Mint-3 Regulates the Retrieval of the Internalized Membrane-type Matrix Metalloproteinase, MT5-MMP, to the Plasma Membrane by Binding to Its Carboxyl End Motif EWV*

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Matrix metalloproteinases (MMPs) have emerged as critical molecules that mediate the remodeling of the extracellular matrix (1–4). The MMP family is composed of at least 21 MMPs that fall into two major groups according to their cellular localization: secreted MMPs and membrane-anchored MMPs (also known as membrane-type MMPs, MT-MMPs) (5, 6). While virtually all members of the MMP family have been implicated in the progression of cancer, the MT-MMPs have been demonstrated, both in vitro and in vivo, to play a more critical role than their soluble counterparts (2). Our previous study has shown that internalized MT1-MMP could be recycled back to the cell surface through the trans-Golgi network (12). Although the carboxyl-terminal three residues have been identified as the recycling signal (12), little is known about the cellular machinery that controls the dynamic trafficking of MT-MMPs.

As important cell surface molecules, a critical question concerning the mechanisms by which MT-MMPs are regulated is how the cells deliver them to specialized areas through trafficking events. Recently, we and others (9–11) have shown that MT1-MMP is internalized by a dynamin-dependent process. Deletion or mutation of the cytoplasmic tail of MT1-MMP significantly impairs its internalization. Moreover, our recent study has shown that internalized MT1-MMP could be recycled back to the cell surface through the trans-Golgi network (12). The MT5-MMP is its ability to activate pro-MMP2, a secreted pro-peptide, is expressed predominantly in the brain and at low levels in the kidney, pancreas, and lung (14). Moreover, the expression of MT5-MMP can be detected in various cancer cells and tissue, suggesting that MT5-MMP may play a role in the progression of cancer (14). MT5-MMP has been shown to play a role in axonal growth (15). However, the most established function for MT5-MMP is its ability to activate pro-MMP2, a secreted proteinase implicated widely in physiological and pathological conditions such as tumor invasion and metastasis (16). While sharing similar domain structure with other MT-MMPs, the cytoplasmic tail of MT5-MMP is the most divergent, having only 50% identity with those of MT1-MMP, MT2-MMP, and...

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† The abbreviations used are: MMPs, matrix metalloproteinases; MT, membrane-type; TM, transmembrane domain; TGN, trans-Golgi network; MDCK, Madin-Darby canine kidney; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; AP, adaptor protein; siRNA, small hairpin inhibitory RNA; HEK, human embryonic kidney.
MT5-MMP (16). Indeed, Uekita et al. (10) concluded that MT5-MMP is not internalized, given the fact that it lacks the conserved LLY motif shown to interact with the μ2 subunit of AP2. In this report, we analyzed the trafficking of MT5-MMP and present evidence that it is internalized, albeit slower than MT1-MMP. More interestingly, we present evidence that the internalized MT5-MMP recycles back to the cell surface in a process dependent on its last three residues EWV, a motif shown to bind protein. With two type III PDZ domains, Mint-3 is located to the TGN with MT5-MMP, where it mediates the recycling of MT5-MMP to the cell surface. This represents the first report that a PDZ protein regulates the trafficking of MT-MMPs.

**Materials and Methods**

**Reagents**—All general chemicals were purchased from Sigma or Fisher. Rabbit anti-MT5-MMP antibodies were raised against the purified catalytic domain of mouse MT5-MMP and affinity-purified as described previously (17). Mouse anti-α-hemoglobin monoclonal antibody was purchased from Sigma. Anti-Mint-3 and anti-p230 antibodies were obtained from BD Transduction Laboratories (Lexington, KY). Goat anti-rabbit or mouse secondary antibodies were purchased from Sigma. Alexa 488- and 595-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). BB94 was a gift from British Biotechnology (Oxford, UK).

**Yeast Two-hybrid System**—The MATCHMAKER GAL4 two-hybrid System3 and the human leukocyte cDNA library were purchased from Clontech (Palo Alto, CA). The bait for library screening was the cytoplasmic tail of mouse MT5-MMP. Two-hybrid screening was done according to the protocol provided by the manufacturer.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells, N2A cells and MDCK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum. All cells were transfected using the calcium phosphate-DNA co-precipitation method as described previously (6, 16). For all transfection experiments, pcDNA3 or PC3R.1 vector was used to equalize total DNA input to the same level.

