Brief report

Molecular mechanisms of the defective hepcidin inhibition in *TMPRSS6* mutations associated with iron-refractory iron deficiency anemia

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Matriptase-2 is a transmembrane serine protease that negatively regulates hepcidin expression by cleaving membrane-bound hemojuvelin. Matriptase-2 has a complex ectodomain, including a C-terminal serine protease domain and its activation requires an autocatalytic cleavage. Matriptase-2 mutations have been reported in several patients with iron-refractory iron deficiency anemia. Here we describe a patient with 2 missense mutations in the second class A low-density lipoprotein receptor (LDLRA) domain. Functional studies of these 2 mutations and of a previously reported mutation in the second C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1 (CUB) domain were performed. Transfection of mutant cDNAs showed that membrane targeting of the 2 LDLRA mutants was impaired, with Golgi retention of the variants. The activating cleavage was absent for the LDLRA mutants and reduced for the CUB mutant. All 3 mutated proteins were still able to physically interact with hemojuvelin but only partially repressed hepcidin expression compared with wild-type matriptase-2. Our results underline the importance of LDLRA and CUB domains of matriptase-2. (Blood. 2009;113:5605-5608)

Introduction

Hepcidin, a circulating peptide synthesized by hepatocytes, is a major regulator of iron homeostasis.[1] When hepcidin is produced in excess, it limits the amount of ferroportin present at the plasma membrane of enterocytes and macrophages and induces iron-restricted erythropoiesis.[2] Hemojuvelin (HJV) is a coreceptor for bone morphogenetic proteins[3] that is required for hepcidin expression. Biallelic inactivation of the *Tmprss6* gene in mice leads to excessive hepcidin production and consequently to iron-deficient erythropoiesis.[4,5] In humans, mutations of *TMPRSS6* have been reported in patients with iron-refractory iron deficiency anemia (IRIDA).[6-8] *TMPRSS6* is highly expressed in the liver and encodes the membrane serine protease matriptase-2 (MT2), homologous to the ubiquitous matriptase-1.[9,10] MT2 represses hepcidin expression by cleaving membrane-bound HJV (m-HJV).[11] MT2 has a short N-terminal cytoplasmic tail, a single transmembrane domain, and a composite ectodomain with 2 C1r/C1s, urchin embryonic growth factor and bone morphogenetic protein 1 (CUB) domains, 3 class A low density lipoprotein receptor (LDLRA) domains, and a C-terminal serine protease domain.[12] In this paper, we report a new case of IRIDA in a patient with compound heterozygosity for 2 mutations in the second LDLRA domain of the protein.[6] The aim of the present work was to characterize the functional consequences of these and one other missense mutations outside the serine protease domain.

Methods

The proband originated from France. He was 10 months old when microcytic anemia was first diagnosed. Hemoglobin was 10 g/dL and mean corpuscular volume was 63 fL. Low serum iron (2.6 μM) and transferrin saturation (5%) were observed as well as low serum ferritin (4 μg/L). Anisocytosis and poikilocytosis were noticed. He was the third child of the family. A response to oral iron therapy was observed with a rise in ferritin (up to 180 μg/L) and hemoglobin (up to 11.8 g/dL) concentrations. After a few months, both ferritin and hemoglobin levels decreased despite ongoing treatment. He was given a course of intravenous iron (100 mg once a week for 8 weeks) when he was 7 years old that induced a rapid rise in ferritin and plasma iron. Hemoglobin increased up to 12.9 g/dL 3 months after the first iron infusion and remained normal for several months (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). However, the highest plasma iron concentration obtained at the end of the course of iron therapy was only 12 μM. Two other courses of intravenous iron produced similar results. Plasma hepcidin was measured on one occasion using an enzyme-linked immunosorbent assay[13] (Intrinsic LifeSciences, La Jolla, CA) and found at 443 μg/L (normal range, 29-254 μg/L in men[13]) despite a hemoglobin concentration of 10.2 g/dL.

DNA analyses

Blood samples of family members were obtained for genetic diagnosis after written informed consent of the parents in accordance with "Agence de Biomedecine" and the Declaration of Helsinki. Genomic DNA was extracted and *TMPRSS6* gene was explored as previously described.[7] The Service de Biochimie Hormonale et Génétique has received an approval for the practice of genetic diagnosis.

