Phosphorylation of the type II transmembrane serine protease, TMPRSS13, in hepatocyte growth factor activator inhibitor-1 and -2-mediated cell-surface localization

Andrew S. Murray§#, Fausto A. Varela‡**, Thomas E. Hyland§, Andrew J. Schoenbeck§, Jordan M. White∥§¶, Lauren M. Tanabe‡, Sokol V. Todi‡‡, and Karin List∥§¶

From the Departments of Pharmacology, Oncology, and Neurology, Cancer Biology Graduate Program, and Pharmacology Graduate Program, and the Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201

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TMPRSS13 is a member of the type II transmembrane serine protease (TTSP) family. Although various TTSPs have been characterized in detail biochemically and functionally, the basic properties of TMPRSS13 remain unclear. Here, we investigate the activation, inhibition, post-translational modification, and localization of TMPRSS13. We show that TMPRSS13 is a glycosylated, active protease and that its own proteolytic activity mediates zymogen cleavage. Full-length, active TMPRSS13 exhibits impaired cell-surface expression in the absence of the cognate Kunitz-type serine protease inhibitors, hepatocyte growth factor activator inhibitor (HAI)-1 or HAI-2. Concomitant presence of TMPRSS13 with either HAI-1 or -2 mediates phosphorylation of residues in the intracellular domain of the protease, and it coincides with efficient transport of the protease to the cell surface and its subsequent shedding. Cell-surface labeling experiments indicate that the dominant form of TMPRSS13 on the cell surface is phosphorylated, whereas intracellular TMPRSS13 is predominantly non-phosphorylated. These data provide novel insight into the cellular properties of TMPRSS13 and highlight phosphorylation of TMPRSS13 as a novel post-translational modification of this TTSP family member and potentially other members of this family of proteases.

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To whom correspondence should be addressed: Depts. of Pharmacology and Oncology, Gordon H. Scott Hall of Basic Medical Sciences, Rm. 6332, 540 East Canfield, Detroit, MI 48201. Tel.: 313-577-1034; E-mail: klist@med.wayne.edu.

The abbreviations used are: TTSP, type II transmembrane serine protease; TMPRSS, transmembrane protease, serine; HAI, hepatocyte growth factor activator inhibitor; HAT, human airway trypsin-like protease; IDR, intrinsically disordered region; HMW, higher molecular weight; LDLA, low-density lipoprotein receptor class A; αM, α2-macroglobulin; CM, conditioned media; SP, serine protease; CIP, calf intestinal alkaline phosphatase; EV, empty vector; PNGase, peptide/N-glycosidase; pNA, p-nitroanilide; Suc, succinyl.

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tein can exist in the cell (16). Here, we describe a comprehensive study on the biochemical and cell biological properties of TMPRSS13 and report for the first time that TMPRSS13 is a phosphorylated TTSP. Importantly, our results show that HAI-1 and HAI-2 enhance TMPRSS13 phosphorylation and facilitate cell-surface localization.

Results

**TMPRSS13 is expressed as a 70-kDa glycosylated active protease in mammalian cells**

Like other TTSPs, TMPRSS13 contains a C-terminal catalytic serine protease (SP) domain, an N-terminal intracellular domain, a transmembrane signal anchor, and a stem region. The stem region in TMPRSS13 is composed of a group A scavenger cysteine-rich receptor domain, preceded by a single low-density lipoprotein receptor class A domain (LDLA) (Fig. 1A, panel I). The catalytic domain of human TMPRSS13 contains the essential serine protease catalytic triad residues, His\(^{361}\), Asp\(^{409}\), and Ser\(^{506}\), and the substrate-binding pocket residues, Asp\(^{500}\), Ser\(^{525}\), and Gly\(^{527}\) (8). TTSP’s are universally synthesized as inactive zymogens that are activated by cleavage at a conserved activation site motif, predicted to be 320RIVGG in TMPRSS13 (indicated with an arrow in Fig. 1A, panel I) (5, 8). Upon activation, the catalytic domain remains tethered via a
disulfide bond to the stem region of the protease. Therefore, the active SP-domain can be visualized by SDS-PAGE and Western blotting under reducing conditions. In a previous study, using recombinant TMPRSS13 in which the putative activation cleavage sequence was replaced with the enterokinase recognition sequence (DDDDK), activation with enterokinase under cell-free conditions was achieved, and TMPRSS13 displayed proteolytic activity toward synthetic peptide substrates (17). However, this earlier work did not examine whether this phenomenon applies in a cellular environment. To determine whether TMPRSS13, containing its native activation sequence, is expressed as an active protease in mammalian cells, we expressed full-length human TMPRSS13 in HEK293T cells and analyzed whole-cell lysates by Western blotting (Fig. 1B) using an anti-TMPRSS13 polyclonal antibody raised against the extracellular part of TMPRSS13 (α-extra-TMPRSS13, indicated in Fig. 1A, panel I). The predicted molecular masses of the 586 amino acid full-length protease and the 266 amino acid SP-domain are 61 and 27 kDa, respectively. Two major proteins of ~70 and 32 kDa were detected in lysates (Fig. 1B). These species migrated at higher molecular masses than predicted for the full-length and the SP-domain, suggesting that TMPRSS13 could be post-translationally modified. Analysis of the amino acid sequence of human TMPRSS13 using the NetNGlyc 1.0 Server revealed four potential N-glycosylation sites, two of which are located in the serine protease domain (Fig. 1A). In addition to the 70- and 32-kDa species, two forms of ~60 and 50 kDa were recognized by the α-extra-TMPRSS13 antibody in whole-cell lysates (Fig. 1B). To minimize the risk of cleavage events occurring as a result of cell lysate preparation, or due to high levels of recombinant TMPRSS13 expression, we analyzed cell lysates with low expression levels and lysates prepared using highly stringent conditions (boiling in 1.5% SDS with 100 mM DTT; supplemental Fig. S1, A and B). We did not observe any discernible differences in protein band patterning by Western blotting between TMPRSS13 low- and high-expressing cells (supplemental Fig. S1A). Furthermore, no noticeable differences were observed when cell lysis was performed in RIPA buffer versus stringent SDS buffer (supplemental Fig. S1B).

To examine whether TMPRSS13 is post-translationally modified by glycosylation in mammalian cells, protein extracts from transfected HEK293T cells were subjected to enzymatic deglycosylation by PNGaseF prior to Western blot analysis and probed with α-extra-TMPRSS13 (Fig. 1C). Upon deglycosylation and probing with α-extra-TMPRSS13, we observed a reduction in the molecular mass of the proposed full-length 70-kDa form, to a form closer to the predicted lower molecular mass (~60 kDa); this band coincides with the 60-kDa species seen before deglycosylation. These results indicate that the full-length form of TMPRSS13 exists as two different glycosylation variants (Fig. 1C). The presumed SP-domain shifted to the predicted molecular mass upon deglycosylation, indicating that one or both of the potential N-linked glycosylation sites in the SP-domain are utilized (Fig. 1C). The 50-kDa band migrated at ~40 kDa and may represent an ectodomain cleavage product generated upon cleavage in the LDLA domain of the stem region. Several TTSPs have been shown to be cleaved in the stem region, including corin, where two fragments result from corin autocleavage in the frizzled 1 domain and LDLA 5 domain, respectively (18). The cleavage event generating the 50-kDa TMPRSS13 species appears to be dependent on TMPRSS13 proteolytic activity because the fragment is not detected in cells transfected with a catalytically inactive form of TMPRSS13 and may result from autocleavage (Fig. 3A). Deglycosylated lysates were also analyzed using an antibody raised against a peptide localized within the first 60 (N-terminal) amino acids (α-intra-TMPRSS13) as indicated in the schematic representation of TMPRSS13 in Fig. 1A. Similarly to α-extra-TMPRSS13, we observed the 70-kDa form drop to 60 kDa upon deglycosylation (Fig. 1D). In addition, other N-terminal processed forms of ~43 and 22 kDa were observed. The latter may result from cleavage within the LDLA domain, i.e. represent the N-terminal half of the C-terminal 50-kDa half detected with α-extra-TMPRSS13 in Fig. 1C.