**Plasmids**—Full-length mouse MT5-MMP was cloned into expression vector pCR3.1 as described (16). The deletion and point mutation of MT5-MMP were generated by PCR using this plasmid as template. Rat Mint-3 (NM_031781), human Mint-3 (NM_004886), and Mint-2 (NM_005503) were cloned into pcDNA3.1 vector in-frame with HA at the amino terminus. Mint-3 deletion mutants were subcloned from PCR rat Mint-3 into pcDNA3.1 with the HA tag at the amino terminus. The CMV-GST fusion proteins were expressed from subcloned pcDNA3 by PCR. The vector-based small hairpin inhibitory RNA (shRNA) of Mint-3 was cloned into pcDNA-1. The targeted sequence was in the middle of the open reading frame for human Mint-3 (5'-AGG-TACCTGGGTTCCA-3'). Expression constructs for dynamin and dynamin II were generously provided by Dr. Lefkowitz (Duke University, Durham, NC) and characterized as described (9). The RUPY expression vector is a gift from Dr. Qiu (18).

**Immunoprecipitation**—48 h after transfection, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture) for 10 min, and the samples were cleared by centrifugation at 4 °C overnight. The beads were washed three times and boiled in SDS sample buffer. The prepared samples were subjected to Western blotting analysis.

**GST Pull-down Assay**—HEK293 cells were co-transfected with Mint-3 using HA-tagged GST, GST-V614G, or GST-CT. Cells were lysed with lysis buffer, and cell extracts were cleared and incubated with GST beads at 4 °C overnight. The beads were washed three times and boiled in SDS sample buffer. The prepared samples were analyzed by Western blotting with anti-HA antibody.

**Zymography and Western Blotting**—Zymography was performed as previously described (7). Briefly, cells were cultured in 6- or 12-well plates. After transfection, cells were washed three times with PBS, and the medium was changed to 5% fetal bovine serum (the source of pro-MMP2) (0.5 ml per well for a 12-well plate or 1 ml for a 6-well plate). After 24 h of incubation, the medium was harvested and cleared by centrifugation at 12,000 rpm for 10 min and subjected to analyze by SDS-PAGE impregnated with 1 mg/ml gelatin as described (6, 16). The gels were incubated at 37 °C overnight, stained with Coomassie Blue, destained, and images were captured by scanning. The active species of MMP2 were quantified by densitometry using an Eagle-Eye system (Stratagene). For Western blotting, cells were lysed in lysis buffer, cleared by centrifugation, and analyzed using specific antibody.

**Immunostaining and Confocal Microscopy**—Cells were grown on glass coverslips and transfected with indicated plasmids. 24 h later, cells were fixed with 4% paraformaldehyde for 20 min and incubated with PBS containing 0.1% Triton X-100 for 5 min. HA-tagged fusion protein was detected with rhodamine-conjugated goat anti-mouse IgG, and MT5-MMP was detected using fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody. Coverslips were analyzed on a confocal microscope in the Biomedical Image Processing Laboratories (BIPL) at the University of Minnesota as described (9). For internalization experiments, cells on coverslips were washed three times with PBS and kept at 4 °C. Anti-MT5-MMP antibodies were added to the cells at 0.2 μg/ml for 2 h at 4 °C. Antibodies were subsequently removed, and cells were washed before being heated to 37 °C with prewarmed medium for the indicated time. Cells were then processed as described previously (9). The coverslips were mounted with NO-FADE (10% glycero in PBS, 0.1% p-phenylenediamine, pH 8.0). Confocal images were collected from a Bio-Rad MRC 1024 system attached to an Olympus microscope (Melville, NY) with a ×60 objective at the BIPL, University of Minnesota. Quantification was performed with Openlab (Impro vision, Coventry, UK). The statistical analysis was carried out with GraphPad Prism software (San Diego, CA).

**Cell Surface Labeling with Biotin**—Briefly, cells were grown in 6-well plates and washed three times with ice-cold PBS. Then the cells were incubated with 0.5 mg/ml Sulfo-NHS-Biotin (Pierce) for 30 min on ice. After extensive washing with ice-cold PBS containing 50 mM glycine, the cells were lysed and incubated with streptavidin-conjugated beads overnight at 4 °C. The beads were washed with lysis buffer, boiled in SDS sample buffer, and analyzed by Western blotting as previously described (9).

