT2. Experiments were repeated four times with consistent results. E, quantification of MT2 bands in D. The intensities of MT2 bands in D were quantified by LiCor using immunofluorescent goat anti-rabbit antibody. The MT2 band intensities at 0 h for each cell line were regarded as 100%. The MT2 band intensities at 1-, 2-, 4-, and 6-h time points were calculated as the percentage of 0 h for each form of MT2, respectively. Results are from four different experiments. F, Western blot analysis of MT2 at about 48 h after transient transfection of MT2, MT2ΔCD9, and MT2ΔCD46 in HepG2 cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Low Iron Increases Matriptase-2

IRON (IRE) in either the 5’ end or the 3’ end of the untranslated regions (UTR) of target mRNAs. IRP binding to the 5’ UTR blocks the initiation of ferritin translation (42). *m*rnase mRNA does not contain any predicted IRE sequence, but this does not rule out the possibility that its translation could be modulated through an undefined iron-regulated mechanism. We generated models of acute and chronic iron depletion by feeding weanling male rats an iron-deficient diet (less than 2 mg of iron/kg diet) for 3 and 14 days, respectively. In comparison with their corresponding control groups feeding a control diet (50 mg of iron/kg diet, group control), animals with either acute or chronic iron depletion had marked decreases in Tf saturation (31) and hepatic hepcidin mRNA expression (Fig. 7A), unchanged hepatic *mprss6* mRNA levels (Fig. 7A), but significantly increased MT2 protein in the liver by Western blot (Fig. 7B) (17). Thus rats on a low iron diet do show increased

**FIGURE 6.** Iron depletion does not affect the internalization of cell surface MT2. A, depletion of cellular iron does not affect the internalization of biotinylated cell surface MT2. HepG2-MT2 cells were incubated with or without 100 μM SIH for 18 h. Cell surface proteins were biotinylated at 4 °C, followed by incubation at 37 °C for 0, 10, and 20 min. The biotinylated proteins remaining on cell surface were removed by Pronase digestion at 4 °C. The internalized proteins were isolated by neutravidin-agarose beads for immunodetection of MT2 and Na⁺,K⁺ ATPase (NeuATPase). Three MT2 images with different exposure are presented. B, quantification of the internalized MT2 in A. The intensities of MT2 bands in A were quantified by LiCor using immunofluorescent goat anti-rabbit antibody. The intensities at 0-min warm-up with no Pronase digestion for each condition were regarded as 100%. The MT2 band intensity at 0 min warm-up time with Pronase digestion was regarded as the background for the internalization at 10 and 20 min warm-up. The rates of MT2 internalization were expressed as the percentage of a 0-min warm-up with no Pronase digestion for MT2-HepG2 cells with or without SIH treatment, respectively. Results are from four different experiments. C, depletion of cellular iron does not alter the ratios of cell surface versus total MT2. HepG2-MT2 cells were incubated with or without 100 μM SIH for about 18 h, followed by biotinylatation of cell surface proteins at 4 °C. Biotinylated proteins from equal amounts of cell lysate proteins were pulled down using neutravidin-agarose beads. The eluted cell surface proteins and about 10% of input lysate were subjected to SDS-PAGE and immunodetection of MT2, Na⁺,K⁺ ATPase (NeuATPase), and β-actin using specific antibodies. HepG2-Ctrl cells (Ctrl) were included as a negative control for MT2. D, quantification of MT2 band in C. The intensities of MT2 bands in C were quantified by LiCor using immunofluorescent goat anti-rabbit antibody. The MT2 band intensities in the lysate input (×10) for each condition were regarded as 100%. The relative amounts of cell surface MT2 were expressed as the percentage of total lysate MT2 for HepG2-MT2 cells with or without SIH treatment, respectively. Results are from 10 different experiments. E, depletion of cellular iron decreases the degradation of cell surface MT2. HepG2-MT2 cells were incubated with or without 100 μM SIH for 18 h, followed by biotinylatation of cell surface proteins at 4 °C. Cell lysates were collected after 0, 0.5, 1–3 h of incubation at 37 °C in the same medium for pulldown of biotinylated proteins by neutravidin-agarose beads and immunodetection of MT2, Na⁺,K⁺ ATPase (NeuATPase), and TfR1. F, quantification of MT2 bands in E. The intensities of MT2 bands in E were quantified by LiCor using immunofluorescent goat anti-rabbit antibody. The MT2 band intensities at 0 h for each condition were regarded as 100%. The relative amounts of MT2 at 0.5- and 1–3-h time points were expressed as the percentage of 0 h for HepG2-MT2 cells with or without SIH treatment, respectively. Results are from four different experiments. Two-tailed Student’s t test was used to evaluate the differences for all the above studies. *, p < 0.05; ***, p < 0.001.
MT2 levels independent of *Tmprss6* mRNA and were similar to what we observed in iron-deprived HepG2-MT2 cells (Fig. 1).