Figure 1. TMPRSS13 is a 70-kDa active glycoprotein in human cells. A. schematic representation of the four different recombinant TMPRSS13 proteins generated for this study. Panel I, full-length human TMPRSS13 (WT-TMPRSS13); TM, transmembrane domain; L, lipoprotein receptor class A domain; scavenger cysteine-rich receptor (SRCR); G, group A scavenger cysteine-rich receptor; N = predicted N-glycosylation sites, the activation cleavage site is indicated with an arrow, and S–S represents the disulfide bridge linking the stem region to the serine protease (SP) domain. The epitopes for the two anti-TMPRSS13 antibodies used are indicated. A polyclonal antibody was raised against a recombinant protein fragment corresponding to a region within amino acids 195 and 562 of human TMPRSS13 (α-extra-TMPRSS13) that recognizes the extracellular part of the protease. An additional polyclonal antibody (α-intra-TMPRSS13) was raised against a peptide within residues 1–60, which recognizes the N-terminal intracellular domain. Panel II, C-terminally tagged full-length human TMPRSS13 (WT-TMPRSS13-V5); VS–H = VS–His epitope. Panel III, active soluble TMPRSS13 serine protease domain protein generated in P. pastoris. Panel IV, N-terminally HA-tagged full-length human TMPRSS13 (HA-WT-TMPRSS13); HA, human influenza hemagglutinin tag. B, whole-cell protein lysates from HEK293T cells expressing non-glycosylated full-length human TMPRSS13 were separated by SDS-PAGE under reducing conditions. TMPRSS13 was detected by Western blotting using the rabbit α-extra-TMPRSS13 antibody against the extracellular part of TMPRSS13. Non-transfected cells (NT) and cells transfected with EV were included as controls. The full-length TMPRSS13 and SP-domain are indicated with open arrowheads. C and D, proteins were separated by SDS-PAGE and analyzed by Western blotting using α-extra-TMPRSS13 (C) and α-intra-TMPRSS13 (D), respectively. Lanes with protein extracts treated with PNGaseF prior to SDS-PAGE are indicated with “+” and control extracts treated and incubated in parallel without PNGaseF with “−”. NT, no treatment. The white arrowheads connected to black arrowheads indicate the reduction in apparent molecular weight of the glycosylated forms of TMPRSS13. E, conditioned media from untreated cells (NT) or cells transfected EV or non-tagged full-length human TMPRSS13. Only the SP-domain is detected. F, to determine whether the TMPRSS13 SP-domain is secreted into conditioned medium as an active protease, an α-M capture experiment was performed, and samples were separated by SDS-PAGE under reducing conditions, and detected by Western blotting using α-extra-TMPRSS13. The positions of the non-complexed TMPRSS13 SP-domain (black arrowhead) and the SP-domain–α-M complex (open arrowhead) are indicated. G, detection of the SP-domain in conditioned media from P. pastoris expressing cleaved, active TMPRSS13 with (+) and without (−) PNGaseF treatment by reducing SDS-PAGE and Western blotting (left panel). The white arrowheads connected to black arrowheads indicate the reduction in molecular mass of the glycosylated form of the SP-domain. Bar graph, right panel, conditioned media samples from P. pastoris clones transfected with either the expression vector without protease insert (EV), TMPRSS13 SP-domain, or matrithase SP-domain were incubated at 37 °C for 60 min with the synthetic chromogenic peptide Suc-Ala-Ala-Pro-Arg-pNA (100 μM), and the absorbance at 405 nm was recorded. Negative control (Neg. control) lane contains buffer and peptide with no media added.
In addition to whole-cell lysates, conditioned media (CM) samples collected from the same cells were analyzed. One band corresponding to the predicted SP-domain was detected in cells transfected with full-length TMPRSS13 (Fig. 1E) suggesting that TMPRSS13 is shed mainly in its active form. To validate that the SP-domain is in fact a catalytically active serine protease, we performed an α,β-macroglobulin (α,βM) capture assay (Fig. 1F). Incubation of α,βM with CM from cells expressing full-length TMPRSS13 resulted in the formation of a TMPRSS13 high molecular weight complex, consisting of the TMPRSS13 SP-domain covalently linked to α,βM subunits, indicating that expression of TMPRSS13 in mammalian cells produces a catalytically active serine protease (Fig. 1F). Additionally, lysates from cells expressing a C-terminal V5-tagged version of full-length TMPRSS13 (Fig. 1A, panel II) were analyzed under reducing and non-reducing conditions, respectively (supplemental Fig. S2). Under non-reducing conditions, the SP-domain was not detected, indicating that the C-terminal SP-domain remains tethered to the N-terminal part of TMPRSS13 via a disulfide bond after activation cleavage as predicted.

To further confirm the enzymatic activity of the TMPRSS13 SP-domain, the Pichia pastoris expression system, which utilizes the intracellular yeast protease KEX2, was employed. The KEX2 transmembrane serine protease belongs to the subtilisin-like pro-protein convertase family with specificity for cleavage after paired basic amino acids and is localized in the late Golgi compartment. By cloning the TMPRSS13 SP-domain into the PIC9 vector with the TMPRSS13 active serine protease domain sequence (S21IVG) immediately following the LGKR KEX2 cleavage site encoded by the vector, a novel fusion cleavage site was generated (Fig. 1A, panel III, see arrow indicating cleavage site). The new LGKR I/VG sequence is cleaved between Arg and Ile by KEX2, generating a secreted active TMPRSS13 SP-domain with the same IVG N terminus as the mammalian active SP-domain (Fig. 1A, panels I and III). The presence of TMPRSS13 serine protease in conditioned media from P. pastoris clones transfected with the PIC9-TMPRSS13 vector was confirmed by Western blotting using the polyclonal α-extra-TMPRSS13 antibody (Fig. 1G, left panel). Deglycosylation of the untagged, cleaved, and active form of the TMPRSS13 SP-domain, secreted by transfected P. pastoris, results in a form with an apparent molecular mass of ~29 kDa (Fig. 1G, left panel). This indicates that both the human- and yeast-expressed active serine protease domains migrate at the same approximate molecular mass upon deglycosylation. The catalytic activity of the TMPRSS13 SP-domain was confirmed using the serine protease chromogenic peptidolytic substrate Suc-Ala-Ala-Pro-Arg-pNA (Fig. 1G, right panel). Conditioned media from cells transfected with empty PIC9 vector were included as a negative control to ensure the absence of interfering secreted yeast proteases, and the well-characterized TTSP, matriptase, which was produced in parallel in P. pastoris, was included as a positive control. Collectively, these data demonstrate that TMPRSS13 is a glycosylated protease with peptidolytic activity.

**Catalytic inactivation of TMPRSS13 promotes TMPRSS13 cell-surface localization**

Analysis of the TMPRSS13 protein sequence using the TMHMM Server version 2.0 reveals the presence of a single transmembrane domain, predicting that the TMPRSS13 protein, similar to previously characterized members of the TTSP family, will localize on the cell surface (1–3, 8). To investigate the cellular localization of TMPRSS13, HEK293T cells transiently expressing the wild type (WT) full-length protease with a C-terminal V5-tag (WT-TMPRSS13-V5) (Fig. 1A, panel II) were analyzed by fluorescent immunocytochemistry. To detect the localization of WT-TMPRSS13-V5, an anti-V5 antibody was used on non-permeabilized cells in combination with a FITC-conjugated secondary antibody to visualize cell-surface TMPRSS13. Interestingly, little to no WT-TMPRSS13-V5 was detected under non-permeabilized conditions (Fig. 2A, top left panel). In contrast, an intense green fluorescent signal confined to the cytoplasm in permeabilized cells was observed, indicating that the majority of WT-TMPRSS13-V5 is retained within the cells (Fig. 2A, bottom left panel). When primary antibodies were substituted with non-immune IgG, no detectable staining was observed (Fig. 2A, right panels). Because the untagged WT-TMPRSS13 appeared to be present in its active form in lysates and CM as demonstrated by Western blotting above (Fig. 1, B and E), we speculated that the active protease fails to properly localize to the cell surface due to the deleterious effects of unopposed proteolytic activity. To test this possibility, we utilized a catalytically inactive form of TMPRSS13 (S506A-TMPRSS13-V5) where the serine in the catalytic triad is mutated to alanine (Fig. 2B). In addition, we also generated a “zymogen-locked” form (R320Q-TMPRSS13-V5) in which the conserved activation site is mutated to prohibit activation cleavage. Upon transfection, both S506A- and R320Q- TMPRSS13-V5 clearly localized to the cell surface in both permeabilized and non-permeabilized cells (Fig. 2, B and C). This observation is in agreement with the expected distribution of the predicted membrane-anchored topology of TMPRSS13, indicating that catalytically incompetent TMPRSS13 primarily localizes to the cell surface.

Lysates of transfected cells were then examined by Western blotting to analyze the state of WT-TMPRSS13-V5 compared with the two mutant versions. To ensure that WT-TMPRSS13-V5 exists in its intact full-length form with both the N and C terminus present, we probed with α-intra-TMPRSS13 and α-V5 (Fig. 2D). The two full-length glycosylation variant forms (~65 and ~75 kDa, which include the ~5 kDa V5-tag) were detected in WT-TMPRSS13 as well as in S506A- and R320Q-TMPRSS13-V5 with both antibodies. Thus, the C-terminal V5 tag appears intact, and the N terminus is most likely intact because the α-intra-TMPRSS13 antibody was raised against an N-terminal peptide. Surprisingly, in lysates from cells expressing S506A- or R320Q-TMPRSS13-V5 (Fig. 2D), an additional prominent and higher molecular mass (HMW) TMPRSS13 form of ~95 kDa was observed. This HMW species was resistant to extraction in RIPA buffer followed by boiling in Laemmli sample buffer with 5% 2-mercaptoethanol (Fig. 2D) and to extraction under stringent conditions (1.5% SDS with
100 mM DTT) (supplemental Fig. S1B), suggesting the possibility of covalent post-translational modifications, in addition to glycosylation, in two different catalytically incompetent forms of TMPRSS13. To further validate these findings, we analyzed lysates from cells expressing TMPRSS13 with an N-terminal HA-tag (depicted in Fig. 1A, panel IV, HA-WT-TMPRSS13) in combination with cell-surface biotinylation analysis using a membrane-impermeable biotinylation reagent (Fig. 2E). Cell-surface biotinylated proteins were eluted from the beads by addition of Laemmli sample buffer with 5% 2-mercaptoethanol. Samples were separated by SDS-PAGE and analyzed by Western blot using an α-HA antibody.