**Growth of MCF-7 Cells in the Three-dimensional Collagen Gel**—MCF-7 stable transfectants (1.2×10⁶) were mixed with 300 μl of 1% collagen (2 mg/ml, Collaborative Research, Bedford, MA) and allowed to gel at 37 °C in 24-well plates to give rise to a three-dimensional collagen matrix. Fresh medium containing 10% fetal bovine serum with or without 5 μM BB94 were added to the wells and changed every 2 days. After 12 days, cells were photographed by a video camera at the University of Minnesota BIPL as described (7).

**Results**

MT5-MMP Recycles through the trans-Golgi Network—Recently, we have shown that MT1-MMP is internalized constantly through dynamin-regulated endocytosis such that most of the MT1-MMP resides intracellularly in the trans-Golgi network, and little MT1-MMP activity is detected on the cell surface as measured by pro-MMP-2 activation (9). In a similar study, Uekita et al. (10) identified a di-leucine motif as the signal for MT1-MMP internalization. Interestingly, while MT2-MMP and MT3-MMP have similar di-leucine motifs at their cytoplasmic tails, MT5-MMP lacks such a di-leucine motif and was reported to be defective in internalization as well (10). We re-evaluated the internalization of MT-MMPs and discovered that MT5-MMP was internalized efficiently, albeit slower than MT1-MMP (data not shown). As shown in Fig. 1A, MT5-MMP labeled with anti-MT5-MMP antibodies commenced internalization around 30 min (panel c) at permissive temperature and reappears on the cell surface between 70 and 90 min (panels e and f), compared with 60 min observed for MT1-MMP (data not shown). To track the intracellular compartments that MT5-MMP is routed through, we co-stained the internalized MT5-MMP with various markers at different time points. As shown in Fig. 1B, internalized MT5-MMP was co-localized with RUPY1, an early endosomal marker, suggesting that MT5-MMP is delivered to the early endosomes after internalization. Furthermore, the internalized MT5-MMP was also routed to the TGN evidenced by their co-localization with p230, a well-known marker for the TGN (Fig. 1C). Together, these data demonstrate for the first time that MT5-MMP is not only...
internalized, but also recycled back to the cell surface via the trans-Golgi network.

MT5-MMPΔ3 Is Defective in Recycling—To evaluate the signals responsible for the internalization and recycling of MT5-MMP, we performed systematic deletion experiments on the cytoplasmic tail of MT5-MMP. Among the mutants analyzed, a deletion mutant lacking the last three residues, EWV, is shown in Fig. 2A. While wild-type MT5-MMP was internalized and recycled as shown in Fig. 1A, MT5Δ3 was internalized rapidly, appearing 10 min at the permissible temperature and remained intracellular even at prolonged periods of time (Fig. 2B, panels a–f), suggesting that it does not recycle back to the cell surface. Indeed, careful quantification of the cell surface MT5-MMP demonstrates that MT5Δ3 is indeed defective in recycling (Fig. 2C).

Recycling-defective MT5-MMPΔ3 Has Diminished Activity on the Cell Surface—The lack of recycling of MT5-MMPΔ3 should diminish the level of MT5-MMP on the cell surface. To test this possibility, we performed dose response experiments by transfecting increasing amounts of both wild-type and mutant MT5-MMP into HEK293 cells, and the resulting MT5-MMP proteins (and their cell surface activity against pro-MMP-2) were analyzed by Western blotting and zymography. As shown in Fig. 3B, while both wild-type and MT5Δ3 were expressed at similar levels as expected (upper panel), the deletion mutant exhibited significant reduction in its ability to activate pro-MMP-2 (lower panel, lanes 1–6 versus 7–12).

To further refine the requirement for the EWV motif, a point mutation V614G of MT5-MMP was generated. In a similar dose-dependent pro-MMP-2 activation assay, this point mutant behaved similar to MT5-MMPΔ3 (Fig. 3C, lower panel, lanes 1–6 versus 7–12), suggesting that Val614 is a critical residue of the EWV motif. Since MT-MMPs have been shown to enhance the growth of tumor cells in type I collagen lattice (2, 7), we
have generated stable cell lines that express wild-type and MT5Δ3 in MCF-7 cells, and their ability to grow in threedimensional type I collagen gel was analyzed. As shown in Fig. 4, while MCF7 cells harboring wild-type MT5-MMP grew into significantly larger cysts than MCF7 cells with only the expression vector (B versus A), the cells with MT5Δ3 grew less robustly than those with wild-type MT5-MMP (C versus B). As expected, the MMP inhibitor BB-94 inhibited the growth of MCF-7 cells grown in type I collagen three-dimensional gels (Fig. 4, panels E–G versus A–C). Thus, these data suggest that the EWV motif behaves as a positive signal for MT5-MMP-mediated pro-MMP2 activation and growth in the type I collagen gel.

