Shedding of Membrane Type Matrix Metalloproteinase 5 by a Furin-type Convertase

A POTENTIAL MECHANISM FOR DOWN-REGULATION*

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The shedding of membrane-associated proteins has been recognized as a regulatory mechanism to either up-regulate or down-regulate cellular functions by releasing membrane-bound growth factors or removing ectodomains of adhesion molecules and receptors. We have reported previously that the ectoenzyme of membrane type matrix metalloproteinase 5 (MT5-MMP) is shed into extracellular milieu (Pei, D. (1999) J. Biol. Chem. 274, 8925–8932). Here we present evidence that MT5-MMP is shed by a furin-type convertase activity in the trans-Golgi network. Among proteinase inhibitors screened, only decanoyl-Arg-Val-Lys-Arg-chloromethylketone, a known inhibitor for furin-type convertases, blocked the shedding of MT5-MMP in a dose-dependent manner. As expected, decanoyl-Arg-Val-Lys-Arg-chloromethylketone also prevented the activation of MT5-MMP, raising the possibility that the observed shedding could be autolytic. However, an active site mutant devoid of any catalytic activity, is also shed efficiently, thus ruling out the autolytic pathway. The shedding cleavage was subsequently mapped to the stem region immediately upstream of the transmembrane domain, where a cryptic furin recognition site, 540RRKERR, was recognized. Indeed, MT5-MMP and furin are co-localized in the trans-Golgi network and the shed species could be detected inside the cells. Furthermore, deletion mutations removing this cryptic site prevented MT5-MMP from shedding. The resulting mutants express a gain-of-function phenotype by mediating more robust activation of pro-MMP-2 than the wild type molecule. Thus, shedding provides a potential mechanism to regulate proteolytic activity of membrane-bound MMPs.

Shedding or proteolytic release of membrane-bound molecules has been established as an important regulatory mechanism to down-regulate cell adhesive receptors such as 1-selectin (1–3), generate soluble ligands such as tumor necrosis factor α (4), heparin-binding epidermal growth factor (5), and Delta ligand for Notch (6), or release dormant transcriptional factors (7, 8). Given the diverse molecules shed from membranes, specific mechanisms must have been evolved to handle the specific shedding needs in various biological processes. For proteins bound to plasma membrane, two members of the α-disintegrin and metalloproteinase (ADAM)1 family, tumor necrosis factor α-converting enzyme/ADAM17 and Kuz/ADAM10, have been identified as efficient sheddases (see review in Ref. 9). The availability of inhibitors against the ADAMs, e.g. hydroxamate-based synthetic compounds or tissue inhibitor of metalloproteinase-3 (9, 10), should allow rapid determination if a shedding process is ADAM- or metalloproteinase-dependent, thus facilitating the identification and characterization of alternative shedding pathways.

Matrix metalloproteinases are a family of zinc-dependent and neutral pH optimal endopeptidases believed to play a critical role in the remodeling of extracellular matrix under both physiological as well as pathological conditions (11, 12). To date, ∼25 MMPs have been reported and confirmed by cDNA cloning and chromosomal localizations (for review, see Refs. 11 and 12). Although the majority of the MMPs are secretory in nature, a growing list of newly identified MMPs appear to be membrane-bound by at least three distinct anchoring mechanisms: 1) type I transmembrane domains for MT1, -2, -3, and -5-MMPs (13–16); 2) glycosyl phosphatidylinositol linkage for MT4 and 6-MMPs (17, 18); and 3) type II transmembrane domain for MMP-23/cysteine-array MMP (19). MT1-MMP, the archetypal membrane-bound MMP, mediates proMMP-2 activation, cell invasion, migration, fibronolysis, collagenolysis, and angiogenesis when anchored on plasma membrane (13, 20–24). Truncation of the transmembrane (TM) domain renders MT1-MMP incapable of activating proMMP-2 in transfected cells (23), whereas a similarly TM-truncated MT1-MMP is capable of processing proMMP-2 when purified and assayed in vitro (25). Thus, the transmembrane domain along with its cytosolic domain may confer unique cellular localization required for the proper function of these membrane-bound MMPs (21, 23, 24).