Functional characterization of matriptase-2 mutants

Expressing vectors encoding MT2 variants (G442R, D521N, and E522K) were obtained by mutagenesis of wild-type cDNA as described in the

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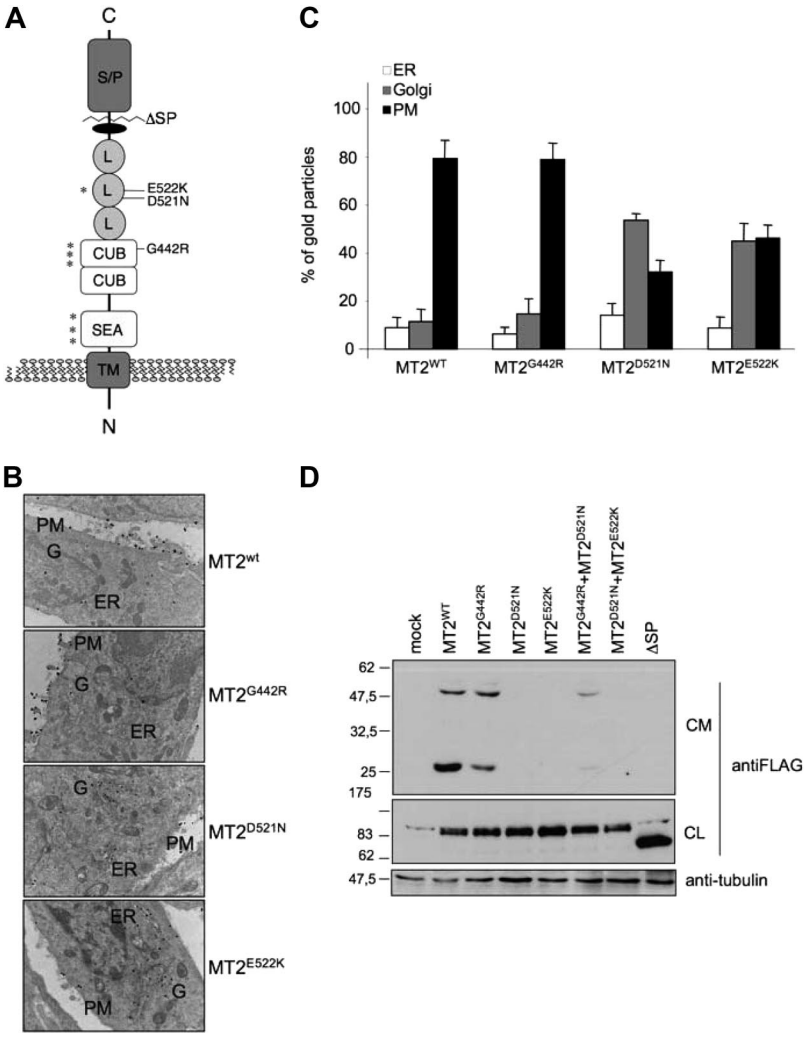

Figure 1. Morphometric analysis and processing of MT2 variants. (A) Schematic representation of MT2 functional domains and localization of the studied mutations. TM indicates transmembrane domain; SEA, sea urchin sperm protein, enteropeptidase agrin; CUB, complement protein subcomponents C1r/C1s, urchin embryonic growth factor, and bone morphogenetic protein 1 domain; L, low-density lipoprotein receptor class A domain; S/P, serine protease domain. *Predicted consensus N-glycosylation sites. (B) Electron microscopy and (C) morphometric analysis of MT2 variants. HeLa cells were transiently transfected with Lipofectamine 2000 using pcDNA3.1 expressing WT and mutant MT2. After 18 hours, cells were fixed, labeled with a polyclonal rabbit anti-FLAG using the gold-enhance protocol, embedded in Epon-812, and cut. Immunoelectron microscopy (EM) images were acquired from thin sections under a Philips Tecnai-12 electron microscope (Philips, Eindhoven, The Netherlands) using an ULTRA VIEW CCD digital camera (Philips). Images were acquired using AnalySIS software (Soft Imaging System, Lakewood, CO; original magnification ×23 000; B). Thin sections were used to quantify gold particles residing within different compartments of the secretory pathway (C). PM indicates plasma membrane; ER, endoplasmic reticulum; and G, Golgi. (D) Characterization of wild-type and mutant MT2. Whole-cell extracts and concentrated media of transiently transfected HeLa cells were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blot was performed following standard procedures; MT2 was revealed by the anti-FLAG antibody. CL indicates cellular lysates; and CM, conditioned medium. The equal loading was verified by antitubulin. Scales refer to relative molecular mass (in kilodaltons).