Identification of Mint-3 as a Binding Partner for MT5-MMP—Our data strongly suggest that the terminal motif, EWV, plays a key role in mediating MT5-MMP trafficking. We hypothesized that this motif binds to proteins in the trafficking pathway through its carboxy-terminal valine to regulate the surface expression and activity of MT5-MMP. To identify those proteins that directly bind to MT5-MMP, we used the cytoplasmic tail of MT5-MMP fused to the GAL4 DNA binding domain as bait in the yeast two-hybrid system to screen a human leukocyte complementary DNA library, which we have demonstrated to be an efficient library. About thirty positive clones were obtained in the screening from 11 million transformants. Sequencing revealed that one positive clone encoded fusion proteins of the GAL4 activation domain with a portion of Mint-3 (residues 421–575). Mint-3 belongs to the Mint protein family with three members (Mint 1–3, also known as X11/H9251, H9252, H9253) that all contain a conserved PTB domain and two carboxy-terminal PDZ domains (19). It has been reported that Mints can bind to some membrane proteins, and overexpression of the Mints protein leads to an increase of APP production in cells (20). The insert we obtained encoded a part of PDZa and the entire PDZb domains of Mint-3, suggesting that the PDZ domains may bind to the EWV motif in MT5-MMP.

To confirm the interaction between MT5-MMP and Mint-3 in intact cells, we co-expressed an HA-tagged version of Mint-3
with MT5-MMP in HEK293 cells. The HA-Mint-3 was immunoprecipitated with anti-HA antibody, and the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting analysis demonstrated that MT5-MMP co-precipitates with Mint-3 (Fig. 5A). To our surprise, three MT5-MMP species were detected in Mint-3 immunoprecipitates (Fig. 5A) and all appear to be insensitive to both Endo H and PNGase F, which can remove the N-linked sugars (data not shown). Reciprocal assays also demonstrated that Mint-3 was present in the MT5-MMP immunoprecipitates (Fig. 5B). Moreover, another member of the Mint protein family, Mint-2, could also bind to MT5-MMP in transfected cells efficiently (data not shown).

Mint-3 contains one PTB and two PDZ domains, all known for their ability to interact with motifs in membrane proteins (19, 21). To further map the region of Mint-3 that interacts with MT5-MMP in cells, a series of Mint-3 deletion mutants were generated and characterized by coimmunoprecipitation assays (Fig. 5C). Deletion of both PDZ domains almost completely abolished their interactions (Fig. 5C, lanes 5 versus 2). In contrast, mutants lacking either PDZ domain remain associated with MT5-MMP comparable to the full-length Mint-3 (Fig. 5C). These data indicate that either of the PDZ domains is sufficient for binding MT5-MMP.

To further demonstrate whether Mint-3 binds to the PDZ binding motif of MT5-MMP, we constructed two GST fusion proteins with wild type (GST-CT) or the V614G point-mutated cytoplasmic tail (GST-V614G) of the MT5-MMP. An HA tag was engineered to the amino terminus of GST in order to detect expression of the fusions in cells. Then Mint-3 was co-expressed with GST, GST-V614G, and GST-CT in cells, and GST fusion proteins were purified. As shown in Fig. 5D, Mint-3 was only pulled down by GST-CT (lane 3, upper panel), but not by GST
or GST-V614G. These data indicate that Mint-3 indeed binds to the PDZ motif, EWV, of MT5-MMP.

Biphasic Dose Response of MT5-MMP Activity to Mints—Does Mint-3 regulates MT5-MMP activity? We co-expressed MT5-MMP with increasing amounts of Mint-3, and its close relative Mint-2, in HEK293 cells. As shown in Fig. 6A, low levels of Mint-3 significantly increased the MT5-MMP activity toward pro-MMP-2 on the cell surface. Surprisingly, increasing doses of Mint-3 exhibited strong inhibitory effects on MT5-MMP-mediated activation of pro-MMP-2 (Fig. 6A, lanes 5–7 versus 2–4). Similar biphasic responses were also observed when Mint-2 was co-expressed with MT5-MMP (Fig 6A, lanes 10–12 versus 8–9). These results were duplicated in a neuronal cell line, Neuro2A (data not shown).