MT5-MMP is a brain-specific MT-MMP closely related to MT1, -2, and -3-MMPs both structurally and functionally (16, 26). For example, it activates proMMP-2 when co-transfected in various cells (16, 26). Like MT1-MMP, recombinant MT5-MMP expresses proteolytic activities against extracellular matrix components such as proteoglycans (25, 27). On the other hand, MT5-MMP appears to have several unique features. It has a short half-life of ∼30 min at 37 °C (27). In fact, a synthetic inhibitor, BB-94, has to be included in conditioned media to keep the enzyme from autocatalytic decay, thus ensuring its integrity throughout the purification process at 4 °C (27). Furthermore, it is shed readily from cell surface (16). The shed

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1 The abbreviations used are: ADAM, a disintegrin and metalloproteinase; MMP, matrix metalloproteinase; MT, membrane-type; MT5-MMP, membrane type matrix metalloproteinase 5; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; CMK, chloromethylketone; TGN, trans-Golgi network; ER, endoplasmic reticulum; GFP, green fluorescent protein; BFA, brefeldin A; TM, transmembrane.
species behaves like a secretory MMP and can be detected by gelatin zymography (16). Interestingly, BB-94, an inhibitor known to block both MMPs and ADAMs, did not inhibit the shedding process, suggesting that MT5-MMP be shed by a novel mechanism independent of metalloproteinase activity. In this report, we demonstrate that MT5-MMP is shed by a furin-type convertase activity cleaving a cryptic furin recognition motif $^{545}$RRKERR within its stem region. We propose that shedding provides a potential mechanism of down-regulation for MT5-MMP activity.

MATERIALS AND METHODS

Cell Lines, Chemicals, and Immunological Reagents—Cell lines including MDCK and its derivatives were obtained and maintained as described (16, 28). The following stable lines were used: a stable cell line expressing full-length mouse MT5-MMP (F951) and a cell line expressing MT51–570F (16, 28). Stable cell lines, EA20 and EA24, were generated by stable transfection of pCR3.1MT5-MMPPE252A into MDCK cells and characterized as described (28). Laboratory chemicals and protease inhibitors were from Sigma or Calbiochem (San Diego, CA). The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) was purchased from Bachem (Philadelphia, PA). Cell culture shop version 6. Laboratory. The images were then further processed using Adobe Photoshop version 6.

RESULTS

Profile of MT5-MMP Protein Products in Vivo—We have demonstrated previously that MDCK cells expressing the brain-specific MT5-MMP shed a gelatinolytic species into conditioned media at ~27 kDa on zymography and a major species at ~34 kDa plus several minor ones on Western blots in the absence of any protease inhibitor, a pattern similar to that generated by autocatalytic fragmentation of the 56-kDa full-length recombinant ectoenzyme (27). To test the hypothesis that MT5-MMP is also shed in natural settings, we profiled the expression of MT5-MMP in various regions of mouse brain and revealed by reverse transcription-PCR that it is expressed highly in cerebellum, modestly in cerebrum, but minimally in heart (Fig. 1A, middle panel of lanes 1–3). Consistently, Western blot analysis with anti-MT5-MMP antisera detected a major species at approximately 34 kDa in supernatants of tissue homogenates from cerebellum and cerebrum, but not heart (Fig. 1A, upper panels, lanes 1 and 2 versus lane 3). This soluble species from tissue homogenates is almost identical to the shed fragment of MT5-MMP from recombinant cells (16), suggesting that MT5-MMP is shed in vivo. When equivalent amounts of supernatants and pellets freshly prepared from cerebellum were analyzed simultaneously, the same 34 kDa was detected in the supernatants while a ~65-kDa species along with several smaller and minor ones was detected in the membrane
pellet (Fig. 1B, lanes 2 versus 1, arrow). These products were also detected in recombinant cells expressing MT5-MMP (16, 26), thus supporting the idea that MT5-MMP is shed in vivo in a similar fashion as observed in vitro. Furthermore, it is estimated that the 34-kDa species amounts to ~40% of the total MT5-MMP present in the pellets and supernatants. These data argue that MT5-MMP is shed in natural settings as well.

**Shedding of MT5-MMP Is Resistant to a Broad Spectrum of Protease Inhibitors**—Since the MT5-MMP protein products detected in recombinant cells appear to recapitulate those detected in cerebellum as demonstrated in Fig. 1, we decided to focus on this established in vitro system to dissect the shedding process (16). In fact, this strategy remains the only viable alternative since the primary cells isolated from mouse cerebellum apparently lost the expression of MT5-MMP at the mRNA and protein levels in culture (data not shown).

