Essential Role of Endocytosis of the Type II Transmembrane Serine Protease TMPRSS6 in Regulating Its Functionality*

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The type II transmembrane serine protease TMPRSS6 (also known as matriptase-2) controls iron homeostasis through its negative regulation of expression of hepcidin, a key hormone involved in iron metabolism. Upstream of the hepcidin-regulated signaling pathway, TMPRSS6 cleaves its target substrate hemojuvelin (HJV) at the plasma membrane, but the dynamics of the cell-surface expression of the protease have not been addressed. Here, we report that TMPRSS6 undergoes constitutive internalization in transfected HEK293 cells and in two human hepatic cell lines, HepG2 and primary hepatocytes, both of which express TMPRSS6 endogenously. Cell surface-labeled TMPRSS6 was internalized and was detected in clathrin- and AP-2-positive vesicles via a dynamin-dependent pathway. The endocytosed TMPRSS6 next transited in early endosomes and then to lysosomes. Internalization of TMPRSS6 is dependent on specific residues within its N-terminal cytoplasmic domain, as site-directed mutagenesis of these residues abrogated internalization and maintained the enzyme at the cell surface. Cells coexpressing these mutants and HJV produced significantly decreased levels of hepcidin compared with wild-type TMPRSS6 due to the sustained cleavage of HJV at the cell surface by TMPRSS6 mutants. Our results underscore for the first time the importance of TMPRSS6 trafficking at the plasma membrane in the regulation of hepcidin expression, an event that is essential for iron homeostasis.

The serine protease superfamily of hydrolytic enzymes is composed of ~200 members (MEROPS Database (1)), most of them being secreted soluble proteins. TMPRSS6 (matriptase-2), a member of a novel family of type II transmembrane serine proteases that function at the cell surface (2), was originally characterized as a protease expressed mostly in the liver (3) and capable of processing proteins such as type I collagen, fibronectin, and fibrinogen (4, 5). The protease is a mosaic protein composed of multiple domains, including a 52-residue N-terminal cytoplasmic domain (6), a transmembrane domain that anchors the enzyme within membranes, and an extracellular domain itself constituted of multiple regions, including the catalytic region. Until now, no function had been associated with the cytoplasmic tail of the enzyme. TMPRSS6 is synthesized as an inactive zymogen with the catalytic region being disulfide-linked to the main chain following activation. Moreover, the enzyme undergoes cell-surface shedding, releasing a soluble and active form in the extracellular milieu (7). It exhibits many of the enzymatic specificities of other members of the type II transmembrane serine protease family, preferring arginine residues in P4, P3, and P1 positions relative to the cleaved peptide bond (5, 8).

Although TMPRSS6 has been associated with breast and prostate cancer (9, 10), recent reports have demonstrated its direct involvement in iron homeostasis. Indeed, genetic analysis of kindred suffering from iron-refractory iron deficiency anemia identified sequence variants in the TMPRSS6 gene, conclusively demonstrating that its loss is causative for this disease (11). Concomitantly, another group using chemically induced mouse models that showed progressive loss of body hair and microcytic anemia (12) found that the phenotype was caused by high levels of hepcidin, the major hormonal regulator of iron in mammals, this itself due to a splicing defect in the TMPRSS6 gene. Other nonsense mutations within the TMPRSS6 gene were also found in patients suffering from microcytic anemia and iron deficiency (13, 14). The involvement of TMPRSS6 in hepcidin regulation and iron homeostasis was initially discovered in a mouse mutant (mask), the phenotype of which resulted from reduced absorption of dietary iron caused by high levels of hepcidin due to a splicing defect of TMPRSS6 (12). Mechanistically, TMPRSS6 controls iron homeostasis by repressing expression of the HAMP gene, which encodes hepcidin, the major hormonal regulator of iron metabolism (15). The link between TMPRSS6 and hepcidin involves the cleavage by TMPRSS6 of hemojuvelin (HJV)(5) (16), which acts as a bone morphogenetic protein coreceptor (17), thereby affecting the bone morphogenetic protein/SMAD signaling pathway and activation of the HAMP gene. Mutations present in iron-refractory iron deficiency anemia patients within specific TMPRSS6 extracellular domains affect either 1) translocation of the enzyme to the cell surface, which leads to increased

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intracellular retention, resulting in the impairment of efficient HJV cleavage at the cell surface, or 2) the capacity of the enzyme to be activated (18).