We then evaluated the effect of endogenous Mint-3 on MT5-MMP activity employing an RNA-interfering technique to knockdown the endogenous protein level of Mint-3. As shown in Fig. 6B, the expression level of endogenous Mint-3 was significantly decreased by Mint-3 siRNA (upper left panel, lanes 2 versus 1), while the expression level of actin was not affected by siRNA (upper left panel, lanes 2 versus 1), suggesting the specificity of the siRNA. Then the effect of the siRNA on the MT5-MMP activity was tested. Shown in Fig. 6B, MT5-MMP activity was significantly inhibited by Mint-3 siRNA (right panel, lanes 3 versus 2). These data suggest that Mint-3 is required for MT5-MMP-mediated pro-MMP-2 activation on the cell surface. One likely explanation for the observed inhibition of MT5-MMP function by high levels of Mints is the titration of downstream factors by overexpressed Mints. Data presented so far are consistent with the notion that Mint-3, and perhaps Mint-2, is a positive regulator of MT5-MMP activity on the cell surface.

The PDZ Domains Are Required for Mint-3-mediated Regulation of MT5-MMP Activity—We then tested whether Mint-3 regulates the activity of MT5-MMP via direct interaction. The wild-type MT5-MMP and MT5V614G were co-expressed with increasing amounts of Mint-3. As expected, mutation of the last residue V almost completely impaired the ability of MT5-MMP to respond to low levels of Mint-3 (Fig. 7A, lanes 2–4 versus 1).
However, high levels of Mint-3 remained inhibitory to MT5-V614G (Fig. 7A, lanes 11–13), suggesting that the inhibitory effect is independent of MT5-MMP/Mint-3 interaction. To further test the effect of Mint-3 on the MT5-MMP activity, the wild-type MT5-MMP, MT5ΔC with deletion of the entire cytoplasmic tail, and MT5Δ3 were also co-expressed with increasing amounts of Mint-3. As shown in Fig. 7B, MT5ΔC expressed a much higher activity than wild type (lane 2, middle panel versus upper panel), consistent with the idea that the cytoplasmic tails of MT-MMPs negatively regulate their activities by mediating internalization (8, 9). As expected, deletions of cytoplasmic or the last three residues in MT5-MMP also completely impaired the enhancement of MT5-MMP activity by low levels of Mint-3 (Fig. 7B, lanes 3–5), while the inhibitory effect of Mints was not affected by any deletion (Fig. 7B, lanes 6 and 7).

These results suggest that low levels of Mint-3 stimulate MT5-MMP through direct interaction between Mint-3 and the EWV motif of MT5-MMP, while high levels of Mint-3 inhibit MT5-MMP activity independent of this interaction; perhaps, by titrating out a positive factor in the Mint-3 pathway.

To further understand the effect of Mint-3, the MMP2 was co-expressed with MT5-MMP with increasing amounts of Mint-3 in cells. As shown in Fig. 7C, MT5-MMP efficiently activated MMP2 in cells. Compared with cell surface-mediated activation by MT5-MMP, Mint-3 appeared to have no effect on the MMP2 activation in cells. However, high levels of Mint-3 decreased the secretion of either pro- or activated form of MMP2 while low levels of Mint-3 did not affect the secretion of MMP2 (Fig. 7C, lanes 6 versus 2–5). Then we asked whether high levels of Mint-3 decrease the secretion of proteins in general. We tested the effect of Mint-3 on another secreted protein, clusterin. As shown in Fig. 7D, low levels of Mint-3 had no effect on the secretion of clusterin, while high levels of Mint-3 decreased the clusterin level in media and increased the clusterin in cell lysates. These data further support the idea that enhancement of MT5-MMP activity by Mint-3 is specific, whereas the inhibitory effect was nonspecific. These results also suggest that Mint-3 plays an important role in the default secretory pathway of cells.

Identification of Domains in Mint-3 Required for the Regulation of MT5-MMP Activity—To understand the structural basis of Mint-3-mediated regulation of MT5-MMP activity, we constructed a series of deletion mutants for Mint-3 as shown in Fig. 8A. These mutants were then tested against MT5-MMP in co-transfection experiments. As shown in Fig. 8B, ΔPDZab, ΔPDZb, or PDZab all failed to enhance MT5-MMP activity by low levels of Mint-3 (Fig. 7B, lanes 3–5), while the inhibitory effect of Mints was not affected by any deletion (Fig. 7B, lanes 6 and 7). These results suggest that the PTB and PDZ domains are required for both the negative and positive effects on MT5-MMP.