To determine the extent to which *Tmprss6* mRNA is translationally regulated by iron depletion, we performed polysome profile analysis of *Tmprss6*, mitochondrial aconitase (m-acon), and β-actin mRNA in the liver extracts. We chose m-acon mRNA because it is more weakly repressed relative to ferritin mRNAs that are strongly repressed in untreated cells and in rat liver,3 making the former a better target to view the impact of iron deficiency on the mRNA translation state. Repressed mRNA in ribonucleoprotein particles and the 80 S monosomes (fraction 1–4) were separated from the translationally active polysomes (Fig. 7, C–H, fractions 5–11), qRT-PCR was used to quantify the distribution of specific mRNA between translational states.

3 R. S. Eisenstein, unpublished data.
Low Iron Increases Matriptase-2

tionally inactive RNP fractions and translationally active polyribosomal fractions. Here m-acn mRNA was used as a positive control. It contains an IRE in its 5’ UTR, and variations of iron load are positively associated with changes in m-acn abundance in the liver of rats (43–45). β-Actin mRNA, whose expression is not regulated by iron, was included as a negative control. Similar to Tmprss6, neither acute iron deprivation nor chronic iron deprivation altered the total levels of m-acn mRNA expression (Fig. 7A). However, acute iron deprivation resulted in a mild but significant decrease of m-acn mRNA in the polyribosome fraction 9 and a concomitant increase in RNP fraction 2 (Fig. 7C). In the case of chronic iron deprivation, we detected a striking decrease of m-acn mRNA in polyribosome fractions 2 and 3 (Fig. 7F). However, no significant change was detected for Tmprss6 under either acute or chronic iron deprivation (Fig. 7, D and G), which is similar to β-actin (Fig. 7, E and H). Interestingly, the majority of Tmprss6 mRNA was detected in the polyribosomal fractions (Fig. 7, D and G), indicating that Tmprss6 mRNA is actively translated regardless of iron load. Together, these observations fail to support increased Tmprss6 regulation of mRNA levels or a translational control of Tmprss6 by cellular iron deprivation.

No Detectable Control of MT2 mRNA by BMP6, ID1, BMP Signaling, or Iron Load in the Liver—A recent study indicates that the transcription of the TMPRSS6 gene is induced by BMP6 likely through ID1 (a direct target of BMP signaling) in Hep3B cells, and in mice through chronic iron loading or by injection of high concentrations of BMP6 (27). We examined whether this represents a global mechanism for the control of TMPRSS6 expression. Incubation of HepG2 cells with BMP6 at 5, 25, or 50 ng/ml for 16 h was able to robustly induce the expression of hepcidin, ID1, and SMAD7 mRNA (Fig. 8A). ID1 is a widely used indicator for BMP signaling, and SMAD7 (an inhibitory SMAD) is induced by BMP signaling providing a negative feedback mechanism to limit BMP signaling. No significant change of endogenous TMPRSS6 mRNA expression was detected (Fig. 8A). These results suggest a lack of regulation of TMPRSS6 mRNA by BMP6 via the BMP signaling and ID1 in HepG2 cells.

We compared the hepatic Tmprss6 mRNA levels between 10-week-old male wild-type and Hjv−/− mice to determine the role of BMP6 in the regulation of Tmprss6 expression in vivo. In comparison with the former, Hjv−/− mice had ~28-fold lower hepcidin mRNA, a severe iron overload with about 14-fold higher liver non-heme iron (34), and an approximate 4-fold higher Bmp6 mRNA in the liver (Fig. 8B). No significant difference of Tmprss6 mRNA expression was detected in the liver between wild-type and Hjv−/− mice (Fig. 8B), thus the increase in Bmp6 expression was not sufficient to induce Tmprss6 expression.