Figure 2. Catalytic inactivation of TMPRSS13 promotes its cell-surface localization and produces a higher molecular weight TMPRSS13 species. HEK293T cells were transiently transfected with full-length human WT-TMPRSS13-V5 (A), S506A-TMPRSS13-V5 (B), or R320Q-TMPRSS13-V5 (C) expression plasmids. Non-permeabilized cells (upper panels) or permeabilized cells (lower panels) were incubated with an anti-V5 antibody, followed by incubation with FITC-labeled secondary antibodies and DAPI staining to visualize nuclei. The cells were visualized by confocal fluorescence microscopy at 351–364 nm excitation wavelength (DAPI) or 488 nm (FITC). Merged images obtained at the two excitation wavelengths are shown. Non-immune IgG was included instead of anti-V5 as negative controls. D, protein lysates from HEK293T cells expressing EV, WT-TMPRSS13-V5, S506A-TMPRSS13-V5, or R320Q-TMPRSS13-V5 were analyzed by Western blotting using the α-intra-TMPRSS13 antibody recognizing the N terminus or α-V5 recognizing the C terminus. The HMW species is indicated with gray arrowhead. E, cell-surface proteins of HEK293T cells expressing either HA-WT-TMPRSS13 or HA-S506A-TMPRSS13 were biotin-labeled at room temperature for 30 min. Biotin-labeled cell-surface proteins were separated from non-labeled proteins by incubation with streptavidin-agarose for 60 min at 4 °C. The streptavidin-agarose beads were then pelleted, and the supernatant-containing proteins not precipitated by streptavidin-agarose were collected (wash lane). Biotin-labeled proteins were eluted from the beads by addition of Laemmli sample buffer with 5% 2-mercaptoethanol. Samples were separated by SDS-PAGE and analyzed by Western blot using an α-HA antibody.

Phosphorylation of the TTSP family member TMPRSS13


TMPRSS13 is capable of auto-activation

We consistently observed decreased detection of the cleaved active SP-domain in S506A-TMPRSS13-expressing cells in comparison with the levels of the active species seen in cells transfected with WT-TMPRSS13 (Fig. 3A). Quantitative ratio comparisons of the active (SP-domain) versus inactive full-length forms of TMPRSS13 confirmed that the majority of TMPRSS13 in S506A-TMPRSS13-expressing cells is in its inactive full-length forms (Fig. 3B). These data indicate that TMPRSS13 proteolytic activity is important for its own activation and that TMPRSS13, like several other TTSPs, is likely
Phosphorylation of the TTSP family member TMPRSS13

Figure 3. TMPRSS13 proteolytic activity promotes its own activation. A, protein lysates from HEK293T cells expressing EV, WT-TMPRSS13, or S506A-TMPRSS13 were analyzed by reducing SDS-PAGE and Western using the α-extra-TMPRSS13 antibody. B, graph depicting the relative ratios of staining intensity after Western development of active TMPRSS13 (SP-domain) compared with the inactive (full-length) species from three separate experiments. Asterisk indicates significant difference, p < 0.05, Student’s t test. C, cells expressing S506A-TMPRSS13-V5 were mechanically lifted from the plates by gentle pipetting in PBS, pelleted by centrifugation at 1000 × g, and resuspended in PBS pH 7.4 (41). Cells were then incubated with 100 nm active recombinant matriptase, prostasin, or TMPRSS13 or left untreated (no treatment lane) and incubated at 37 °C for 30 min. After incubation, cells were pelleted by centrifugation at 1000 × g, and supernatant was collected. Cells were then washed five times with PBS. After the last wash, cells were lysed with RIPA lysis buffer with protease inhibitor mixture and analyzed by Western blotting under reducing conditions.

capable of auto-activation. To further explore the possibility of auto-activation, intact cells transfected with S506A-TMPRSS13-V5 were treated with the soluble recombinant SP-domain of TMPRSS13, prostasin, or matriptase (Fig. 3C). After protease treatment, cells were pelleted by centrifugation, and the supernatant was collected to detect TMPRSS13 released by shedding. Treatment of cells with exogenous, active recombinant SP-domain led to robust activation as evidenced by shedding. Treatment of cells with exogenous, active SP-domain from prostasin, a non-TTSP glycosylphosphatidylinositol-anchored cell-surface serine protease, had no effect, whereas a low level of TMPRSS13 SP-domain was detected upon treatment with the TTSP, matriptase, in lysates with abundant SP-domain in the supernatant. Interestingly, the HMW form of TMPRSS13 appears to be preferentially cleaved by both TMPRSS13 and matriptase, which indicates that this form is likely accessible on the cell surface to exogenously added proteases. This is in agreement with the biotinylation assay using HA-S506A-TMPRSS13, which demonstrates that the HMW species is the primary species observed at the cell surface (Fig. 2E).

Using a different approach, HEK293T cells were co-transfected with V5-tagged S506A-TMPRSS13 and either empty vector or HA-tagged WT-TMPRSS13 (supplemental Fig. S3). Upon co-transfection with HA-WT-TMPRSS13, cleavage of V5-S506A-TMPRSS13 was observed. Lower signal of the HMW species was observed upon co-expression when compared with the ~75-kDa species, suggesting that the HMW species is preferentially cleaved and activated upon co-expression. Together, the data from these different assays support the notion that TMPRSS13 is capable of auto-activation and that the HMW species is preferentially converted into the two-chain species.

TMPRSS13 is phosphorylated

To characterize the HMW form of TMPRSS13, we first performed deglycosylation of lysates from cells expressing untagged S506A- or R320Q-TMPRSS13, which resulted in the HMW species to migrate at ~73 kDa, which is ~13 kDa greater than the full-length deglycosylated TMPRSS13 (Fig. 4A). This confirms that the HMW form is subject to additional post-translational modification(s). Initial tests were conducted to determine whether the HMW form represents a ubiquitinated species of TMPRSS13, because mono-ubiquitination is a common covalent post-translational modification of cell-surface proteins that results in the attachment of the ~8.5-kDa ubiquitin protein (19). Deubiquitination of whole-cell lysates using the recombinant catalytic domain of deubiquitinase USP2 (20–22) did not result in a mobility shift of the HMW species (supplemental Fig. S4A). Only two lysine residues exist in the intracellular domain of TMPRSS13, and upon mutation to prevent ubiquitin modification (K151R and K159R), no effect on the abundance or size of the HMW species was observed (supplemental Fig. S4B). Taken together, these observations strongly indicate that ubiquitination does not contribute to the HMW form of TMPRSS13. Currently, the only reported post-translational modifications of other hepsin/TMPRSS family members are glycosylation and proteolytic cleavage (3). Other types of post-translational modifications, including phosphorylation, are often found in transmembrane proteins that contain IDR s (23, 24). Intrinsic disorder occurs in ~50% of transmembrane proteins, and proteins with IDR s are more likely to be phosphorylated than proteins that lack them (23). Therefore, we conducted computational prediction of IDR s within the intracellular domain of TMPRSS13 and four additional TMPRSS/hepsin family members using PONDR-FIT (25) to determine whether there is a higher degree of disorder in the intracellular domain of TMPRSS13 compared with the other family members (Fig. 4B). The comparative analysis revealed that a large segment of TMPRSS13, the first 125 amino acids, displays a high degree of disorder followed by a sharp decline to a lower disorder disposition before the beginning of the transmem-
brane domain (Fig. 4B). In comparison, the intracellular domain of TMPRSS2, which contains the second longest intracellular domain of the hepsin/TMPRSS family, contains a short segment of only 10 amino acids with a high degree of disorder before a sharp decline (Fig. 4B). IDRs are also often enriched in disorder-promoting amino acid residues such as proline and serine (24); the intracellular domain of TMPRSS13 contains 15% proline and 19% serine residues (Fig. 4C). In sum, TMPRSS13 contains the largest intracellular domain with a higher degree of intrinsic disorder compared with other hepsin/TMPRSS family members. Based on this observation and on the fact that transmembrane proteins that contain IDRs are