Here, we show that TMPRSS6 is constitutively internalized and that its endocytosis is dependent on motifs found within its cytoplasmic tail. Our results demonstrate that a member of the type II transmembrane serine protease family undergoes dynamic trafficking at the cell surface, thereby suggesting a way by which accessibility to its substrate can be controlled.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—HepG2 and HEK293 cells were purchased from American Type Culture Collection (Manassas, VA), and human primary hepatocytes were from Zen-Bio (Chapel Hill, NC). Cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin (WISENT, St-Bruno, Quebec, Canada). Serum-free 293 SFM II medium was from Invitrogen, and primary hepatocyte plating and maintenance media were from Zen-Bio. Cells were transfected using PEI (Polysciences, Warrington, PA) as described previously (19). Anti-V5 monoclonal antibody (mAb) was from Invitrogen. Anti-HA (HA.11) mAb and polyclonal antibody (pAb) were from Covance (Emeryville, CA). Anti-Na+/K+-ATPase pAb, anti-clathrin heavy chain (D3C6) and anti-caveolin-1 (D46G3) rabbit mAbs were from Cell Signaling Technology (Danvers, MA). Anti-EEA1 (early endosomal antigen 1) mAb was from BD Transduction Laboratories, and the pAb (PA1-063A) from Thermo Scientific. Anti-LAMP-2 mAb (H4B4) was from the University of Iowa (Iowa City, IA), and the pAb (ab37024) from Abcam (Cambridge, MA). Anti-actin (A3853) and anti-HJV (HPA014472) mAbs were from Sigma. Anti-TMPRSS6 pAb was developed in collaboration with 21st Century Biochemicals (Marlboro, MA). The tyramide signal amplification (TSA™) kit with HRP-labeled goat anti-rabbit IgG and Alexa Fluor 488-labeled tyramide was from Invitrogen.

Biochemicals—AME, including 20 mM Tris, 50 mM sodium 2-mercaptoethanesulfonate (pH 8.6), were used in complete DMEM for various periods of time, washed, and then left untreated or treated with biotin cleavage solution (150 mM NaCl, 20 mM Tris, 50 mM sodium 2-mercaptopetanesulfonate (pH 8.6)). The detection signal of endogenous TMPRSS6 in HepG2 and human primary hepatocytes was amplified with the TSA™ kit.

Plasmid Constructions and Site-directed Mutagenesis—Human TMPRSS6 cDNA was obtained from C. López-Otín (Universidad de Oviedo, Oviedo, Spain) and inserted in a modified form of pcDNA6/V5- His (Invitrogen) in which a stop codon has been inserted to block His-tag translation. HA-tagged dominant-negative dynamin-1 mutant K44A (pcDNA3.1/HAl-dynamin-1 K44A) was obtained from Dr. Sandra Schmid (The Scripps Research Institute, La Jolla, CA). TMPRSS6 mutants were generated using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. YFP-tagged β2-adaptin (pYEFP-N1) was described previously (20). Plasmid encoding human HJV vari-A was from OriGene Technologies, Inc. (Rockville, MD). Expression of all proteins was under the control of the human CMV immediate-early promoter.

Biotinylation Assays—HEK293 cells were transfected with 1 μg of TMPRSS6-V5 plasmid using 4 μg of PEI in 6-cm plates. After a 24-h transfection, biotinylation of HEK293 surface proteins was performed with Pierce cell-surface protein isolation kit (Thermo Scientific). Cells were incubated at 37 °C in complete DMEM for various periods of time, washed, and then left untreated or treated with biotin cleavage solution (150 mM NaCl, 20 mM Tris, 50 mM sodium 2-mercaptopetanesulfonate (pH 8.6)) for 2.5 h prior to lysis. Biotinylated proteins were precipitated with avidin, and the resulting samples were loaded on a 10% SDS-polyacrylamide gel and then analyzed by immunoblotting using anti-V5 (TMPRSS6) and anti-Na+/K+-ATPase antibodies.

Immunofluorescence—Cells were seeded on 12- or 22-mm coverslips (polylysine-coated for HepG2 cells and collagen I-coated for human primary hepatocytes) placed in 6-well plates. Where indicated, cells were transfected with 1 μg of plasmid using 4 μg of PEI. After a 24-h transfection, cell-surface TMPRSS6 was labeled with anti-V5 or anti-TMPRSS6 antibody for 1 h at 4 °C in serum-free DMEM. Cells were then washed and incubated in complete DMEM at 37 °C for various periods of time. The protocol for cell preparation has been described previously (21). The detection signal of endogenous TMPRSS6 in HepG2 and human primary hepatocytes was amplified with the TSA™ kit.

Treatment with Lysosomal Inhibitors—Cells were incubated for 6 h at 37 °C in complete DMEM containing 1 mg/ml leupeptin, 10 μg/ml E-64 (HEK293 cells) or E-64d (hepatocytes), and 70 μg/ml pepstatin as described previously (21).

Expression of TMPRSS6 Mutants—HEK293 cells were transfected with 1 μg of plasmid using 4 μg of PEI in 6-wells plates. pcDNA6-V5 plasmids containing inserts coding for WT TMPRSS6, catalytically dead mutant S762A, penta-alanine mutants, or single-alanine mutants of region 2–11 of the cytoplasmic tail of TMPRSS6 were used. After a 24-h transfection, cell media were replaced with serum-free 293 SFM II medium for another 24 h. Cells were then lysed, and cell media were collected and concentrated with centrifugal filters with a molecular weight cutoff of 3000. The resulting samples were loaded on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-V5 antibody.