To determine whether an increase in Id1 mRNA expression up-regulates Tmprss6 expression, we re-examined the liver tissues collected in our previous studies (34), in which exogenous Hjv cDNA was introduced specifically into the liver of 8-week-old male Hjv−/− mice using AAV8 vector, and animals were euthanized for analysis after 14 days. Compared with the parallel controls, introduction of Hjv cDNA into the liver of Hjv−/− mice led to significant increases in phosphorylated Smad1/5/8 (a direct indicator of BMP signaling) by ~2.5-fold (34) and hepcidin mRNA by ~65-fold in the liver (Fig. 8B, right panel). No significant decrease in Bmp6 mRNA was detected because of the mild amount of iron unloaded from the liver (Fig. 8B) (34). In agreement with the induction of BMP signaling, the expression of both Id1 and Smad7 mRNA was markedly elevated by the exogenous Hjv (Fig. 8B). No significant induction of Tmprss6 mRNA expression was observed (Fig. 8B). These results suggest the lack of correlation between the Tmprss6 mRNA, Bmp-mediated signaling, and Id1 expression in vivo.

Additionally, we also examined the effects of chronic iron loading on Tmprss6 expression in the liver tissues as well as in isolated liver cell populations from wild-type 129/S mice fed either a high iron rodent diet with 2% carbonyl iron or a control rodent diet with 48 ppm iron for 3 weeks. Analysis of these samples in our previous studies revealed that feeding a high iron diet led to significant elevation of non-heme iron content (an indicator of iron load), hepcidin mRNA, and Id1 mRNA by about 12.5-, 4.4-, and 11.9-fold in the liver when compared with the animals fed the control diet (35). Interestingly, no significant change of Tmprss6 mRNA was detected in both the isolated hepatocytes and the whole liver tissues between the control and high iron groups (Fig. 8C). Consistent with our previous studies in rats, Tmprss6 mRNA was detected predominantly in the hepatocyte population (Fig. 8C) (17). As expected, chronic iron loading increased the mRNA levels of hepcidin, Id1, and Smad7 in the isolated hepatocytes (Fig. 8D). These results indicate a lack of Tmprss6 regulation by chronic iron loading in 129/S mice. Together, these results indicate that the level of Tmprss6 mRNA is not regulated by BMP6, ID1, the BMP signaling, or iron.

DISCUSSION

Hepatic hepcidin expression is positively regulated by increased iron loading in the body as part of a negative feedback mechanism to limit further iron uptake. HJV, MT2, BMP6, holo-Tf, TfR1, TfR2, HFE, neogenin, and a subset of BMP receptors all appear to be involved in the control of hepcidin expression (2, 46). This study focused on the iron-mediated control of MT2. We investigated the mechanism for an increase of hepatic MT2 protein in response to acute iron depletion. Our results indicated that the decreased intracellular iron, rather than reduced serum Tf saturation, is responsible for the increase of MT2 levels. This increase is accomplished by decreasing the lysosomal degradation of MT2 protein, instead of inhibiting its shedding from the cells or increasing the translation of TMPRSS6 mRNA. We also showed that the cytoplasmic domain of MT2 is necessary for the increased cell surface MT2 under low iron conditions. Further studies in mice showed that the BMP-mediated signaling did not regulate TMPRSS6 mRNA levels. Changes in Bmp6 or ID1 mRNA did not lead to changes in TMPRSS6 mRNA in either HepG2 cells or in mice. Together, these results support the idea that the iron regulation of MT2 occurs at the level of protein degradation rather than by translational or transcriptional mechanisms.

We previously showed that the rapid decrease of Tf saturation in rats with acute iron deprivation is associated with a robust reduction of hepcidin expression and increases in MT2
increased TfR1 is an indirect indicator of cellular iron depletion. The level of TfR1 is post-transcriptionally regulated. TfR1 mRNA contains multiple IREs in the 3’ UTR region. Cellular iron deprivation increases the levels of IRPs. IRP binding to the IREs in the 3’ UTR of TfR1 mRNA stabilizes TfR1 mRNA for translation (42).

In this study, we provide the first evidence for the close correlation of reduced cellular iron load with the increased stability of the MT2 protein. However, our data do not exclude the existence of other mechanisms for apo-Tf regulation of MT2.

MT2 and its putative substrate, HJV, traffic to the plasma membrane via distinct pathways (28), which suggests that the cleavage and inactivation of HJV by MT2 take place at the cell surface or after internalization. MT2 protein undergoes active
shedding of its C-terminal catalytic domain from the cells (19, 20, 40). Evidence from two groups suggests that the shed MT2 catalytic domain has no activity toward HIV (20, 36). Shed MT2 catalytic domain does not cleave HJV (20). Our previous co-culture studies indicate that only the cell-associated MT2 is able to cleave HJV (36). Thus, it is likely that the rapid suppression of hepcidin expression upon acute iron depletion at least partially results from cleavage of HIV by increased levels of MT2. Additionally, our results also imply that the suppression of hepcidin by chronic iron deprivation is possibly accomplished through the same mechanism.