Figure 4. TMPRSS13 is a phosphorylated TTSP family member. A, protein lysates from cells expressing S506A- or R320Q-TMPRSS13 were either untreated (NT) or treated with PNGaseF (+) or without (−) (incubated in deglycosylation buffer minus PNGaseF) for 60 min at 37 °C prior to reducing SDS-PAGE and Western blotting using α-extra-TMPRSS13. The white arrowheads connected to black arrowheads indicate the reduction in apparent molecular weight of the glycosylated forms of TMPRSS13. B, graph representing the intrinsic disorder of hepsin/TMPRSS family member’s intracellular domains. Full-length FASTA sequences of hepsin, TMPRSS2, TMPRSS3, TMPRSS4, spinesin, and TMPRSS13 were analyzed using PONDR-FIT (25), and the intracellular domains were plotted on a single graph for comparison. The blue line represents TMPRSS3 overlaying TMPRSS4 (turquoise line) because the profiles of the two are virtually identical. The final x axis data point for each protein indicates the last amino acid of the intracellular domain. C, amino acid sequence of the 160 amino acid proline (26 residues, red) and serine-rich (30 residues, blue) intracellular domain of TMPRSS13. D, two different exposures of Western blot analysis of lysates from cells expressing WT-TMPRSS13, S506A-TMPRSS13-V5, or R320Q-TMPRSS13-V5 that were treated with or without CIP and incubated at 37 °C for 60 min. E, lysates from HEK293T cells expressing HA-S506A-TMPRSS13 were immunoprecipitated (IP) using an α-HA or an α-IgG control antibody. Samples were separated in parallel and analyzed by Western blotting (WB) by probing with α-phosphoserine/threonine or α-HA antibodies.
frequently phosphorylated (3, 23, 24), we investigated whether the HMW species represents a phosphorylated form of full-length TMPRSS13.

Phosphorylation of proteins occasionally causes mobility shifts in Western blot analysis due to the negative charge of the covalently attached phosphate group, thereby reducing negatively charged SDS from interacting with the phosphorylated protein (26). Whole lysates from cells expressing WT, R320Q, or S506A-TMPRSS13-V5 were dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIP) and separated by SDS-PAGE. Western blot analysis of the CIP-treated samples revealed a mobility shift of the HMW form (Fig. 4D) indicating that the higher molecular weight product is a full-length phosphorylated and glycosylated TMPRSS13 species. Of note, dephosphorylation did not reduce the HMW species to the identical molecular mass of the non-phosphorylated V5-tagged full-length species of ~75 kDa. A possible reason for this may be that the inactive phosphorylated full-length protein is differentially glycosylated compared with the non-phosphorylated inactive full-length protein or that dephosphorylation of lysates results in incomplete removal of total phosphorylated residues of the intracellular domain.

The intracellular domain of TMPRSS13 contains a total of 30 serine residues, 12 threonine residues, and 1 tyrosine residue. Previous reports indicate that transmembrane proteins that contain IDRs, like TMPRSS13, are more likely to have greater than 10 phosphorylation sites than proteins that lack IDRs (23). Because it is likely that one or more serine or threonine residues are phosphorylated, and to confirm the phosphorylation of the HMW TMPRSS13 species, immunoprecipitation of lysates from cells expressing HA-S506A-TMPRSS13 was performed. Affinity pulldowns of HA-S506A-TMPRSS13, with α-HA followed by immunoblotting with an α-HA antibody, and an antibody specifically recognizing phosphoserine/threonine residues confirmed that the HMW species is phosphorylated (Fig. 4E). As expected, probing with α-HA reveals both full-length TMPRSS13 and phosphorylated TMPRSS13 (Fig. 4E, left panel). When α-phospho-Ser/Thr was used for detection, a single band corresponding to HMW TMPRSS13 was detected (Fig. 4E, right panel). Together, these data show that HMW TMPRSS13 represents a phosphorylated form of the protease.

**Endogenous TMPRSS13 is phosphorylated and glycosylated**

To determine whether endogenous TMPRSS13 is present in a phosphorylated form, the human breast cancer cell lines HCC1937, MCF-7, and BT-20, as well as the colorectal adenocarcinoma cell line DLD1, were screened for expression of TMPRSS13 by Western blotting. Lysates were analyzed side-by-side with untagged WT- or S506A-TMPRSS13 for comparison and detected with the α-intra-TMPRSS13 (Fig. 5A and supplemental Fig. S5A). We observed bands corresponding in size to the glycosylated full-length (~70 kDa), a non-glycosylated full-length (~60 kDa), as well as an HMW TMPRSS13 (~90 kDa) in all four cell lines, with varying amounts of HMW TMPRSS13 relative to total TMPRSS13 among the different cell lines (Fig. 5A). We also analyzed the expression of the endogenous Kunitz-type serine protease inhibitors HAI-1 and HAI-2 in these cell lines, which are critical regulators of proteolytic activity of several TTSP family members (1–3). Lysates of cell lines were analyzed by Western blotting and probed for TMPRSS13, HAI-1, and HAI-2. Both inhibitors were detected in all four cell lines (Fig. 5B). To ensure α-intra-TMPRSS13 antibody specificity, TMPRSS13 was knocked down in the MCF-7 (Fig. 5C, left) and DLD1 cell lines (Fig. 5C, right) using two different non-overlapping siRNAs targeting the TMPRSS13 transcript. At 72 h after siRNA transfection, cell lysates were analyzed by Western blotting. Levels of endogenous, full-length TMPRSS13 and the HMW species of ~90-kDa TMPRSS13 were significantly reduced upon TMPRSS13 silencing, confirming that the antibody is suitable to study endogenous TMPRSS13 (Fig. 5C).

To determine whether the ~90-kDa product of the endogenous form of TMPRSS13 is a phosphorylated species in MCF-7 and DLD1 cells, whole-cell lysates were dephosphorylated with λ protein phosphatase (Fig. 5D). Dephosphorylation of lysates resulted in a mobility shift of the ~90-kDa band, indicating that the HMW species is a phosphorylated endogenous TMPRSS13 species. To additionally validate that endogenous HMW TMPRSS13 represents the phosphorylated form of the protease, immunoprecipitation of whole lysates from DLD1 cells using the α-intra-TMPRSS13 was performed. Isolated proteins were analyzed by Western blotting, and upon probing with anti-Phospho-Ser/Thr, a band corresponding to HMW TMPRSS13 was detected (Fig. 5E, right panel). Taken together, these data indicate that the ~90-kDa species is representative of an endogenous phosphorylated TMPRSS13 species.

To assess the glycosylation status of endogenous TMPRSS13, lysates from MCF-7 and DLD1 cells were deglycosylated with PNGaseF for 60 min (Fig. 5F). In the control lane (−) two bands of ~70 and ~60 kDa are observed; upon deglycosylation (+ lane), two bands of ~60 and 58 kDa are observed. It is likely that the ~70-kDa band is reduced to the ~60-kDa species because similar observations are seen upon deglycosylation of TMPRSS13 in HEK293T cells (Fig. 1D). Notably, upon deglycosylation is the presence of two bands, which may represent two different TMPRSS13 isoforms rather than two different glycosylated full-length species. Currently, there are two known TMPRSS13 isoforms that have been identified in lung, placenta, pancreas, prostate, and testis (5). Deglycosylation of lysates that expressed high levels of detectable, endogenous, and phosphorylated TMPRSS13 revealed that the phosphorylated species shifts from ~90 to ~73 kDa (supplemental Fig. S5B), which is similar to what we observed upon deglycosylation of S506A-TMPRSS13 (Fig. 4A). Collectively, these results demonstrate that endogenous TMPRSS13 is post-translationally modified by glycosylation and phosphorylation.

**HAI-1 and HAI-2 mediate TMPRSS13 cell-surface localization and phosphorylation**

The data above indicate that loss of TMPRSS13 proteolytic activity, either directly by mutation of a residue within the catalytic triad or indirectly by mutation of the activation site, results in a detectable phosphorylated full-length form of TMPRSS13 and its cell-surface localization. The Kunitz-type serine protease inhibitors, HAI-1 and HAI-2, are critical regu-
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Figure 5. Endogenous TMPRSS13 is phosphorylated. A, lysates from HEK293T cells expressing untagged WT-TMPRSS13 or SS06A-TMPRSS13 were analyzed side by side with lysates from the breast cancer cell lines MCF-7, BT20, and HCC1937 and the colorectal carcinoma cell line DLD1. The figure shows a short exposure (left) for the exogenously expressed TMPRSS13 and a long exposure (right) for the endogenously expressed TMPRSS13 to compare sizes. Original Western blot exposures can be found in supplemental Fig. S5A. Asterisk indicates nonspecific bands. B, lysates from the indicated cell lines were analyzed for expression of TMPRSS13, HAI-1, and HAI-2. Asterisk indicates nonspecific bands. **C**, MCF-7 and DLD-1 cells were treated with two non-overlapping RNA duplexes (siRNA 31 and 33) targeting the TMPRSS13 transcript or a scrambled GC-matched control (SCRM). After 72 h post-treatment, cells were lysed, and Western blotting was done to analyze decreased expression of TMPRSS13. Asterisk indicates nonspecific bands. D, MCF-7 and DLD-1 cell lysates were treated with (+) or without (−) λ phosphatase and analyzed by Western blotting (WB) to detect endogenous TMPRSS13 using the α-intra-TMPRSS13 antibody under reducing conditions. E, DLD-1 cell lysates were immunoprecipitated (IP) with either α-intra-TMPRSS13 or α-IgG control. Precipitated samples were separated in parallel and analyzed by Western blotting by probing for either α-phosphoserine/threonine or α-intra-TMPRSS13. Input lane contains samples from lysates prior to immunoprecipitation. F, lysates from DLD1 or MCF-7 cells were treated with (+) or without (−) PNGaseF and analyzed by Western blotting under reducing conditions.