Proteolytic Activity Measurements—HEK293 cells were transfected with 1 μg of plasmid using 4 μg of PEI in 6-wells plates. After a 24-h transfection, the medium was replaced with serum-free 293 SFM II medium for 72 h. Enzymatic assays were then performed with serum-free medium in 100 mM Tris-HCl (pH 9) containing 500 μg/ml bovine serum albumin. Enzyme activity was monitored by measuring the release of fluorescence (excitation, 360 nm; and emission, 441 nm) from T-butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (Alexa Fluor 488-labeled tyramide was from Invitrogen. t-Butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin was from Bachem Bioscience, Inc. (King of Prussia, PA). Amicon Ultra centrifugal filters (Ultracel 3K) with a molecular weight cutoff of 3000 were from Millipore (Cork, Ireland). Poly-L-lysine- and collagen I-coated glass coverslips (BD BioCoat) were collected and concentrated with centrifugal filters with a molecular weight cutoff of 3000. The resulting samples were loaded on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-V5 antibody.
**Hemojuvelin Processing by TMPRSS6**—HEK293 cells were cotransfected with 1 μg of HJV and 1 μg of TMPRSS6-V5 plasmid using 8 μg of PEI in 6-well plates. The plasmids used were WT TMPRSS6-V5 or single-alanine mutants of region 2–11 of the cytoplasmic tail. After a 24-h transfection, cell media were replaced with serum-free 293 SFM II medium for another 24 h. Cells were then lysed, and media were collected and concentrated with centrifugal filters with a molecular weight cutoff of 3000. The resulting samples were loaded on a 10% SDS-polyacrylamide gel and analyzed by immunoblotting with anti-V5 and anti-HJV antibodies.

**siRNA Transfection**—siRNAs were transfected using DharmaFECT 4 transfection reagents (Dharmacon) according to the manufacturer’s instructions.

**Hepcidin Quantification**—Hepcidin-25 was assessed in concentrated cell media by a competitive ELISA method using a hepcidin-25 enzyme immunoassay commercial kit (Bachem Bioscience, Inc.).

**RESULTS**

**Cell-surface TMPRSS6 Is Internalized**—Because regulation of HAMP involves cleavage of HJV by TMPRSS6, we reasoned that there could be a regulatory step necessary for controlling the levels of HJV, the presence of which is paramount to the downstream signaling and transcriptional activation of HAMP. To determine the dynamics of TMPRSS6 cell-surface expression and its trafficking path, we examined the endocytic/internalization capacity of both an epitope-tagged form of TMPRSS6 heterologously expressed in HEK293 cells and the endogenous protein in HepG2 cells and human primary hepatocytes. To examine whether TMPRSS6 is present at the plasma membrane and is internalized, we used a cell-surface biotinylation assay. HEK293 cells transiently transfected with a C-terminal extracellular V5-tagged TMPRSS6 (TMPRSS6-V5) construct were labeled with membrane-impermeable biotin (sulfo-NHS-SS-biotin) and kept at 4 °C (0 min) to prevent internalization or incubated at 37 °C for various periods of time (5, 10, 20, 30, and 60 min) to allow internalization (Fig. 1A). Cells were then kept at 4 °C to prevent further internalization; cell-surface biotin was cleaved with sodium 2-mercaptoethanesulfonate, a membrane-impermeant reducing agent; and total cell lysates were analyzed by SDS-PAGE and immunoblotting. Fig. 1A shows that TMPRSS6 migrated as a 125-kDa band, corresponding to the predicted molecular mass of the N-linked glycosylated protease. We compared the biotinylation of the TMPRSS6 population with that of Na+/K+-ATPase, a protein that does not internalize under our conditions. (Na+/K+-ATPase undergoes internalization only when HEK293 cells are stimulated (see Ref. 22)). As expected, biotinylated Na+/K+-ATPase was not protected from sodium 2-mercaptoethanesulfonate cleavage, indicating that this protein was not internalized. However, we found that, after 5 min of incubation, a subset of biotinylated TMPRSS6 was protected from cleavage, which suggested internalization within intracellular compartments; further time points (10–60 min) led to the detection of increased levels of protected TMPRSS6.