To probe the proteolytic mechanism of shedding, we attempted to block shedding with various protease inhibitors. Control as well as MT5-MMP-transfected MDCK cells (F591) constitutively expressed MMP-9 migrating at approximately 92 kDa on zymography in Fig. 2A (lanes 1–14). A 27-kDa gelatinolytic species was secreted into conditioned media by MT5-MMP-transfected cells as reported previously (Fig. 2A, lane 6, arrowhead) (16). Proteinase inhibitors including aprotinin for serine proteinases (10 or 100 μg/ml), E64 for cysteine proteinases (5 or 50 μM), BB-94 for metalloproteinases (5 or 50 μM), and pepstatin A for aspartyl proteinases (5 or 50 μM) (30) in serum-free culture media failed to block the shedding of the ~27-kDa gelatinolytic species (Fig. 2A, lanes 6–14). BB-94 did not inhibit the shedding process as reported previously, even at concentrations as high as 50 μM, but apparently converted the smaller fragments into a ~52-kDa species as detected by both zymography and Western blotting (Fig. 2A, lanes 7, 8, 17, and 18 marked by arrows). Thus, this 52-kDa species should be considered as the primary product of shedding, which was autocatalytically fragmented into the smaller ones at 27 or 34 kDa, in agreement with our previous report that BB-94 stabilizes active MT5-MMP (27). Consequently, BB-94 was always included in culture media to prevent autolysis when analyzing MT5-MMP shedding. Since BB-94 blocks the activities of ADAMS, these data would rule out the ADAMs as the MT5-MMP sheddase, thus suggesting that MT5-MMP is shed by an unknown mechanism.

**A Chloromethylketone-based Inhibitor of Furin-type Convertases Blocks the Activation as Well as the Shedding of MT5-MMP**—Like other MT-MMPs, MT5-MMP contains a furin site and may be activated by furin in the TGN as demonstrated previously (29, 31). Indeed, MT5-MMP-transfected cells are capable of activating proMMP-2 (16, 26), indicating that MT5-MMP must have been processed and activated. We analyzed cell-associated MT5-MMP products by Western blotting. In the absence of BB-94, only the 65-kDa pro species was detected (Fig. 2B, lane 1). The active species at ~58 kDa became detectable with the addition of BB-94, which inhibited autocatalytic decay as described (27) (Fig. 2B, lane 2). The minor and smaller ones are nonspecific and present in MDCK cells as well (data not shown). To implicate furin as the activator, a furin-type convertase inhibitor, CMK, was included in the culture media as indicated in Fig. 2B, lane 14, BB-94 dose-dependent inhibition of MT5-MMP processing was observed (lanes 2–6), suggesting that MT5-MMP is processed by a furin-type convertase (32). In the supernatants, we observed not only the expected conversion of the smaller species into the 52-kDa one (Fig. 2B, lanes 7 and 8) but also a dose-dependent decrease of the shed species (Fig. 2B, lanes 8–12) by Western blotting, indicating for the first time that a furin-type convertase activity is required for the observed shedding. Although CMK is expected to block the activation of MT5-MMP due to the presence of a consensus furin recognition site between its pro and catalytic domains (16), its efficient blocking of shedding is quite unexpected.