Lytators of proteolytic activity of several TTSP family members (1–3) and play a role in proper cell-surface localization (27–29). As shown in Fig. 5B, the cancer cell lines DLD1, BT20, HCC1937, and MCF-7 all express endogenous HAI-1 and HAI-2. Therefore, we reasoned that HAI-1 and/or HAI-2 could facilitate TMPRSS13 phosphorylation and cell-surface localization. To test the inhibitory activity of HAI-1 and HAI-2 toward TMPRSS13, we performed a peptidolytic assay using active recombinant TMPRSS13 SP-domain, generated in P. pastoris, with known inhibitors of TTSP family members, including HAI-1, HAI-2, aprotinin, leupeptin, and benzamidine. The test was performed in parallel with active matriptase SP-domain,
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generated in the same expression system, for comparison with a well-characterized reference protease and to ensure inhibitor activity (Fig. 6A) (12). Active TMPRSS13 or matriptase was incubated with the chromogenic peptide Suc-Ala-Ala-Pro-Arg-pNA in the absence or presence of the indicated serine protease inhibitors (inhibitor and substrate added concomitantly), and changes in absorbance were analyzed. Similar to previous reports, recombinant HAI-1 and HAI-2 both inhibit matriptase activity (12, 30, 31). HAI-1 appeared to be a poor inhibitor of TMPRSS13 activity in this in vitro setting, whereas incubation with HAI-2 resulted in nearly complete loss of measurable proteolytic activity. This is similar to previous reports where HAI-1, containing both Kunitz inhibitory domains, displayed weak inhibitory activity toward recombinant TMPRSS13 compared with HAI-2 in a cell-free assay (17). We also observed TMPRSS13 inhibition with the globular polypeptide, Kunitz-type serine protease inhibitor, aprotinin (bovine pancreatic trypsin inhibitor), and leupeptin, whereas benzamidine was a poor inhibitor of TMPRSS13 in this assay.

To validate the HAI-1/HAI-2 inhibitor observations in a cellular setting, we conducted peptidolytic activity assays utilizing whole-cell extracts of cells expressing untagged TMPRSS13 either alone or together with HAI-1 or HAI-2 (Fig. 6C). No significant inhibition of activity, as measured by decreased absorbance compared with lysates of cells expressing WT-TMPRSS13 alone, was observed upon TMPRSS13/HAI-1 co-expression, whereas significant TMPRSS13 inhibition was seen when co-expressed with HAI-2. As expected, no substrate conversion above mock background levels was observed when HAI-1 or HAI-2 was expressed without TMPRSS13 or when catalytically inactive S506A-TMPRSS13 was expressed. Taken together, these data are in concordance with the results from cell-free assays above (Fig. 6A) and further indicate that HAI-2 is a more efficient inhibitor of TMPRSS13 proteolytic activity than HAI-1.

To determine whether there is a functional link between TMPRSS13 activation, phosphorylation, and HAI, lysates from HEK293T cells co-expressing either HAI-1 or HAI-2 and WT-TMPRSS13 were analyzed by Western blotting (Fig. 6C). Co-expression of HAI-2 and WT-TMPRSS13 resulted in the appearance of an HMW species that corresponds in size to the phosphorylated form of TMPRSS13. This species was verified to be phosphorylated TMPRSS13 by CIP treatment, which led to the expected shift in mobility (Fig. 6D). Co-expression of WT-TMPRSS13 with HAI-1 resulted in no signal or a weak signal of the phosphorylated TMPRSS13 species in whole-cell lysates by Western blotting (Fig. 6C). We suspected this could, at least in part, be due to limited detection capability of the α-extra-TMPRSS13 antibody, because a signal was detected in whole-cell lysates of cells expressing WT-TMPRSS13-V5 and HAI-1 at longer exposure (Figs. 6E, input, and 8A, whole lysate). When WT-TMPRSS13-V5 was immunoprecipitated from lysates of cells co-expressing either HAI-1 or HAI-2, phosphorylated TMPRSS13-V5 was detected in both samples, albeit at lower relative levels for HAI-1 (Fig. 6E, IP: V5), indicating that both HAI-1 and HAI-2 lead to increased TMPRSS13 phosphorylation. Additionally, the co-immunoprecipitation of TMPRSS13-V5 with HAI-1 and HAI-2, respectively, indicates association between each of the inhibitors and TMPRSS13 (Fig. 6E). Taken together, these data indicate that co-expression of HAI-1 or HAI-2 with TMPRSS13 promotes TMPRSS13 phosphorylation.

We consistently observed lower levels of both total and phosphorylated TMPRSS13 when expressed with HAI-1 compared with HAI-2 (Fig. 6, C and E), which could be due to enhanced activation and shedding of TMPRSS13 from the cell surface when co-expressed with HAI-1. Conditioned media analyzed from cells co-expressing untagged WT-TMPRSS13 with either HAI-1 or HAI-2 revealed that co-expression of TMPRSS13 with either inhibitor results in increased shedding of the TMPRSS13 active SP-domain into the media compared with WT-TMPRSS13 without HAI expression (Fig. 6F). A greater relative amount of the shed SP-domain was observed upon HAI-1 co-expression compared with HAI-2 co-expression alongside TMPRSS13. Similar observations were previously described for the TTSP family member, matriptase. When matriptase was co-expressed with either HAI-1 or HAI-2, more shed matriptase was detected in the presence of HAI-1 in MDCK cells (28). These data suggest that the lower levels of the phosphorylated TMPRSS13 observed in cells co-expressing HAI-1 compared with HAI-2 may have resulted from increased activation and shedding of TMPRSS13 due to lower inhibitory potential of HAI-1 toward TMPRSS13 compared with HAI-2 (Fig. 6, A, B, and F).

To further study the role of HAI-1 and HAI-2 in regulating cell-surface localization of TMPRSS13, HEK293T cells were co-transfected with WT-TMPRSS13-V5 and either empty vector, HAI-1, or HAI-2, followed by immunocytochemical analysis (Fig. 7). Visualization of HAI-1 was accomplished using the M19 antibody (32), and HAI-2 was visualized using a vector expressing a HAI-2-EYFP fusion protein (28). As expected, transfection with WT-TMPRSS13-V5 alone resulted in intracellular, perinuclear accumulation of the protease (Fig. 7A). In contrast, co-expression with HAI-1, which itself mainly localizes to the cell surface, led to cell-surface localization of TMPRSS13 as well as some intracellular staining (Fig. 7B). In cells co-expressing HAI-2-EYFP and WT-TMPRSS13-V5, HAI-2 localizes mostly intracellularly (Fig. 7C), in accordance with previous observations in MDCK cells (28). TMPRSS13 appears to co-localize with HAI-2 intracellularly as well as at the cell surface (also refer to the surface biotinylation data below). It is plausible that interactions between TMPRSS13 and HAI-2 take place along the secretory pathway because co-localization of HAI-2 with endoplasmic reticulum and cis-Golgi markers has been previously reported (28, 33).

To verify that TMPRSS13 is localizing to the cell surface, and to identify which form(s) of TMPRSS13 is(are) surface-associated, we performed biotin labeling of cell-surface proteins on intact HEK293T cells expressing WT-TMPRSS13-V5 with either empty vector, HAI-1, or HAI-2 (Fig. 8, A and B). As expected, when WT-TMPRSS13-V5 was expressed without HAI-1 or HAI-2, none or very low levels of cell-surface biotin-labeled protease were detected by Western blotting (Fig. 8A, left) in accordance with the immunocytochemistry data in Figs. 2A and 7A. In contrast, co-expression with HAI-1 or HAI-2 resulted in surface-associated TMPRSS13 (Fig. 8, A, right, and
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A

Percent Activity

Neg Control No Inhibitor Hai-1 Hai-2 Aprtinin Laupetan Benzamidine

B

Absorbance

WT-TMPRSS13 EV WT-TMPRSS13 EV WT-TMPRSS13 EV WT-TMPRSS13 EV WT-TMPRSS13 EV WT-TMPRSS13 EV

C

HEK293T lysate α-extra-TMPRSS13

α-HAI-1

HEK293T lysate WT-13-V5 α-V5 (Short Exposure)

α-HAI-2

D

α-V5 (Long Exposure)