We next examined internalization of TMPRSS6 in transiently transfected HEK293 cells, HepG2 cells, and human primary hepatocytes using confocal microscopy. To observe the internalization phenomenon directly, we first labeled TMPRSS6-V5 transiently expressed in HEK293 cells with a specific anti-V5 monoclonal antibody at 4 °C to prevent any internalization. The antibody-tagged TMPRSS6-V5-express-
ing cells were then incubated at 37 °C to allow internalization (Fig. 1B). At initial time points and for a 5-min period at 37 °C (Fig. 1B), TMPRSS6-V5 was present at the plasma membrane of transfected cells, consistent with the accessibility to cleavage with sodium 2-mercaptoethanesulfonate after biotinylation (Fig. 1A). After 10 and 20 min at 37 °C, labeling was found in vesicular structures near the plasma membrane, with some fluorescence retained at the plasma membrane. Beginning at 30 min and especially at 60 min, the majority of the enzyme was found in large vesicles in the juxtanuclear region, further demonstrating transport of the protein into the cytoplasm. This observed internalization could explain the partial protection from biotin cleavage starting at 10 min of incubation time and through to 60 min (Fig. 1A). To examine this phenomenon in cells endogenously expressing TMPRSS6, we first raised an antibody against a peptide sequence found within the extracellular region of the enzyme (see supplemental Fig. 1A, which shows specificity of the antibody using blocking peptides) and used it to observe similar internalization dynamics in HepG2 cells and human primary hepatocytes endogenously expressing TMPRSS6 (Fig. 1, C and D). Collectively, these results demonstrate that cell-surface TMPRSS6 undergoes internalization.

Characterization of the TMPRSS6 Internalization Pathway—Our observations on the internalization of TMPRSS6 prompted us to investigate the endocytic pathway by which this is mediated. Because dynamin-mediated endocytosis is a major form of endocytosis involved in internalization of cell-surface proteins such as receptors (23), we first examined whether TMPRSS6 endocytosis is dynamin-mediated. As shown in Fig. 2A, the expression of a dominant-negative mutant form of dynamin-1 (K44A), which inhibits clathrin- and caveolin-mediated endocytosis (24), abrogated internalization and maintained TMPRSS6 at the plasma membrane in HEK293 cells transiently expressing TMPRSS6-V5. Similar results were obtained in HepG2 cells. Indeed, Fig. 2A clearly shows that, in HepG2 cells not expressing dynamin-1 K44A, TMPRSS6 was internalized (box 1), whereas TMPRSS6 was maintained at the cell surface in dynamin-1 K44A-expressing cells (box 2).

To define more precisely the compartments where cell surface-labeled TMPRSS6 is internalized, we carried out double labeling for TMPRSS6 and endocytic markers in HEK293 cells transiently expressing TMPRSS6-V5. After short internalization periods (5 min), TMPRSS6 partially colocalized with the clathrin-coated vesicle markers β2-adaptin and clathrin in fixed, permeabilized, and immunostained using anti-HA antibody followed by fluorescent secondary antibodies. Box 1, dominant-1 K44A-untransfected cell; box 2, dynamin-1 K44A-transfected cell (n = 3); B, HEK293 cells transfected with TMPRSS6-V5 in the absence or presence of YFP-tagged β2-adaptin and human primary hepatocytes were co-stained for TMPRSS6 and different endocytosis markers. Cell-surface TMPRSS6 was labeled on ice with anti-V5 (HEK293) or anti-TMPRSS6 (hepatocytes) antibody and incubated at 37 °C for 5 or 10 min. TMPRSS6 immunofluorescence is displayed in green, and YFP, anti-clathrin antibody, or anti-EEA1 antibody is shown in red (n = 3). TMPRSS6 immunofluorescence is displayed in green, LAMP-2 in red, and the Hoechst-stained nucleus in blue. Yellow color and white arrowheads indicate colocalization. Scale bars = 50 μm.
punctate structures along the plasma membrane (Fig. 2B). No colocalization was observed with caveolin-1 (supplemental Fig. 2). To determine the nature of the compartments to which TMPRSS6 next traffics, we carried out double labeling of TMPRSS6 and EEA1, a marker for early endosomes (25). TMPRSS6 colocalized with EEA1 after a 10-min internalization period in transfected HEK293 cells and human primary hepatocytes (Fig. 2B), suggesting its transport to early endosomes.

To determine whether TMPRSS6 further transits to lysosomes, we next compared its localization with LAMP-2, a lysosomal marker. Fig. 2C shows that, after 6 h of internalization and in the presence of lysosomal inhibitors, TMPRSS6 partially colocalized with LAMP-2-labeled structures both in transfected HEK293 cells and in human primary hepatocytes. Taken together, our results suggest that TMPRSS6 traffics to early endosomes via clathrin-coated vesicles in a dynamin-dependent manner, where it is then sorted to the lysosomes.

**The Cytoplasmic Tail of TMPRSS6 Is Involved in Its Internalization**—To understand better the molecular determinants involved in TMPRSS6 internalization, we initially alanine-scanned the 52-residue N terminus by constructing 10 penta-Ala mutants that cover the entire cytoplasmic tail and expressing them in HEK293 cells (Fig. 3A). We also constructed a catalytically inactive TMPRSS6 mutant (S762A) in which the catalytic serine of the protease was replaced with Ala to examine whether proteolytic activity plays a role in the internalization process.