MT2 interacts with neogenin (28). Disruption of the MT2-neogenin interaction results in MT2 accumulation in HepG2 cells (28). Iron deprivation did not alter either the total cellular neogenin or its cell surface expression in HepG2-MT2 cells (Figs. 1 and 3). Acute iron deprivation in rats does not affect neogenin levels in the liver extracts of rats (31). Thus, it is unlikely that the increased MT2 by iron deprivation is due to a change in neogenin levels.

MT2 undergoes constitutive endocytosis via a dynamin-dependent pathway, and it is mainly degraded in the lysosomes (19). The putative internalization residues have been mapped to its N-terminal cytoplasmic domain. Mutations of these residues or expression of dominant-negative dynamin facilitates its functional activity to cleave its substrate HIV (19). Here, we provided additional evidence supporting the previously reported lysosomal degradation of internalized cell surface MT2 (19). More importantly, we found that deletion of the entire endocytosis motif or deletion of most of the cytoplasmic domain was able to largely abolish the increase of MT2 by iron depletion. However, further studies ruled out the possibility that iron depletion increases MT2 by modulating the internalization rate of cell surface MT2. In this study, we also found that iron depletion slows down the degradation of whole cell lysate MT2 as well as biotinylated cell surface MT2, but it does not alter the relative proportion of MT2 on the cell surface. These observations show that iron depletion increases MT2 by decreasing the degradation of MT2.

To further explore other mechanisms of MT2 regulation, we asked whether Tmprss6 mRNA was translationally regulated in vivo in conditions of iron deficiency where MT2 protein was accumulated. Because the IRE-IRP mechanism is the paradigm for iron regulation of mRNA translation, we used it as a control for these studies (42). Consistent with the fact that m-acon mRNA contains a 5’ IRE, we found that m-acon mRNA was translationally repressed in iron-deficient liver. In contrast, Tmprss6 mRNA translation was not altered even though Tmprss6 protein accumulated. This observation strengthens the idea that the major iron-mediated regulation of MT2 is at the level of protein stability.

Our results also rule out the possibility for the transcriptional regulation of TMPRSS6 by BMP6, BMP signaling, or iron. We previously showed that the rapid suppression of hepcidin expression in rats with acute iron deprivation is associated with a decrease of BMP signaling in the liver but with no change in Tmprss6 mRNA (17). A recent study reports that TMPRSS6 mRNA could be up-regulated by BMP6 in Hep3B cells and chronic iron load in mice (27). Here, we re-examined this regulatory mechanism employing several different models, and we revealed no correlation of Tmprss6 mRNA expression with the status of BMP6, BMP signaling, or chronic iron load. Thus our results do not support the transcriptional regulation of TMPRSS6 under physiological conditions. Consistent with our results, incubation of HepG2-HJV cells with BMP6 induces the BMP signaling, but does not increase the release of MT2-cleaved HJV product.4

Studies in animal models support the idea that MT2 is a limiting factor in the suppression of hepcidin expression. Mice with a single copy of Tmprss6 have an increased susceptibility to iron deficiency when iron demands are high or when dietary iron is restricted (47). Thus, iron regulation of MT2 levels may represent an important mechanism for a rapid modulation of hepcidin expression according to the bodily iron load. However, this does not rule out the involvement of other molecules in this process. For example, a recent study showed that the hepatocyte growth factor activator inhibitor-2 (HAL-2), a serine protease inhibitor, can form a complex with MT2 to inhibit its enzymatic activity, which indirectly increases the hepcidin expression in hepatic cell lines (48). HAL-2 is a type I transmembrane glycoprotein with two Kunitz-type serine protease domains in their extracellular portion (49–52). The iron regulation of HAL-2 in the liver has not been reported.

MT2 and HIV are co-expressed in hepatocytes (17, 23). Hepatic HIV is essential for the induction of hepcidin expression (25, 34, 53). HJV is the only identified iron-regulated substrate of MT2 to date (20, 26). MT2 likely suppresses hepcidin expression by binding to and cleaving HIV into an inactive form. On the basis of the findings in this study, we hypothesize a mechanism for the suppression of hepcidin expression in response to iron deprivation, by which the decreased iron load in hepatocytes increases the stability of MT2 protein by inhibiting its trafficking to lysosomes via an undefined machinery. Increased levels of MT2 lead to the inactivation of HIV and the subsequent suppression of HIV-induced hepcidin expression.

In summary, this study uncovered an important mechanism for the suppression of hepcidin expression by acute iron deprivation.

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Low Iron Increases Matriptase-2