E

Input

IP: V5

WT-13-V5 EV WT-13-V5 + HAI-1

F

HEK293T Conditioned Media

α-TMPRSS13

α-HAI-1

α-HAI-2

α-V5
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Figure 6. HAI-1 and HAI-2 mediate TMPRSS13 phosphorylation. A, recombinant protease (5 nM) from P. pastoris clones transfected with either TMPRSS13 (black bars) or matrkapase (white bars) was incubated at 37 °C for 60 min with the synthetic chromogenic peptide Suc-Ala-Ala-Pro-Arg-pNA (100 μM) in the absence or presence of the inhibitor indicated (inhibitor and substrate added concomitantly). The absorbance at 405 nm was recorded, and enzyme activities indicated were incubated with the chromogenic peptide Suc-Ala-Ala-Pro-Arg-pNA (100 μM) at 37 °C for 60 min, and absorbance at 405 nm was recorded. Asterisks indicate significant decrease in proteolytic activity compared with WT-TMPRSS13 + EV measurement (analysis of variance multiple comparisons (p < 0.0001) compared with “no inhibitor” measurements. B, 200 μg of whole-cell protein lysates from HEK293T cells transfected with the plasmids indicated were incubated with the chromogenic peptide Suc-Ala-Ala-Pro-Arg-pNA (100 μM) at 37 °C for 60 min, and absorbance at 405 nm was recorded. Asterisks indicate significant decrease in proteolytic activity compared with WT-TMPRSS13 + EV measurement (analysis of variance multiple comparisons (p < 0.01)), n.s., no significance. C, lysates from HEK293T cells that were transiently transfected with full-length human HAI-1 alone, HAI-2 alone, or together with WT-TMPRSS13 or EV were separated by SDS-PAGE and analyzed by Western blotting. D, protein lysates (5 μg) from cells expressing WT-TMPRSS13-V5 with HAI-2 were treated with CIP for 60 min at 37 °C and analyzed by Western blotting under reducing conditions. WT-13-V5 = WT-TMPRSS13-V5. E, cells expressing EV, WT-TMPRSS13-V5/EV, HAI-1/EV, HAI-2/EV, or WT-TMPRSS13-V5 with either HAI-1 or HAI-2 were immunoprecipitated with a-V5 antibody and analyzed by Western blotting (right panels). Whole-cell lysates were analyzed by Western blotting to verify expression before precipitation (input) (left panels). F, conditioned media from HEK293T cells expressing the indicated proteins were collected 24 h after addition of serum-free media and analyzed by Western blotting under reducing conditions.

Figure 7. HAI-1 and HAI-2 mediate TMPRSS13 cell-surface localization. Permeabilized cells transfected with WT-TMPRSS13-V5 alone (A) or co-transfected with HAI-1 (B), or HAI-2-EYFP (C) were stained with anti-V5 (A and C) or anti-HA and the anti-HAI-1 M19 antibody (B). The cells were visualized by confocal fluorescence microscopy. Nuclei (DAPI) (blue, A–C), WT-TMPRSS13-V5 (red, A–C), HAI-1 (green, B), and HAI-2-EYFP (green, C). Merged images are shown in panels on the right.

Interestingly, the V5-tagged phosphorylated, full-length inactive (~95 kDa) and active protease (~38 kDa) SP-domain species appeared to be predominantly localized at the cell surface, because those TMPRSS13 species were readily detected in the biotin-labeled samples, and little to no phosphorylated or active TMPRSS13 was observed in the corresponding non-biotinylated fractions (Fig. 8, wash lanes). Correspondingly, the non-phosphorylated, full-length inactive TMPRSS13 species (~75 kDa) was detected in the wash lanes (Fig. 8, A and B). This experiment was repeated with co-transfection of N-terminal HA-tagged (HA-WT-TMPRSS13) and HAI-2 with similar results (supplemental Fig. S6). According to these data, the non-phosphorylated, inactive full-length species localizes primarily intracellularly. Notably, cells expressing TMPRSS13 with HAI-1 display the highest active SP-domain to total TMPRSS13 ratio comparatively to TMPRSS13 with HAI-2 on the cell surface. It is possible that this could, at least in part, be attributed to the poor inhibitory capability of HAI-1 toward TMPRSS13, which may lead to increased auto-activation of TMPRSS13. Importantly, both HAI-1 and HAI-2 are detected in the biotinylated samples indicating that they both localize to the cell surface. Collectively, these data indicate that HAI-1 and HAI-2 promote TMPRSS13 phosphorylation and cell-surface localization of the protease and that phosphorylated TMPRSS13 is the predominant form present at the cell surface.

Discussion

As an ongoing effort to characterize the members of the TTSP family, we performed biochemical and cell biological analyses of the hepatim/TMPRSS subfamily member, TMPRSS13. Expression of human full-length TMPRSS13 cDNA in mammalian cells led to the production of a recombinant glycoprotein that could be detected in both cell lysates and as a shed form in conditioned media. Full-length TMPRSS13 displayed proteolytic activity both in whole-cell extracts and in conditioned media. Like various other TTSPs, including matriptase, matriptase-2, hepsin, TMPRSS2, TMPRSS3, and TMPRSS4 that are capable of auto-activation (2), the proteolytic activity of TMPRSS13 also appears to be involved in its own activation. Mutation of the active-site serine residue led to decreased levels of detectable active SP-domain by Western blotting. Addition of active, recombinant TMPRSS13 to cell-surface catalytically deficient TMPRSS13 (S506A-TMPRSS13-V5) also led to TMPRSS13 two-chain conversion. Intriguingly, the well-studied TTSP family member, matriptase, is also capable of cleaving TMPRSS13 at its activation site. Therefore, matriptase may function as an activator of TMPRSS13 in certain conditions; however, the physiological relevance of this phenomenon awaits further study.

Cell imaging studies revealed that catalytic inactivation of TMPRSS13 by either mutation of the catalytic serine or of the arginine residue in the zymogen activation site results in efficient cell-surface localization of TMPRSS13. WT-TMPRSS13 failed to localize at the cell surface and was largely detected intracellularly. However, when TMPRSS13 is expressed with either HAI-1 or HAI-2, efficient cell-surface localization is observed using both immunocytochemistry and cell-surface protein biotinylation assays. Therefore, the cognate inhibitors HAI-1 and HAI-2 may facilitate TMPRSS13 localization by preventing aberrant intracellular activation. In this regard, it is interesting to note that HAI-1 displays weak inhibitory activity toward TMPRSS13. Previous reports examining the transport of the TTSP family member matriptase to the cell surface have proposed that HAI-1 interacts with the zymogen form of matriptase, thus preventing intracellular activation and to aid in its transport to the cell surface (27–29, 34). Therefore, HAI-1 may function in a similar manner by interacting with the zymo-
gen form of TMPRSS13 to prevent artificial intracellular activation of TMPRSS13.

One of the most intriguing findings of our studies is the presence of a phosphorylated form of TMPRSS13. To our knowledge, this is the first time a TTSP, or any other membrane serine protease, has been demonstrated to be modified by phosphorylation. Biotinylation studies revealed that the phosphorylated species primarily localizes at the cell surface, presenting the possibility that phosphorylation of TMPRSS13 promotes its translocation to the cell surface. Additionally, in the experiments designed to determine whether TMPRSS13 is capable of auto-activation, the full-length phosphorylated species is the predominant species to be converted into the two-chain form. The mechanism of TTSP auto-activation is not known; however, it is thought to involve oligomerization (2). Therefore, one possibility is that phosphorylation of TMPRSS13 promotes oligomerization and cleavage into the two-chain species, thus linking phosphorylation events to zymogen conversion of TMPRSS13. Indeed, previous reports on proteins that contain IDRs have demonstrated that phosphorylation of disordered regions allows for tight control over protein–protein interactions (35, 36). Additional studies are needed to gain a better understanding of the consequences of TMPRSS13 phosphorylation.

Previous studies of protease phosphorylation mainly focused on intracellular proteases, including caspas and deubiquitinating enzymes (37). ADAM (A Disintegrin and Metalloprotei-

case) proteases have also been shown to be phosphorylated (38). The C-terminal domain of the transmembrane ADAM17 undergoes phosphorylation at different sites, including Thr

and Ser

, and phosphorylation at Thr

 was found to be necessary for ADAM17-catalyzed shedding of TGF-α. With ADAM15, phosphorylation of the cytoplasmic domain resulted in interaction with several potential signaling proteins, including the Src kinases Hck and Lek (39). It is plausible that phosphorylation of TMPRSS13 regulates similar and/or additional processes, including localization, protein–protein interactions, and activation.

Currently, the kinases responsible for TMPRSS13 phosphorylation are unknown. Phosphorylation software tools predict potential candidates for multiple serine residues within the intracellular domain, including the stress-activated kinases JNK and p38 MAPK, the cyclin-dependent kinases 2 and 5, and ERK1. Other candidates have been suggested including calmodulin kinase II and protein kinase C (6, 8). Future studies to identify the kinase(s) responsible for TMPRSS13 phosphorylation may reveal novel insights into the cellular pathways in which TMPRSS13 is involved.