Constructs for all 11 mutants were transfected in HEK293 cells, and the cells were labeled with anti-V5 antibody. Cells were further incubated in complete DMEM for 30 min at 37 °C to allow internalization. Interestingly, two of the 11 mutants (2–6A and 7–11A) failed to undergo intracellular sequestration and remained mostly at the cell surface, whereas all other mutants behaved similarly to the WT in their internalization capacity (Fig. 3B). Furthermore, the S762A mutant exhibited similar internalization properties to the WT, suggesting that the proteolytic activity of the enzyme is not involved in internalization. Immunoblot analysis of cell lysates of these mutants revealed that they were equally expressed (Fig. 3C, upper panels). Cell-surface TMPRSS6 is known to undergo a complex maturation process that includes activation and subsequent shedding from the plasma membrane (7, 16, 18, 26, 27). Hence, multiple forms are generated, most of them the result of autocatalysis.

Immunoblot analysis of media originating from cells expressing WT and mutant TMPRSS6 (Fig. 3C, middle panels) revealed different shed forms detectable as fragments migrating at 90, 85, 75, 32, and 30 kDa. First, the larger 90-kDa form...
could originate from cleavage within the enzyme’s SEA domain, similar to what has been observed for matriptase (28), whereas the 75- and 85-kDa fragments would be the consequence of proteolysis at Arg 413 and Arg 446 within the stem region, leading to cell-surface release (7). All these larger forms would not carry proteolytic activity because no cleavage has occurred at the activation peptide, an event required for activity. Indeed, it is cleavage at Arg 576 within the conserved activation peptide that yields the 30-kDa active fragment and that converts the single-chain zymogen into the activated two-chain protease (7). Moreover, because TMPRSS6 cleaves after selective basic residues, the 32-kDa fragment could be the result of an alternative cleavage near the activation site (for example, at Arg 576). Compared with the WT, mutants 2–6A and 7–11A exhibited increased cell-surface shedding as shown by the bands migrating at 90, 85, 75, 32, and 30 kDa. Mutant 37–41A also had increased cell-surface shedding but only for the 90-, 32-, and 30-kDa bands. We detected significant increases in proteolytic activity (4–4.5-fold over the WT) in the media of cells expressing mutants 2–6A and 7–11A (Fig. 3D) but, intriguingly, not for mutant 37–41A. These results suggest that cleavages after Arg 413 and Arg 446 (75- and 85-kDa bands, respectively) may be required to obtain active shed forms. Another possibility for the lack of activity of the 37–41A mutant is that cleavage yielding the 32- and 30-kDa forms does not occur at the precise site (Arg 576) required for activation of the protease. Certainly, the higher proteolytic activity detected in the media of cells expressing mutants 2–6A and 7–11A is consistent with immunoblot data showing that impeding cell-surface internalization increases cell-surface shedding, which is associated with higher proteolytic activity in cell media compared with the WT and other mutants.

The results obtained with the 2–6A and 7–11A mutants suggested to us that specific individual residues are involved in TMPRSS6 internalization. We therefore examined the effect of systematically replacing each of the first 10 residues of the protein (excluding the initiator methionine) with alanine. Fig. 4A shows that selective amino acid substitutions led to profound effects on the ability of TMPRSS6 to internalize. Indeed, confoveal analysis revealed that five of the mutants (L2A, L3A, F5A, S7A, and K8A) remained mostly at the cell surface after a 30-min incubation at 37 °C, whereas L4A, H6A, R9A, M10A, and P11A exhibited normal internalization. Immunoblot analysis (Fig. 4B) and proteolytic activity in the cell media (Fig. 4C) showed increased cell-surface shedding of the L2A, L3A, F5A, S7A, and K8A mutants that was associated with higher proteolytic activity in the cell media compared with the WT and other mutants.

**Impairment of TMPRSS6 Internalization Affects Cell-surface Cleavage of HJV and Hepcidin Production**—HJV is a cell-surface protein that is important for HAMP gene expression, and TMPRSS6 plays a regulatory role in this process by cleaving HJV. To determine the functional impact of impairment of TMPRSS6 internalization on this process, we first analyzed the effect of sustained cell-surface expression of the protease and the various protease mutants on HJV cleavage. HEK293 cells were cotransfected with a HJV-encoding plasmid together with empty vector (mock), WT, or single-alanine mutant