Supplemental data. MT2 devoid of the serine protease domain (ΔSP) and HJV^{wt} constructs were as described.[11] Functional studies on hepcidin promoter inhibition, MT2 processing, HJV cleavage, and MT2/HJV coimmunoprecipitation were performed as described.[11]

Results and discussion

We identified 2 missense mutations in exon 13 of the *TMPRSS6* gene in a patient with IRIDA: a heterozygous c.1561G → A substitution leading to the replacement of an aspartic acid by an asparagine at position 521 of the protein (D521N) and a c.1564 G → A substitution leading to the replacement of a glutamic acid by a lysine at position 522 (E522K). Segregation of the mutations within the family is shown in Figure S2. These mutations are both predicted to modify the sequence of the second LDLRA domain of the protein (Figure 1A). The D521N mutation was previously reported in an IRIDA patient in combination with the G442R mutation in the CUB domain.[6] Functional studies have only been performed on the R774C mutation, which affects the serine protease domain.[11] To characterize the effect of D521N, E522K, and G442R mutations on MT2 activity, HeLa cells were transfected with the corresponding cDNA constructs. By electron microscopy, we found that the proportion of protein expressed at the cell membrane was significantly reduced for D521N and E522K mutants, which partially remained in the Golgi apparatus, whereas G442R was normally targeted to the cell membrane (Figures 1B,C, S3). We have previously shown that activation of wild-type MT2 results in the release of shorter fragments in the culture medium, thought to reflect a catalytic cleavage.[11] Such fragments were undetectable in the supernatant of cells transfected with MT2^{D521N} and MT2^{E522K} cDNAs and only detectable as faint bands with MT2^{G442R} (Figure 1D), suggesting that the integrity of the CUB and LDLR domains is required for the activation of MT2.

The ability of MT2 mutants to cleave m-HJV was assessed in cotransfection experiments. HJV cleavage fragments were observed in the culture medium of cells cotransfected with normal MT2 and HJV, as previously described.[11] On expression of MT2^{D521N} and MT2^{E522K}, no cleavage fragments were observed, whereas expression of MT2^{G442R} generated only a small amount of HJV cleavage compared with normal MT2 (Figure 2A, CM). When the amount of HJV remaining at the plasma membrane was evaluated by treatment with phosphatidylinositol-phospholipase C (PI-PLC) that specifically cleaves glycosylphosphatidylinositol-anchored proteins, it appeared that MT2^{D521N} or MT2^{E522K} maintained a greater amount of m-HJV, compared with MT2^{wt} and MT2^{G442R} (Figure 2B PI-PLC). These results were consistent with those obtained with a binding assay measuring the proportion of