**Shedding of Catalytically Inactive MT5E252A**—Given the fact that active MT5-MMP autocatalytically fragments itself into smaller species (27), it is possible that CMK inhibited the shedding by blocking furin-mediated activation of MT5-MMP, thus preventing autocatalytic shedding. To rule out this possibility, we analyzed the shedding profile of a catalytically inactive mutant of MT5-MMP, MT5E252A. As shown in Fig. 3A, this mutant carries a single point mutation converting Glu to Ala at the active site, rendering MT5-MMP inactive, as demonstrated by its inability to activate proMMP-2 (16). Should shedding be autocatalytic, MT5E252A should not be able to shed its ectodomain. However, the ectodomain of MT5E252A was shed very efficiently into conditioned media when two independently derived cell lines, EA24 and EA20, were analyzed (arrows, Fig. 3B, lanes 3, 4, 7, and 8). In fact, the mutant
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Fig. 3. Shedding of a catalytically inactive mutant, MT5E252A. A, schematic illustration of MT5E252A mutant. The domain structure of MT5-MMP is presented. S, signal peptide; Pro, prodomain; C, cysteine-switch; R, furin recognition site; CAT, catalytic domain; H, hinge region. 52-kDa species (Fig. 3A, panel c) raises the possibility that shedding takes place, just like activation, in the TGN. Indeed, a cell-associated 52-kDa species, detected in Fig. 3C (marked by an asterisk), closely resembles the shed species (also 52 kDa), and is sensitive to CMK treatment. Should this 52-kDa protein be the shed species prior to being secreted, it must have lost its transmembrane domain intracellularly. To prove that this 52-kDa species lost its transmembrane domain by shedding prior to secretion, we performed saponin extractions, a technique that can differentially extract soluble proteins from intracellular compartments (19). As shown in Fig. 4B, saponin extraction almost completely removed the 52-kDa species (indicated by asterisk) from EA24 cells and partitioned it into the supernatant (lane 5 versus lane 4), yet, leaving the pro (65 kDa) and active (58 kDa) species with the cell pellets (lane 6 versus lane 4), arguing that the 52-kDa species lost its transmembrane domain, i.e. shed from membrane intracellularly prior to secretion. The same 52-kDa species is then secreted and accumulated in the media (arrow, Fig. 4B, lane 8) as described previously (Figs. 2B and 3C).

To further confirm the intracellular nature of MT5-MMP shedding, we treated the cells with pharmacological agents known to disrupt vesicular trafficking or furin maturation. As shown in Fig. 4C, A23187, a calcium ionophore known to inhibit furin maturation in the ER (35), blocked the shedding by depleting the calcium in ER (lane 8 versus lane 6). Similarly, BFA inhibited the shedding process by interfering with ER to Golgi transport (Fig. 4C, lane 9 versus lane 6) (36). As controls, BB-94 did not and CMK did block the shedding process as described previously (Fig. 4C, lanes 7 and 10; see also Figs. 2B and 3C). Together, these data argue that the ectodomain of MT5-MMP is shed in the TGN.

Mapping of the Shedding Cleavage within the Stem Region—To estimate the approximate location of the shedding
cleavage, we compared the mobility of the shed species with that of MT5-22::GFP, a secretory form generated by deleting its transmembrane domain (27). As shown in Fig. 5A, the shed species is predicted to be approximately 3 kDa smaller than MT5-22::GFP (marked by two small horizontal bars between lanes 1 and 2). The estimated molecular mass for the entire stem region, CKQKEVERRKERRVERLPQDDVDIMVTIDDVPG SVN570 plus the FLAG epitope, DYKDDDDK, is 4.8 kDa (Fig. 5B). A differential of ~3 kDa would suggest that the shedding cleavage occurs around R550L (Fig. 5C). The estimated molecular mass for the entire stem region containing the cryptic 545RRKERR signal site (Fig. 5B) was identified (16, 32).

The 545RRKERR Motif Is Required for Shedding—The localization of shedding cleavage around the cryptic 545RRKERR motif within the stem region suggests that it may play a critical role in the observed shedding process. To ascertain the contribution of the stem region in regulating the shedding process, we constructed two deletion mutants. We first constructed the deletion removing only the 545RRKERR motif in the stem region, CKQKEVERRKERRVERLPQDDVDIMVTI DDVPG, and analyzed as described in Fig. 1B. The arrow depicts the shed MT5E252A 52-kDa ectodomain. The arrowheads indicate the saponin-extracted, transmembraneless, shed MT5-MMP species from intracellular compartment. C, blockade of MT5-MMP shedding by A23187 and BFA. EA24 cells (see above) were incubated in serum-free media alone (lanes 1 and 6) or with BB-94 (lanes 2 and 7), A23187 (lanes 3 and 8), BFA (lanes 4 and 9), or CMK (lanes 5 and 10) for 48 h. Media (lanes 6–10) were collected, and cells were washed, lysed, and analyzed as described in Fig. 1B. The arrow indicates the shed MT5-MMP ectodomain at 52 kDa, and the horizontal bar denotes the intracellular proMT5-MMP at 65 kDa.