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**FIGURE 4.** **A** TMPRSS6 internalization depends on specific amino acids within region 2–11 of the cytoplasmic tail. HEK293 cells were grown on coverslips and transfected with TMPRSS6-V5 region 2–11 single-alanine mutants. Cells were surface-labeled on ice with anti-V5 antibody and fixed 30 min after incubation at 37 °C (n = 3). Anti-V5 immunofluorescence is shown in green, and the Hoechst-stained nucleus is shown in blue. Scale bars = 50 μm. **B** HEK293 cells were transfected with TMPRSS6-V5 region 2–11 single-alanine mutants. Expression was detected by Western blotting with anti-V5 antibody. An equal amount of cell lysate (CL) was loaded on an SDS-polyacrylamide gel (upper panels). The cell medium (CM) was concentrated and loaded on a 10% SDS-polyacrylamide gel (middle panels). Cell lysate actin was blotted as a loading control (n = 3) (lower panels). **C** Proteolytic activity was measured by cleaving the fluorogenic peptide t-butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (50 μM) and monitoring fluorescence release in media of cells transfected with TMPRSS6-V5 and alanine mutants. Activity was normalized by cell lysate total protein (micrograms) and is presented as -fold over the WT (mean ± S.D., n = 3).
TMPRSS6-V5 constructs (Fig. 5A). HJV cleavage by TMPRSS6 was analyzed by immunoblotting with anti-HJV antibody to detect cleaved soluble HJV in the cell media (Fig. 5A, middle panels). As reported previously (29), shed HJV migrated at ~40 kDa due to proprotein convertase cleavage, whereas cleavage by TMPRSS6 yielded a shorter 36-kDa form. Indeed, in cells transfected with WT TMPRSS6-V5 or single-alanine mutants, the major forms of soluble HJV in the conditioned medium migrated at ~36 kDa. Expression of all non-internalized TMPRSS6 mutants (L2A, L3A, F5A, S7A, and K8A) led to an increase in the intensity of the 36-kDa band in conditioned cell medium, supporting the notion that the sustained presence of the enzyme at the cell surface leads to increased proteolysis of the substrate.

Because the sustained presence of TMPRSS6 at the plasma membrane produced higher levels of soluble HJV and hence decreased cell-surface levels of intact HJV, we examined if hepcidin levels are affected by this event. We measured the effect of expressing TMPRSS6 mutants in HepG2 cells, which endogenously produce and secrete hepcidin (30). Fig. 5B shows that a basal level of secreted hepcidin was detected in the conditioned media. When HJV was overexpressed, a significant increase in hepcidin levels was found, but these levels returned to normal once WT TMPRSS6 was overexpressed, thereby confirming its role as a negative regulator of hepcidin production (Fig. 5B). However, when the cell-surface sustained mutants (L2A, L3A, F5A, S7A, and K8A) were expressed in these cells, hepcidin levels were significantly reduced when compared with the WT. These reductions can be linked to the increased cell-surface cleavage of HJV as shown in Fig. 5A. Using an alternative strategy by transfecting dynamin-1 K44A or siRNA to knock down TMPRSS6 (see supplemental Fig. 1B for controls and efficacy of siRNA on TMPRSS6 expression in transfected cells) in HJV-transfected HepG2 cells, we determined that modulating the cell-surface or overall cellular levels of TMPRSS6 in an endogenous model affected hepcidin levels (Fig. 5C). Maintaining TMPRSS6 at the surface with dynamin-1 K44A led to a sig-
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significant decrease in hepcidin compared with cells transfected only with HJV, whereas knocking down endogenous levels of TMPRSS6 partially restored the signaling capacity of the cell to produce hepcidin.

DISCUSSION

TMPRSS6 is a cell-surface serine protease that plays a critical role in the regulation of iron homeostasis through its proteolytic processing of plasma membrane HIV (31). Normally, intact HJV associates with bone morphogenetic protein to control hepcidin expression via the bone morphogenetic protein receptor/SMAD signaling pathway.

In this study, we have shown for the first time that TMPRSS6 undergoes internalization at the plasma membrane. Using biotinylation and confocal microscopy analysis, we have demonstrated that the enzyme reaches the cell surface and undergoes endocytosis in early endosomes via clathrin-coated vesicles in a dynamin-dependent manner. Internalization was observed not only in transiently transfected HEK293 cells but also in the physiologically relevant HepG2 cells and in human primary hepatocytes that endogenously express TMPRSS6. At later time points, TMPRSS6 was detected in LAMP-2-labeled vesicles, suggesting that the protease eventually transits to lysosomes, where it may be degraded.

Internalization of cell-surface proteins is frequently mediated by motifs found within their cytoplasmic tail (32). Initially, we screened for important regions within the cytoplasmic tail of TMPRSS6 using penta-alanine mutants and identified N-terminal region 2–11 as essential for internalization. Interestingly, this region contains a dileucine motif (Leu2 and Leu3), a determinant that is often found in cell-surface proteins that undergo internalization. These motifs found in membrane proteins have been shown to act as endocytic and/or constitutive sorting signals through interaction with the AP-2 adaptor protein (33). Interestingly, however, the dileucine motif found in TMPRSS6 seems atypical when compared to the canonical (D/E)XXX(L/I) sequence observed in most trafficking proteins, as it lacks an upstream acidic residue. Our data are nonetheless consistent with the recent findings that dileucine motifs on their own can still act as internalization signals (32) and that many other trafficking proteins with such motifs also lack an acidic residue at position 4 but instead rely on other hydrophilic residues downstream of the dileucines (e.g. LLXXXS in TMPRSS6) (33). Using single-alanine mutation of the amino acids in region 2–11, we found that Leu2 and Leu3 play a role in internalization but that three other determinants (Phe5, Ser7, and Lys8) are also involved in this process.