![Fig. 6. Mints affected the MT5-MMP-mediated pro-MMP2 activation on the cell surface.](http://www.jbc.org/)

A. MT5-MMP was transfected into HEK293 cells with different amounts of HA-Mint-3 or HA-Mint-2. The activity was analyzed by gelatin zymography as described in the legend to Fig. 1. Expression of Mint proteins was detected with anti-HA antibody, and MT5 was detected with anti-MT5 antibody. B. HEK293 cells were transfected with U6 vector or Mints siRNA. The endogenous Mint-3 was detected with anti-Mint-3 antibody. The protein level was also monitored by anti-actin. C. MT5-MMP was co-transfected with U6 vector or Mints siRNA. MT5-MMP activity was detected by gelatin zymography, and protein expression was analyzed by Western blotting using anti-MT5 antibody.
To further understand the mechanism by which Mint-3 protein mediates MT5-MMP trafficking, we attempted to detect the co-localization of MT5-MMP with wild-type and mutant Mint-3. As shown in Fig. 8C, only wild-type Mint-3 co-localized with MT5-MMP in TGN (panels a–c). The mutants, ΔPDZb, ΔPDZb, or PDZab are distributed diffusely and lost the TGN concentration observed for the wild-type Mint-3 (Fig. 8C, panels d–l versus a–c). Based on these observations, we concluded that the localization of Mint-3 is required for the observed enhancement of MT5-MMP activity.

Mint-3 Enhances MT5-MMP Activity Independent of Dynamin-dependent Endocytosis—Our previous report demonstrated that the cellsurface expression of MT1-MMP was regulated by dynamin-dependent internalization and K44A, a dominant negative form of dynamin, can increase the MT1-mediated pro-MMP2 activation on the cell surface (9). To determine if Mint-3 regulates MT5-MMP activity through the endocytic pathway, both Mint-3 and K44A were co-expressed with MT5-MMP in cells. As shown in Fig. 9A, co-expression of either Mint-3 (low level) or K44A stimulated MT5-MMP-mediated pro-MMP2 activation on the cell surface (lanes 3 and 4 versus 2). Interestingly, the K44A-mediated enhancement of MT5-MMP activity can be further augmented by wild-type Mint-3, but not the PDZ deletion Mint-3 mutant (Fig. 9A, lanes 5 and 6 and B). This result suggests that

FIG. 7. Enhancement of MT5-MMP-mediated activation by Mint-3 was dependent on the PDZ binding motif. A, wild-type or V614G-mutated MT5-MMP were co-transfected with different amounts of Mint-3. The activity was analyzed by gelatin zymography. B, wild-type (MT5wt), cytoplasmic tail-deleted MT5-MMP (MT5Δc), or 3-terminal amino acid-deleted MT5-MMP (MT5Δ3) were co-transfected with increasing amounts of Mint-3. The pro-MMP2 activation was analyzed by gelatin zymography. C, MMP2 was co-transfected with MT5-MMP and increasing amounts of Mint-3 into HEK293 cells. Cells were grown in serum-free Dulbecco's modified Eagle's medium for 24 h. The conditioned medium was analyzed by gelatin zymography. D, GFP-tagged clusterin was co-transfected with increasing amounts of Mint-3. Both the medium and cell lysates were subjected to analyze the expression of clusterin.

FIG. 8. Map of the domain of Mint-3 required for its effect on MT5-MMP activity. A, schematic illustration for Mint-3 constructs. All constructs had the HA tag at the amino terminus. B, MT5-MMP was co-transfected with increasing amounts of Mint-3 and its mutants into HEK293 cells, and the conditioned medium was subjected to zymography analysis. C, MT5-MMP was colocalized with Mint-3 in cells. MT5-MMP was co-transfected with Mints and its mutants in MDCK cells. Cells were fixed and stained with anti-MT5 antibody to detect MT5-MMP (green) and anti-HA antibody to detect Mint-3 (red).
Mint-3 regulates MT5-MMP activity independent of the dynamin-regulated endocytic pathway.