Initial characterization for MT5-MMP activity was demonstrated by Western analysis of the cell lysates (Fig. 6B, lanes 4 and 5 versus lanes 2 and 3), arguing that the stem region regulates MT5-MMP shedding. To test if the 545RRKERR motif is required for the shedding process, we constructed a deletion removing only the 545RRKERR motif in MT5-MMP and named it MT5Δ56 as shown in Fig. 6A. Stable cell lines transfected with MT5Δ56 did not shed any appreciable amount of MT5-MMP ectodomain, while the wild type MT5-MMP did as demonstrated in Fig. 6C (lanes 5 and 6 versus lanes 3 and 4). Consequently, the cell-associated MT5Δ56 was higher than its wild type counterpart (Fig. 6C, lanes 11 and 12 versus lanes 9 and 10). Together, we conclude that the cryptic furin recognition site, 545RRKERR, is the cis-acting signal in the stem region required for the observed shedding.

Functional Consequence of MT5-MMP Shedding—Initial characterization for MT5-MMP activity was demonstrated when both MT5-MMP and MMP-2 constructs were co-transfected into MDCK cells (16), because proMMP-2 added to the transfected cells was not activated efficiently (data not shown). In light of the observed shedding of MT5-MMP, this inefficiency could be explained by the near absence of cell surface-associated MT5-MMP due to shedding (Fig. 4A). To monitor the consequence of shedding, we analyzed the localization pattern of GFP-tagged wild type MT5::GFP or the MT5Δ22::GFP mutant (see Fig. 6A for details). As shown in Fig. 7A, deletion of the stem region containing the cryptic 545RRKERR signal significantly enhanced the cell surface expression of MT5-MMP (panel b versus panel a). Prominent signals were observed on plasma membrane for MT5Δ22::GFP in almost every cells (Fig. 6A, panel b), whereas the wild type MT5::GFP is sequestered in

Fig. 4. Localization of shedding in the TGN. A, colocalization of MT5-MMP and furin in the TGN. Cells from the stable line F591 were grown on coverslips, fixed and stained with anti-MT5-MMP (panel a) and anti-furin antibodies (panel c) followed by fluorescein isothiocyanate- or Rhodamine Red X-conjugated secondary antibodies, respectively, and analyzed by confocal microscopy as described (41). Overlay of panels a and c gave rise to panel b depicting the co-localizations of MT5-MMP and furin in orange color. The arrows indicate identical regions of the cell in panels a–c. B, extraction of shed MT5-MMP ectodomain from intracellular compartments. MDCK (CK, lanes 1–3 and 7) or EA24 (lanes 4–6 and 8) were incubated with serum-free media for 48 h. The media (lanes 7 and 8) were collected, and cells (lanes 1–6) were washed three times with PBS before being lysed (lanes 1 and 4) or extracted with saponin as supernatants (lanes 2 and 3) or remaining pellets (lanes 5 and 6) as described (19). The collected samples were analyzed by Western blotting as described in Fig. 1B. The arrow depicts the shed MT5E252A 52-kDa ectodomain. The arrowheads indicate the saponin-extracted, transmembraneless, shed MT5-MMP species from intracellular compartment. C, blockade of MT5-MMP shedding by A23187 and BFA. EA24 cells (see above) were incubated in serum-free media alone (lanes 1 and 6) or with BB-94 (lanes 2 and 7), A23187 (lanes 3 and 8), BFA (lanes 4 and 9), or CMK (lanes 5 and 10) for 48 h. Media (lanes 6–10) were collected, and cells were washed, lysed, and analyzed as described in Fig. 1B. The arrow indicates the shed MT5-MMP ectodomain at 52 kDa, and the horizontal bar denotes the intracellular proMT5-MMP at 65 kDa.

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in intracellular vesicles and compartments as observed for the native MT5-MMP by immunofluorescence staining (Fig. 7A (panel a) versus Fig. 4A (panel a)), suggesting that the attachment of GFP did not alter the trafficking of MT5-MMP. In a time-course study of proMMP-2 activation by MT5::GFP and MT5Δ22::GFP mutant, we observed more robust activation of proMMP-2 by MT5Δ22::GFP than the wild type protein (Fig. 7B, lanes 2, 5, 8, and 11 versus lanes 3, 6, 9, and 12). The difference was most dramatic in some of early time points such as the 4-h mark when MT5Δ22::GFP activated a significant portion of proMMP-2 while wild type MT5-MMP::GFP did not (Fig. 7B, lane 2 versus lane 3). Taken together, these data demonstrate that shedding down-regulates the activity of MT5-MMP and the deletion mutants, MT5Δ22::GFP and MT5Δ6, could be considered as gain-of-function mutants.