Internalization is a well known regulatory process for G protein-coupled receptors as well as for growth factor receptors such as tyrosine kinase receptors, where it plays a role in desensitization and signaling (34). However, internalization of membrane proteases and the consequences thereof have not been extensively studied. An example of a serine protease that undergoes internalization is furin, a type I transmembrane protein in which routing is a dynamic event shown to be dependent on the phosphorylation state of its cytoplasmic tail (35). Reports have also shown that membrane type 1 matrix metalloproteinase, an integral type I transmembrane metalloproteinase involved in cleaving extracellular matrix proteins, undergoes dynamin-dependent endocytosis using both clathrin-mediated and clathrin-independent pathways (36). Finally, very recent data reveal how another member of the type II transmembrane serine protease family, matripsase, accumulates in intracellular structures (37), bringing support to the notion that these proteins undergo dynamic trafficking.

Recently, it was determined that cell-surface shedding is the only known mechanism by which TMPRSS6 activity could be regulated (18). Our results reveal that, in addition to shedding, internalization of TMPRSS6 can also act as a cellular mode of TMPRSS6 regulation with direct consequences on the processing of specific cell-surface substrates such as HIV. Identifying and understanding the molecular determinants and mechanisms controlling this event can lead to the potential modulation of internalization rates and hence cell-surface levels of TMPRSS6. Modulation of the levels of TMPRSS6 at the cell surface could conceivably lead to the eventual control of systemic iron levels. Interestingly, use of peptide aptamers that can bind the LLY internalization motif located in the membrane type 1 matrix metalloproteinase cytoplasmic tail has prevented its internalization, causing accumulation at the cell surface (38). Thus, one could envisage the modulation of TMPRSS6 cell-surface localization and proteolytic activity by targeting its cytoplasmic tail.

In this study, we have revealed a novel mechanism for TMPRSS6 regulation through plasma membrane internalization and identified key residues within its cytoplasmic tail that are important in sequestering the enzyme within the cell. Further studies will be needed to understand fully the underlying mechanisms regulating TMPRSS6 trafficking and to determine the role of such regulation in iron homeostasis and in situations of iron imbalance.