TMPRSS13, HAI-1, and HAI-2 are co-expressed in many developing and adult mammalian epithelia, including simple, transitional, and multilayered squamous epithelia (7, 31, 40). This study provides novel information about the regulation of TMPRSS13 activity, HAI–mediated cell-surface localization, and phosphorylation, and it may represent a new post-transla-

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Figure 8. Localization of phosphorylated TMPRSS13 on the cell surface. Cell-surface proteins of HEK293T cells expressing WT-TMPRSS13-V5 with either EV (A, left), HAI-1 (A, right), or HAI-2 (B) were biotin-labeled at room temperature for 30 min. Biotin-labeled proteins were precipitated with streptavidin-agarose for 60 min at 4 °C. Streptavidin-agarose beads were then pelleted, and the supernatant containing proteins that were not precipitated with streptavidin-agarose were collected (wash). Biotin-labeled proteins were eluted from beads by treatment with Laemmli sample buffer with 5% 2-mercaptoethanol and run side-by-side with non-labeled proteins and whole lysates prior to streptavidin treatment. Different exposures for V5 Western blotting membranes are displayed.
tional mechanism critical for cellular regulation and function of TMPRSS13 and other similar proteases.

Experimental procedures

Cloning of full-length TMPRSS13 plasmid constructs

The human TMPRSS13 plasmid construct was obtained from GeneCopia with accession number BC114928.1 (Rockville, MD). The full-length human TMPRSS13 was PCR-amplified using a high-fidelity Platinum® Taq polymerase (Invitrogen, Life Technologies, Inc.) with the following primers: 5'-GCCACCATGAGGGAGACGGACGAGG-3' and 5'-AGATTTTGCTGACCTGAGCT-3'. The resulting PCR fragment was cloned into the pcDNA 3.1/V5-His TOPO® TA (Invitrogen, Life Technologies, Inc.) in-frame with a C-terminal His-tag and V5 epitope using standard TA cloning techniques. Point mutations for S506A-TMPRSS13 and R320Q-TMPRSS13 were generated using a QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer’s protocol. Primers used for S506A mutagenesis were 5'-AAGAGGCCCCGGCGGTCTCCCTGGCA-3' and 5'-CTGACAGGGAGACGGG-3'. Primers used for R320Q mutagenesis were 5'-CCATGACGGCGAGATCGTGAGG-3' and 5'-'GCCACCATGAGGGAGACGGACGAGG-3'. Primers used for K151R mutagenesis were 5'-GAGCCAGTGAACCTTGAGGGGCGTC-3' and 5'-CGAGCTGCTGCCGGATCTGG-3'. Primers used for K159R mutagenesis were 5'-GTAGCTGCTCTCGCCGCTCCGGC-3' and 5'-GGGAGGCGACGACAGCTC-3'.

UnTagged TMPRSS13 constructs were generated by PCR amplification of WT, S506A, and R320Q expression vectors using the Platinum® Taq polymerase. The primers used for PCR amplification were 5'-AACCGGATCATGAGGGAGACGGACGAGG-3' and 5'-AACCGGATCATGAGGGAGACGGACGAGG-3'. The resulting PCR fragment was cloned into the pcDNA 3.1/V5-His TOPO® TA. The human TMPRSS13 active serine protease domain was produced in yeast using a Pichia expression kit (Invitrogen, Life Technologies, Inc.).

Transfection of HEK293T (ATCC, Manassas, VA) was performed using Lipofectamine® LTX according to the manufacturer’s instructions (Invitrogen, Life Technologies, Inc.). HEK293T cells were cultured in Dulbecco’s modified Eagle’s media (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Transfection was performed with 1 µg of plasmid DNA for single transfections or 2 µg of total plasmid DNA for co-transfections. Vectors included in transfections were pcDNA3.1-TMPRSS13 vectors, empty vector pcDNA3.1, pcDNA3.1-HAL-1, pcDNA3.1-HAL-2, or pEYFP-N1-HAL-2. The HAL vectors were kindly provided by Dr. Stine Friis, University of Copenhagen. To analyze samples by Western blotting using the polyclonal anti-TMPRSS13 antibody (α-extra-TMPRSS13) (PA5-30935, Thermo Fisher Scientific and Life Technologies, Inc.), 24 h post-transfection cells were washed three times with PBS, and serum-free DMEM was added for 6 h when analyzing cell lysates and conditioned media from cells expressing untagged TMPRSS13 constructs. Cells were then washed again three times with PBS after 6 h, and 1 ml of serum-free media were added. Twenty four h after addition of serum-free media, conditioned media and lysates were collected. Cells were lysed using RIPA buffer: 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, pH 7.4, supplemented with protease inhibitor mixture (Sigma) and cleared by centrifugation at 12,000 × g at 4°C. Protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). For stringent lysis condition, SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 0.25% bromphenol blue, 5% glycerol, 1.5% SDS, and 100 mM DTT) was boiled for 10 min. Cells were washed 1× with PBS, and then boiling stringent lysis buffer was added to cells. Cells were scraped, collected, and boiled for 10 min. After boiling, cell lysates were centrifuged at 16,000 × g for 10 min at room temperature. Samples were then boiled again for 5 min, and 15 µl of each sample was loaded onto 10% SDS gels. Protein samples were separated by SDS-PAGE using 10% Mini-PROTEAN® TGX precast gels (Bio-Rad) under reducing conditions and analyzed by Western blotting. Primary antibodies used in Western blotting were polyclonal rabbit anti-TMPRSS13 raised against a recombinant protein fragment corresponding to a region within amino acids 195 and 562 of human TMPRSS13 (anti-extra-TMPRSS13) (PA5-30935, Thermo Fisher Scientific and Life Technologies, Inc.); rabbit anti-TMPRSS13 intracellular domain antibody (anti-intra-TMPRSS13) raised against an epitope within the first (N-terminal) 60 amino acids (ab59862, Abcam, Cambridge, MA); V5 mouse monoclonal antibody (R96025, Thermo Fisher Scientific and Life Technologies, Inc.); HA rabbit polyclonal antibody (NB600-363, Novus Biologicals, Littleton, CO); rabbit anti-phospho-(Ser/Thr) Phe antibody (catalog no. 9631, Cell Signaling Technology, Danvers, MA); and goat anti-HAI-1 (AF1048, R&D Systems Inc., Minneapolis, MN); goat anti-HAI-2 (AF1106, R&D Systems Inc., Minneapolis, MN). Secondary antibodies included goat anti-rabbit, goat anti-mouse (Millipore, Billerica, MA), and rabbit anti-goat (Dako, Carpinteria, CA) HRP-conjugated antibodies.

Cloning and expression of the TMPRSS13 active serine protease domain in P. pastoris

The human TMPRSS13 active serine protease domain was produced in yeast using a Pichia expression kit (Invitrogen, Life Technologies, Inc.). Human TMPRSS13 serine protease
domain was amplified and cloned into the pPIC9 vector at the Xhol and NotI sites, using the primers 5’-TCTCTCTGAGAAAAAGAATCTGGGAGGGGCGCTGGCCTCG-3’ and 5’-ATTCGCCGCGGCTTAGATTTTCTGAATCGCAC-3’. Cloning resulted in TMPRSS13 serine protease domain sequence (Ile-Val-Gly) being inserted immediately following a Leu-Glu-Lys-Arg KEX2 cleavage site encoded by the vector. The Leu-Glu-Lys-Arg-Ile-Val-Gly is cleaved between Arg and Ile by the yeast protease KEX2, which is a transmembrane protease located in the Golgi, rendering a secreted active SP-domain. Transcribed active TMPRSS13 SP-domain contains the same domain sequence (Ile-Val-Gly) being inserted immediately followed by a Leu-Glu-Lys-Arg KEX2 cleavage site encoded by the domain sequence.

For transformation of clones were isolated and amplified using standard techniques.

**Chromogenic proteinase assays**

The assays were performed in 96-well plates in a total reaction volume of 100 μl using 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% Tween 20, 0.01% BSA for dilution of all samples. 5 nM active recombinant TMPRSS13, matriptase, or empty vector control media was incubated at 37 °C for 60 min with 100 μM of the synthetic peptide L-1720 Suc-Ala-Ala-Pro-Arg-(Bachem, Bubendorf, Switzerland) in the absence or presence (inhibitor and substrate added concomitantly) of recombinant soluble HAI-1 (60 nM) (R&D, Minneapolis, MN), HAI-2 (40 nM) (R&D, Minneapolis, MN), aprotinin (2 μM), leupeptin (20 μM), or benzamidine (2 mM) (Thermo Fisher Scientific). Changes in absorbance at 405 nm were monitored using a Magellan NanoQuant Infinite M200 Pro plate reader (Tecan U.S., Inc., Morrisville, NC). For measurement of chromogenic activity in cell extracts, samples were prepared and analyzed according to (28).

**Deglycosylation of TMPRSS13**

Proteins in lysates or conditioned media prepared as indicated above were deglycosylated using the PNGaseF deglycosylation kit according to the manufacturer’s instructions (New England Biolabs, Ipswich, MA).