**DISCUSSION**

The plasma membrane of a cell is populated with various surface molecules that can (i) receive specific extracellular signals, (ii) instruct neighboring cells to proliferate or differentiate, or (iii) remodel or modify the neighboring microenvironment. To properly execute their physiological functions, cells must regulate their plasma membrane contents with precision and efficiency. In general, cell surface molecules are synthesized in the ER, processed and packaged in the TGN and then delivered via secretory vesicles to the plasma membrane (37). For cell surface receptors, ligand binding usually triggers rapid internalization (38). The internalized receptors may be recycled back to the cell surface or delivered to lysosomes for degradation (38). For molecules with no obvious ligands, their fates at cell surface are less well understood. With increasing frequency, cell surface molecules are found to be shed into extracellular milieu to either down-regulate their function such as the shedding of 1-selectin from leukocytes or generate functional forms, as exemplified by the release of soluble tumor necrosis factor α (1, 4). Thus, shedding has been recognized as an important regulatory mechanism for cells to control its microenvironment. The MT-MMPs qualify as cell surface molecules that can modify or remodel the extracellular environment. Destructive in nature, these enzymes like all other MMPs are regulated at multiple levels, including transcriptional and translational controls, zymogen activation, and inhibition by endogenous inhibitors such as tissue inhibitor of matrix metalloproteinases (11, 12). Being membrane-anchored, MT-MMPs are subject to additional regulations such as vesicular trafficking. At the present, little is known about how MT-MMPs are regulated on the plasma membrane. The shedding of MT5-MMP presented in this paper offers a concrete mechanism how membrane-bound MMP activity at the cell surface could be regulated.

**Shedding of MT5-MMP Is Obligatory and Evolutionarily Conserved—**MT5-MMP distinguishes itself being readily shed into extracellular milieu (Figs. 2B and 3C) (16). In this report, we present evidence that the ectoenzyme of MT5-MMP is shed through an obligatory mechanism by a furin-type convertase recognizing a specific motif, 545RRKERR, within its stem region. Since furin-type convertases are ubiquitous, this shedding mechanism should be operational in almost all cell types characterized so far (32, 34, 35). Indeed, we have also transfected MT5-MMP into various cell lines from human, rat, hamster, and canine and observed shedding in all cell lines examined (data not shown). Furthermore, extracts from mouse cerebellum contains a 34-kDa soluble species, almost identical to the shed species identified in transfected cells, suggesting that MT5-MMP is shed in vivo. It is of interest to point out that MT5-MMPs from mouse (16), rat (AB023659), and human (26) all contain an identical RRKERR motif within the stem region, arguing that the shedding process in evolutionarily conserved.

**Modulation of MT5-MMP Level on Cell Surface by Shedding:**—In addition to the 545RRKERR motif in the stem region, MT5-MMP contains a bona fide furin recognition site R6RR-4NK2R-1 sandwiched between its pro and catalytic domains (16, 26), presumably for zymogen activation as described for similar MMPs (25, 29, 31). We envision two scenarios by which furin or related convertases may regulate MT5-MMP activity on cell surface. First, there is a distinct convertase for activation and another one for shedding. This is likely given the fact that the R6RR-4NK2R-1 site between pro and catalytic domains is a perfect consensus for furin, the archetypal proprotein convertase (32), whereas the 545RRKERR in the stem region is sub optimal for furin recognition due to the presence of a Lys at −4 position instead of the preferred Arg (32, 34). In fact, the motif in the stem region could be viewed as two tandem dibasic motifs, 545RR and 549RR, which can be recognized by those proprotein convertases expressed in the regulated secretory pathway in neuro-endocrine cells (32, 34). Thus, furin could be the activating convertase while the
other dibasic convertases could be the sheddases. Alternatively, both activation and shedding may be mediated by the same convertase. Furin, PACE4, or PC7/8 all prefer motifs with the −4 position as Arg and −6 position as Arg in addition to the dibasic Arg/Lys at −2 and Arg at −1 positions (39, 40). A Lys at −4 position lowers the efficiency of cleavage by these three convertases (39, 40), especially when the concentration of the convertase is limited. This difference in processing efficiencies may offer a mechanism for cells to balance the ratio between activated/membrane-bound and the activated/shed MT5-MMP by modulating the concentration of the convertase(s) in the TGN. At relatively high concentration of convertase(s), both the activation site and shedding site may be cleaved, thus, favoring the secretion or shedding of MT5-MMP ectoenzyme. On the other hand, the concentration of the convertase(s) may be relatively low, thus allowing the activation of MT5-MMP zymogen, but not enough to cleave the shedding site, thus favoring accumulation of active MT5-MMP on the cell surface. The fact that both shedding and activation take place in the TGN suggest that, once past this compartment without being shed, MT5-MMP should remain membrane-bound until reaching the plasma membrane. The presence of furin-type convertases in the plasma membrane (34) would also shed MT5-MMP on the cell surface into the extracellular milieu and thus down-regulate its function.