REFERENCES

1. Rawlings, N. D., Barrett, A. J., and Bateman, A. (2010) Nucleic Acids Res. 38, D227–D233
2. Bugge, T. H., Antalis, T. M., and Wu, Q. (2009) J. Biol. Chem. 284, 23177–23181
3. Ramsay, A. J., Reid, J. C., Velasco, G., Quigley, J. P., and Hooper, J. D. (2008) Front. Biosci. 13, 569–579
4. Hooper, J. D., Campagnolo, L., Goodarzi, G., Truong, T. N., Stuhlmann, H., and Quigley, J. P. (2003) Biochem. J. 373, 689–702
5. Velasco, G., Cal, S., Quesada, V., Sánchez, L. M., and López-Otín, C. (2002) J. Biol. Chem. 277, 37673–37666
6. Keshava Prasad, T. S., Goel, R., Kandasamy, K., Keerthikumar, S., Kumar, S., Mathivanan, S., Telikicherla, D., Raju, R., Shafran, B., Venugopal, A., Balakrishnan, L., Marimuthu, A., Banerjee, S., Somamathan, D. S., Sebastian, A., Rani, S., Ray, S., Harys Kishore, C. J., Kanth, S., Ahmed, M., Kashyap, M. K., Mohmood, R., Ramachandra, Y. L., Krishna, V., Rahman, B. A., Mohan, S., Ranganathan, P., Ramabadran, S., Chaerkady, R., and Pandey, A. (2009) Nucleic Acids Res. 37, D767–D772
7. Stirmberg, M., Maurer, E., Horstmeyer, A., Kolp, S., Frank, S., Bald, T., Arenz, K., Janzer, A., Prager, K., Wunderlich, P., Walter, J., and Gutschow, M. (2010) Biochem. J. 430, 87–95
8. Béliveau, F., Désilets, A., and Leduc, R. (2009) FEBS J. 276, 2213–2226
9. Parr, C., Sanders, A. J., Davies, G., Martin, T., Lane, J., Mason, M. D., Mansel, R. E., and Jiang, W. G. (2007) Clin. Cancer Res. 13, 3568–3576
10. Sanders, A. J., Parr, C., Martin, T. A., Lane, J., Mason, M. D., and Jiang, W. G. (2008) J. Cell. Physiol. 216, 780–789
11. Finberg, K. E., Heeney, M. M., Campagna, D. R., Aydino, Y., Pearson, H. A., Hartman, K. R., Mayo, M. M., Samuel, S. M., Strouse, J. J., Marki- nos, K., Andrews, N. C., and Fleming, M. D. (2008) Nat. Genet. 40,
569–571
12. Du, X., She, E., Gelbart, T., Truksa, J., Lee, P., Xia, Y., Khovananth, K., Mudd, S., Mann, N., Moresco, E. M., Beutler, E., and Beutler, B. (2008) Science 320, 1088–1092
13. Guillem, F., Lawson, S., Kannengiesser, C., Westerman, M., Beaumont, C., and Grandchamp, B. (2008) Blood 112, 2089–2091
14. Melis, M. A., Cau, M., Congiu, R., Sole, G., Barella, S., Cao, A., Westerman, M., Cazzola, M., and Galanello, R. (2008) Haematologica 93, 1473–1479
15. Nemeth, E., and Ganz, T. (2009) Acta Haematol. 122, 78–86
16. Silvestri, L., Pagani, A., Nai, A., De Domenico, I., Kaplan, J., and Camaschella, C. (2008) Cell Metab. 8, 502–511
17. Babitt, J. L., Huang, F. W., Wrighting, D. M., Xia, Y., Sidis, Y., Samad, T. A., Campagna, J. A., Chung, R. T., Schneyer, A. L., Woolf, C. J., Andrews, N. C., and Lin, H. Y. (2006) Nat. Genet. 38, 531–539
18. Silvestri, L., Guillem, F., Pagani, A., Nai, A., Oudin, C., Silva, M., Toutain, F., Kannengiesser, C., Beaumont, C., Camaschella, C., and Grandchamp, B. (2009) Blood 113, 5605–5608
19. Ehrehardt, C., Schmolke, M., Matzke, A., Knoblauch, A., Will, C., Wixler, V., and Ludwig, S. (2006) Signal Transduction 6, 179–184
20. Fessart, D., Simaan, M., Zimmerman, B., Comeau, J., Hamdan, F. F., Wiseman, P. W., Bouvier, M., and Laporte, S. A. (2007) J. Cell Sci. 120, 1723–1732
21. Brodeur, J., Larkin, H., Boucher, R., Thériault, C., St-Louis, S. C., Gagnon, H., and Lavioe, C. (2009) Traffic 10, 1098–1114
22. Pierre, S. V., Belliard, A., and Sottejeau, Y. (2011) Am. J. Physiol. Cell Physiol. 300, C42–C48
23. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) J. Cell Biol. 127, 915–934
24. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 37–44
25. Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P., and Toh, B. H. (1995) J. Biol. Chem. 270, 13503–13511
26. Altamura, S., D’Alessio, F., Selle, B., and Muckenthaler, M. U. (2010) Biochem. J. 431, 363–371
27. Ramsay, A. J., Quesada, V., Sanchez, M., Garabaya, C., Sardà, M. P., Baiget, M., Remacha, A., Velasco, G., and Lopez-Otin, C. (2009) Hum. Mol. Genet. 18, 3673–3683
28. Oberst, M. D., Williams, C. A., Dickson, R. B., Johnson, M. D., and Lin, C. Y. (2003) J. Biol. Chem. 278, 26773–26779
29. Maxson, J. E., Enns, C. A., and Zhang, A. S. (2009) Blood 113, 1786–1793
30. Fein, E., Merle, U., Ehehalt, R., Herrmann, T., and Kulaksiz, H. (2007) Peptides 28, 951–957
31. Maxson, J. E., Chen, J., Enns, C. A., and Zhang, A. S. (2010) J. Biol. Chem. 285, 39021–39028
32. Kozik, P., Francis, R. W., Seaman, M. N., and Robinson, M. S. (2010) Traffic 11, 843–855
33. Bonifacino, J. S., and Traub, L. M. (2003) Annu. Rev. Biochem. 72, 395–447
34. Posner, B. I., and Laporte, S. A. (2010) Prog. Brain Res. 181, 1–16
35. Jones, B. G., Thomas, L., Molloy, S. S., Thulin, C. D., Fry, M. D., Walsh, K. A., and Thomas, G. (1995) EMBO J. 14, 5869–5883
36. Remacle, A., Murphy, G., and Roghi, C. (2003) J. Cell Sci. 116, 3905–3916
37. Friis, S., Godiksen, S., Bornholdt, J., Selzer-Plon, J., Rasmussen, H. B., Bugge, T. H., Lin, C. Y., and Vogel, L. K. (2011) J. Biol. Chem. 286, 5793–5802
38. Wickramasinghe, R. D., Ko Ferrigno, P., and Roghi, C. (2010) BMC Cell Biol. 11, 58