**Dephosphorylation of TMPRSS13 in lysates**

For CIP treatment, 5 μg of protein lysates was added to 1× CutSmart® Buffer (New England Biolabs, Ipswich, MA) for a total volume of 50 μl with treated or, without, alkaline phosphatase, CIP (New England Biolabs, Ipswich, MA). Lysates were incubated at 37 °C for 60 min. After 60 min, Laemmli Sample Buffer with 5% 2-mercaptoethanol was added to the reaction, and samples were boiled prior to Western blot analysis. For dephosphorylation of MCF-7 lysates, 40 μg of protein was incubated in 1× NE Buffer for protein metallo phosphatases, 1 mM MnCl₂, and double distilled H₂O for a total reaction volume of 50 μl, and then 800 units of A protein phosphatase (New England Biolabs) or vehicle were added to the sample. Reactions were incubated for 30 min at 30 °C. After 30 min, Laemmli Sample Buffer with 5% 2-Mercaptoethanol was added to the reaction, and samples were boiled prior to SDS-PAGE and Western blot analysis.

**Knockdown of TMPRSS13 expression in cancer cells**

MCF-7, BT20, and HCC1937 breast carcinoma cells and DLD1 colorectal adenocarcinoma cell line were purchased from ATCC (Manassas, VA). MCF-7 cells were cultured in Dulbecco’s modified Eagle’s media (DMEM) (Gibco, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). BT-20 cells were grown in Eagle’s + NEAA media (Eagle’s minimal essential medium with 2 mM l-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% FBS); HCC1937 cells were grown in RPMI + l-GLUT media (RPMI 1640 media with 2 mM l-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% FBS); DLD1 cells were grown in RPMI 1640 media + l-GLUT media adjusted to contain 10% FBS. Transient knockdown of TMPRSS13 expression was performed using Lipofectamine® RNAiMAX according to the manufacturer’s instructions (Invitrogen, Life Technologies, Inc.). Stealth siRNA™ constructs were obtained from Invitrogen, Life Technologies, Inc. (HSS130533 (corresponding to HSS130531 (corresponding to 31 in Fig. 5C)). To detect endogenous TMPRSS13 species by Western blotting, the polyclonal rabbit anti-TMPRSS13 intracellular domain antibody was used (ab59862, Abcam, Cambridge, MA).

**α₂-Macroglobulin capture assay**

30 μl of conditioned media from cells expressing WT-TMPRSS13 were added to 30 μl of buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5 mM CaCl₂) and treated with or without 30 nM α₂-macroglobulin (Calbiochem). Samples were incubated at room temperature for 1 h. Additionally, α₂-macroglobulin was incubated with buffer alone as control. Samples were treated with Laemmli buffer with 5% 2-mercaptoethanol and boiled for 5 min prior to Western blot analysis.

**Immunoprecipitation**

48 h post-transfection, HEK293T cells expressing either empty vector (EV), TMPRSS13, and/or HAI constructs were lysed with RIPA lysis buffer with protease inhibitor mixture. One μl of primary mouse anti-V5 (Invitrogen, Life Technologies, Inc.) was added to 150 μg of protein lysates, and lysis buffer was added for a total reaction volume of 250 μl. Lysates were then rotated at 4 °C for 60 min. For HA immunoprecipitation, HEK293T cells expressing HA-S506A-TMPRSS13 were lysed with RIPA lysis buffer with protease inhibitor mixture, and
equal amounts of lysates were separated and immunoprecipitated with either 1.0 μg of α-HA or α-rabbit mAb IgG (DA1E-Cell Signaling Technology, Danvers, MA) antibodies for 2 h at 4 °C. For anti-intra-TMPRSS13 immunoprecipitation, whole lysates of DLD1 cells were prepared with RIPA lysis buffer with protease inhibitor mixture, and 1.0 μg of anti-intra-TMPRSS13 or α-rabbit mAb IgG was added to an equal volume of lysates and rotated at 4 °C overnight. After all immunoprecipitations, 30 μl of Ezview™ Red Protein A affinity gel (Sigma) were added to the reaction per the manufacturer’s protocol. Samples were then rotated at 4 °C for an additional 60 min at which point beads were pelleted at 4°C and washed five times with cold PBS, pH 7.5. After the final wash, 60 μl of 2× Laemmli buffer with 5% 2-mercaptoethanol was added, and samples were analyzed by SDS-PAGE and Western blotting.

Immunocytochemistry

Cell-surface imaging was performed using HEK293T cells transfected with human full-length TMPRSS13-V5 vectors. Cells were seeded on coverslips coated with rat type-2 collagen (BD Biosciences) and allowed to adhere and grow overnight. Cells were transiently transfected, and 36 h post-transfection, media were removed, and cells were fixed in Z-Fix (ANATECH LTD, Battle Creek, MI) for 15 min at room temperature. In permeabilized samples, cells were treated with 0.05% Triton X-100 in PBS. Cells were then blocked with 5% BSA in PBS for 1 h prior to staining. TMPRSS13-V5 was detected using a monoclonal anti-V5 antibody (Invitrogen, Life Technologies, Inc.) or an isotype control antibody (Sigma). HAI-1 was detected using the M19 antibody (32) kindly provided by Drs. Chen-Yong Lin and Michael Johnson, Georgetown University. Secondary AlexaFluor-488-conjugated goat anti-mouse and AlexaFluor-647-conjugated antibodies (Invitrogen, Life Technologies, Inc.) were used to detect TMPRSS13-V5 and/or HAI-1. HAI-2 was detected using a HAI-2-EYFP fusion protein (kindly provided by Dr. Stine Friis, University of Copenhagen) (28). Cells were washed with PBS and mounted with Prolong gold with DAPI (Invitrogen). Confocal images were acquired on the Zeiss LSM 780 scope at the Microscopy Imaging and Cytometry Resources Core at Wayne State University School of Medicine.

Biotin labeling of cell-surface proteins

HEK293T cells expressing empty vector, TMPRSS13, and/or HAI constructs were washed three times with PBS 48 h post-transfection. Cells were then gently detached and resuspended in 1.0 ml of PBS, and EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Waltham, MA) was added for a final concentration of 800 μM. Cells were biotin-labeled for 30 min at room temperature. After biotin labeling, cells were pelleted and washed three times with PBS containing 50 mM Tris, pH 8.0. Cells were then lysed in RIPA buffer supplemented with protease inhibitor mixture (Sigma), and protein concentrations were quantitated. 150 μg of protein was added to 40 μl of streptavidin-agarose (Sigma) in a final reaction volume of 200 μl and rotated at 4 °C for 60 min. Beads were pelleted by centrifugation at 8800 × g, and supernatant containing non-biotinylated proteins was collected (wash). Beads were washed five times with cold PBS and subsequently treated with 60 μl of Laemmli Sample Buffer with 5% 2-mercaptoethanol and boiled for 5 min prior to SDS-PAGE.

USP2 deubiquitination assay

HEK293T cells transfected with S506A-TMPRSS13-V5 were lysed 48 h post-transfection in 450 μl of RIPA buffer: 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% Nonidet P-40, supplemented with protease inhibitor mixture (Sigma). Lysates were aliquoted into separate 1.5-ml microcentrifuge tubes, and USP2 catalytic domain (BostonBiochem, Cambridge, MA) (final concentration of 2 μM) or vehicle was added. At 10, 30, 120, and 240 min after incubation, aliquots of the reaction were removed and treated with Laemmli buffer supplemented with 5% 2-mercaptoethanol and boiled for 5 min prior to SDS-PAGE and Western blot analysis. Samples were run in parallel, and TMPRSS13 was detected using mouse anti-V5 (Invitrogen, Life Technologies, Inc.), and ubiquitin was detected using rabbit anti-ubiquitin (Dako, Carpinteria, CA).

Glycosylation, intrinsic disorder, and phosphorylation predictions

NetNGlyc 1.0 Server was used for glycosylation prediction (http://www.cbs.dtu.dk/services/NetNGlyc/).3 To predict the disorder disposition we utilized the PONDR-FIT analysis tool (http://disorder.compbio.iupui.edu/pondr-fit.php).3 The full-length FASTA sequence for each corresponding protein was analyzed and the intracellular domains were plotted on a single graph. Kinase Predictions. Three separate kinase prediction tools were used to identify potential kinases responsible for TMPRSS13 phosphorylation. NetPhospho3.1 (http://www.cbs.dtu.dk/services/NetPhos/),2 GPS 3.0 (http://gps.biocuckoo.org/online.php),3 and PhosphoNet (http://www.phospho.net.ca/) (42).3 For NetPhospho3.1 and GPS 3.0, the FASTA sequences of the intracellular domains were analyzed.

Author contributions—A. S. M. and K. L. conceived the idea of the study and planned the experiments. A. S. M. performed the majority of the experiments. F. A. V. assisted with cell transfections, α-galactosidase capture, and Western blot analysis. J. M. W. cloned the HA-TMPRSS13 construct; L. M. T. assisted with immunocytochemistry. S. V. T. assisted with ubiquitination/deubiquitinating enzyme assays and as a consultant on the project. T. H. E. and A. J. S. assisted with deglycosylation, ubiquitination, and phosphatase experiments. A. S. M. and K. L. wrote the manuscript and prepared the figures. All authors contributed to interpretation and discussion of the results and read, edited, and approved the final version.

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Phosphorylation of the TTSP family member TMPRSS13

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