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Fig. 6. Regulation of MT5-MMP shedding by the stem region with the 5⁴RRKERR motif. A, schematic illustrations of MT5-MMP constructs. The top portion depicts a C-terminal fusion between full-length MT5-MMP and the green fluorescent protein, MT5::GFP. A deletion of 22 residues from the stem region is shown below for mutant Δ22, which has the MT5::GFP backbone. The wild type MT5-MMP is shown at the bottom. The mutant Δ6 is depicted immediately above, showing the deletion of RRKERR motif. This mutant has the MT5-MMP backbone without GFP fusion. The sequence for the stem region Cys⁴⁶⁸ through Asn⁴⁸⁴ is presented in the middle with the downward arrow marking the putative shedding cleavage. B, deletion of Glu⁵⁰⁰ through Thr⁵ⁱⁱ from the stem region in Δ22 blocks MT5-MMP shedding. MDCK cells (lanes 1 and 6), or MDCK-derived stable transfectants with the wild type MT5-GFP (lanes 2, 3, 7, and 8) or MT5Δ22::GFP (lanes 4, 5, 9, and 10) were cultured in serum-free media alone (lanes 1, 2, 4, 6, 7, and 9) or supplemented with 5 μM BB-94 (lanes 3, 5, 8, and 10) for 48 h. Culture media (lanes 6–10) and cell lysates (lanes 1–5) were analyzed by Western blotting as described in Fig. 1. The arrow indicates the shed 52-kDa ectoenzyme species. Note the absence of any shed species in the media from cells transfected with MT5Δ22::GFP mutant. C, the 5⁴⁴RRKERR motif is required for MT5-MMP shedding. MDCK cells (CK, lanes 1, 2, 7, and 8), or F591 (lanes 3, 4, 9, and 10) or stable line expressing MT5-MMPΔ6 mutant (lanes 5, 6, 11, and 12) were cultured in serum free media alone (lanes 1, 3, 5, 7, 9, and 11) or supplemented with 5 μM BB-94 (lanes 2, 4, 6, 8, 10, 12) for 48 h. Culture media (lanes 1–12) and cell lysates (lanes 7–12) were analyzed by Western blotting as described in Fig. 1. The arrow indicates the shed 52-kDa ectoenzyme, and the arrowhead marks the intracellular 65-kDa precursor. Note the absence of MT5-MMP species in the media of MT5Δ6 cells (lanes 5 and 6).

Fig. 7. Shedding negative mutant has a gain-of-function phenotype. A, accumulation of MT5Δ22::GFP, not the wild type version, on plasma membrane. Confocal analysis of cells transfected with wild type MT5::GFP (panel a) and its MT5Δ22::GFP mutant (panel b, see Fig. 6) were presented. Note that most of the MT5 wild type signals are in intracellular vesicles and the Golgi apparatus (panel a, arrows) while MT5Δ22 also accumulates on the plasma membrane (arrows, panel b). B, gain-of-function for MT5Δ22::GFP in processing proMMP-2. MDCK (CK, lanes 1, 4, 7, and 10), or stable transfectants for MT5Δ22::GFP (Δ22, lanes 2, 5, 8, and 11) or MT5-GFP (WT, lanes 3, 6, 9, and 12) were grown to confluence and washed with PBS before being supplemented with proMMP-2 in serum-free media. Aliquots sampled at 4 h (lanes 1–3), 8 h (lanes 4–6), 14 h (lanes 7–9), or 24 h (lanes 10–12) were analyzed by zymography as described (16). Note the rapid processing of proMMP-2 by the MT5Δ22::GFP mutant (lane 2) in as early as 4 h when the wild type MT5::GFP has only minimal activity (lane 3).