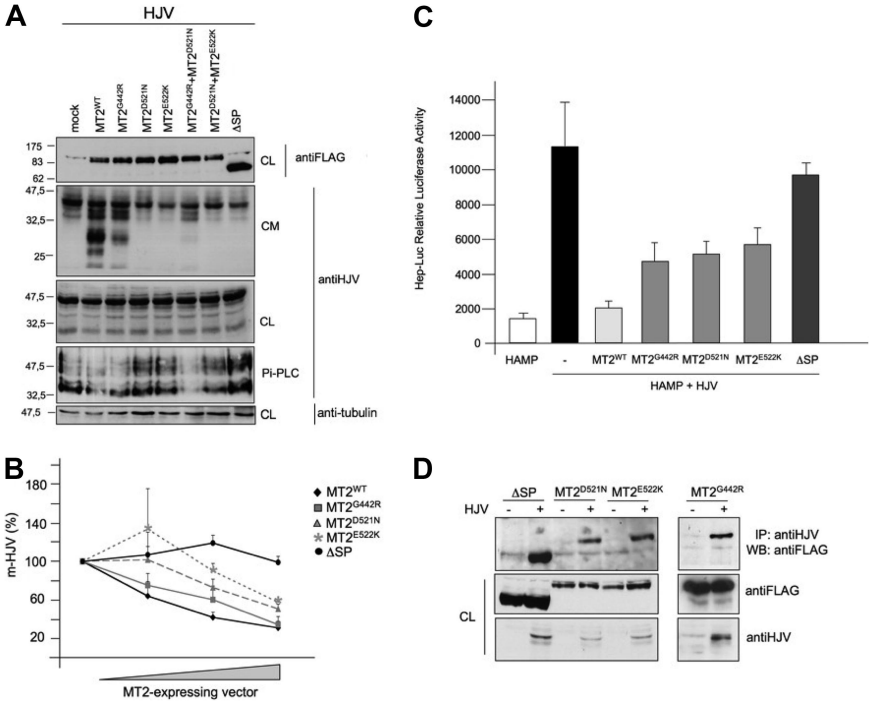

Figure 2. MT2/HJV functional interaction. (A) HeLa cells were transfected with HJV in the presence of the empty vector (mock), matriptase-2^{wt} (MT2^{wt}), MT2^{G442R}, MT2^{D521N}, MT2^{E522K}, MT2$^{G442R/D521N}$, MT2$^{D521N/E522K}$, and MT2$^{\Delta SP}$. Whole-cell extracts (CL), concentrated media (CM), and supernatants after PI-PLC cleavage (PI-PLC) were loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and processed for Western blot analysis. Anti-FLAG and anti-HJV were used to detect MT2 and HJV, respectively. The equal loading was verified by antitubulin. Scales refer to relative molecular mass (in kilodaltons). (B) Binding assay was used to measure m-HJV in the presence of increasing concentrations of wild-type and mutants MT2 expressing vectors and was performed essentially as described in "Functional characterization of matriptase-2 mutants." Experiments were made in triplicate and performed 3 times. Error bars represent SD. (C) Hepcidin promoter responses by HJV, in the presence of MT2. A firefly luciferase reporter driven by 2.9 kb of the proximal hepcidin promoter was cotransfected into Hep3B cells with *Renilla* luciferase vector pRL-TK, either alone (HAMP) or with HJV (HAMP + HJV) combined or not with MT2-expressing vectors. Relative luciferase activity is calculated as reported in "Functional characterization of matriptase-2 mutants" and expressed as a multiple of the activity of cells transfected with the reporter alone. Experiments, made in triplicate, were performed 3 times. Error bars represent SD. (D) HeLa cells were cotransfected with wild-type and mutant matriptase-2 (MT2^{WT}, MT2^{G442R}, MT2^{D521N}, MT2^{E522K}) in the presence of HJV or of an empty vector. Precleared whole-cell extracts were immunoprecipitated with anti-HJV and revealed with the anti-FLAG antibody, which recognizes MT2. To control for transfection, whole-cell extracts (CL) were loaded and revealed with anti-HJV and/or anti-FLAG antibodies.

m-HJV in the presence of escalating doses of normal and mutant MT2 constructs (Figure 2B).

To study the effect of MT2 mutants on hepcidin expression, Hep3B cells were transfected with a hepcidin promoter/firefly luciferase reporter construct.[14] As expected, the presence of HJV enhanced the hepcidin promoter activity in this system, whereas the coexpression of normal MT2 with HJV almost completely prevented this activation. A significantly smaller stimulation of the hepcidin promoter was observed when G442R, D521N, or E522K variants were coexpressed with HJV, compared with the normal construct (Figure 2C).

Altogether, our results indicate that LDLRA mutants are fully, and the CUB mutant partially, defective in their ability to cleave m-HJV. However, all 3 variants are able to interact with HJV with similar efficiency, as assessed by coimmunoprecipitation studies (Figures 2D, S4). Both D521N and E522K substitutions replace highly conserved residues. From known 3-dimensional structures, both residues are predicted to bind Ca^{2+},[15] and amino acid substitutions at homologous positions were shown to affect the folding of the protein.[16,17] Misfolding of the LDLR domain may explain a partial defect in the processing of the protein, resulting in lower expression of mutated MT2 at the plasma membrane and in a defective autocatalytic activation. A similar mechanism may explain the absence of *TMPRSS3* activation in cases of autosomal recessive deafness resulting from a D103G mutation in the LDRA domain of the protein, at a position highly homologous to D521N of MT2. This mutation was shown to abolish the serine protease activity in a yeast expression system.[18] Finally, the decrease in m-HJV cleavage observed with the G442R mutant and its inability to repress hepcidin expression underscore the importance of the CUB domain in the function of MT2.

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Authorship

Contribution: L.S. performed research and contributed to the writing of the paper; F.G., C.O., and C.K. identified the mutations;

A.P. and A.N. performed research; M.S. and F.T. followed up on the patient; C.B. contributed to the writing of the paper; C.C. contributed to the design of the experimental work and to the writing of the paper; and B.G. coordinated the work and contributed to the writing of the paper.

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