**Mint-3 Promotes the Surface Expression of MT5-MMP**—Mint-3 co-localization with MT5-MMP in the TGN suggests that it may function to facilitate the trafficking of MT5-MMP from the TGN to the cell surface. In fact, it has been demonstrated that Mint proteins can regulate the trafficking of some membrane proteins, such as the NMDA receptor (22). Therefore, the surface expression of MT5-MMP with or without Mint-3 expression was tested. As shown in Fig. 10A, K44A can increase the surface expression of MT5-MMP, indicating that MT5-MMP is internalized in a dynamin-dependent fashion as reported for MT1-MMP (9). Moreover, low levels of Mint-3 also greatly enhanced the surface expression MT5-MMP (Fig. 10A, lanes 3 versus 1), consistent with the observed increase in pro-MMP-2 activation (Fig. 9A). As shown in Fig. 10B, the surface expression of V614G mutant was lower than the wild-type MT5-MMP (lanes 4 versus 2), and Mint-3 had little effect on the surface expression of V614G (lanes 5 versus 4). The observed increase of MT5-MMP on the cell surface strongly supports the hypothesis that Mint-3 regulates MT5-MMP activity through trafficking mechanisms between the TGN and cell surface.

**DISCUSSION**

MT1-MMP has been reported to mediate several key functions in tumor progression, cranial facial development, and angiogenesis (1, 2, 17, 23, 24). The defining feature of MT1-MMP and its related type I MT-MMPs is a transmembrane region and a 20-residue cytoplasmic tail at its carboxyl termini. It has been proposed that the cellular machinery regulates these enzymes through interaction with cytoplasmic tails (8–10). Yet, no regulatory proteins have been identified that can regulate MT-MMP function through interaction with cytoplasmic tails. In this study, we have provided the first evidence that a PDZ domain protein Mint-3 regulates MT5-MMP by binding to the conserved PDZ binding motif EWV at its carboxyl end, facilitating the retrieval of internalized molecules to cell surface. Given the fact that all four type I MT-MMPs have similar motifs at their carboxyl ends, it is expected that PDZ domain proteins like Mint-3 interact with these motifs and regulate the trafficking of these MT-MMPs to specialized zones of plasma membrane where focal proteolysis takes place.

Several lines of evidence support our conclusion that Mint-3 specifically regulates MT5-MMP: 1) MT5-MMP recycles through the TGN, 2) MT5-MMP mutants carrying deletion or point mutations of the EWV motif are defective in recycling and less active than the wild-type molecule in mediating pro-MMP2 activation at the cell surface, 3) Mint-3 binds to MT5-MMP through the EWV motif, 4) Mint-3, both endogenous and exogenous, regulates MT5-MMP activity positively at physiological concentrations, 5) Mint-3 and MT5-MMP co-localize in the trans-Golgi network, 6) Mint-3 regulates MT5-MMP trafficking independent of the endocytic pathway, and 7) Mint-3 promotes the cell surface presentation of wild-type MT5-MMP, but not MT5-MMPV614G. Based on this evidence, we concluded that Mint-3 acts at the TGN to facilitate the recycling of internalized MT5-MMP to the cell surface. It would be interesting to see whether other MT-MMPs can be regulated by similar PDZ domain proteins.

Mints proteins are a family of PDZ domain-containing proteins with three members, Mint-1, Mint-2, and Mint-3 (19, 21). All Mints proteins share one conserved PTB domain and two PDZ domains (19, 21). The PDZ domains are well known protein-protein interaction motifs, which often bind to the carboxyl termini of membrane proteins (25). Three PDZ domains can be classified into three principal families according to their specificity for carboxyl-terminal peptides (25): 1) class I PDZ do-
membrane together with unknown proteins (protein X). Mint-3 binds to the cytoplasmic terminus of MT5 in TGN and promotes its recycling back to the plasma membrane together with unknown proteins (protein X).

Regulation of MT5-MMP by PDZ Protein Mint-3

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Fig. 11. Model of MT5-MMP trafficking. MT5-MMP is internalized constitutively through dynamin-dependent pathways and targeted to the early endosome and TGN. Mint-3 binds to the cytoplasmic terminus of MT5 in TGN and promotes its recycling back to the plasma membrane together with unknown proteins (protein X).
Mint-3 Regulates the Retrieval of the Internalized Membrane-type Matrix Metalloproteinase, MT5-MMP, to the Plasma Membrane by Binding to Its Carboxyl End Motif EWV

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