Co-recycling of MT1-MMP and MT3-MMP Through the trans-Golgi Network: Identification of DKV^{582} as a Recycling Signal

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Running title: Recycling of MT1-MMP and MT3-MMP
Abstract:

Members of the membrane-type matrix metalloproteinases (MT-MMPs) have been implicated in a wide range of physiological and pathological processes from normal development to tumor growth. Tethered on plasma membrane, these enzymes are potentially regulated by the trafficking machinery of the cells. Here we demonstrate that both MT1-MMP and MT3-MMP are internalized, transported to the trans-Golgi network through early endosomes and recycled back to cell surface in 60 min, in a manner distinct from the one employed by transferrin receptor. Interestingly, co-expressed MT1-MMP and MT3-MMP are localized and routed in the same vesicles throughout the trafficking process. We further demonstrated that the carboxyl terminal sequence DKV\textsuperscript{582} of MT1-MMP is required for its recycling, thus, defining a novel recycling motif. These results suggest that MT-MMPs may coordinate their proteolytic activities through the cellular trafficking machinery.
Introduction

The cell surface, populated with various protein molecules that mediate communications between the cell and its immediate environment, in many ways defines the cellular phenotype. One class of such molecules are cell surface proteinases that exert functional influence through irreversible cleavage of their substrates (1-4). Therefore, these surface proteinases should be regulated properly, and perhaps, differently from other cell surface molecules. The membrane-type matrix metalloproteinases or MT-MMPs are good examples of cell surface proteinases employed by various cell types to alter their surrounding environment during angiogenesis, tissue remodeling, tumor invasion and metastasis (1,5-10). Displayed on cell surface, these molecules have been shown to mediate a diverse biochemical reactions such as the activation of soluble MMPs, i.e., MMP-2 and MMP-13, or direct degradation of extracellular matrix (ECM) components (6,11-14). Ablation of MT1-MMP in mice validated most of these functions in vivo (15,16). Yet, little is known about the cellular mechanisms regulating the trafficking of MT-MMPs to and from the cell surface.

The MT-MMP subfamily can be divided into two subgroups: MT1, 2, 3, and 5-MMPs with type I transmembrane domains, and MT4 and 6-MMPs with GPI anchors (17-22). Since the first group MT-MMPs have similar structures at their C-termini with transmembrane domains and cytoplasmic tails, it is expected that their activities should be regulated by the trafficking machinery which presumably interacts with their cytoplasmic tails (23,24). Given their apparent divergency in their cytoplasmic domains, the trafficking pattern of each MT-MMP may differ
significantly (23). Indeed, while MT1-MMP and MT3-MMP are regulated by endocytosis, MT5-MMP does not appear to internalize well in the cells analyzed (24). Functionally, we have recently shown that MT1-MMP and MT3-MMP have distinct activities towards two well established substrates: proMMP-2 and type I collagen (25). Furthermore, these distinctions are encoded in their individual domains, i.e., catalytic and hemopexin domains, as analyzed by domain swapping experiments (25). Based on these findings, we hypothesize that the type I MT-MMPs may be regulated coordinately by the cellular trafficking machinery to deliver their distinct proteolytic activities to the cell surface. In this report, we demonstrate that MT1-MMP and MT3-MMP are co-internalized and co-routed through the recycling pathway back to the cell surface, providing the cells a unique way to regulate their activity dynamically under physiological as well as pathological conditions.
Materials and Methods

Cell Culture-- Madin-Darby canine kidney cells (MDCK, ATCC, Manassas, VA) were maintained as described (23,26). Cell culture media and supplements were purchased from Life Technologies (Rockville, MD).

Antibodies and Reagents-- Rabbit anti-MT1-MMP antibody (Ab3) has been described (23,27). FITC-labeled mouse anti-MT1-MMP monoclonal antibody was purchased from R&D Systems (Minneapolis, MN). Rabbit anti-MT3-MMP antibody was from Chemicon (Temecula, CA). Mouse Anti-p230 Ab was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-Furin antibody was from Affinity Bioreagents (Golden, CO). Texas Red conjugated human transferrin, Alexa-488 and -595 conjugated secondary antibodies were from Molecular Probe (Eugene, OR). Proteinase inhibitors and secondary Ab conjugates were from Sigma. BB-94 was a gift from British Biotechnology (Oxford, U.K.). MP070 is a novel MMP inhibitor which will be described (38).

Expression Constructs and Transfection—The expression constructs for MT1-MMP and MT3-MMP have been described (23,26). MT1-MMPΔ3 was constructed by a two-step process using high-fidelity PCR and inserted in the EcoRV site of pCR3.1 expression vector as described (7). RUFY expression construct is a gift from Dr. Qiu (21). The DNA constructs were transfected into various cells by Effectene (Qiagen, Valencia, CA) according to manufacturer’s protocol. Select experiments were performed in the presence of MMP inhibitor, BB-94 and MP-070, to prevent the autocatalytic decay of MT1-MMP.
Western Blotting, Immunoprecipitation, and Gelatin Zymography-- These procedures have been described previously (13,21). In brief, serum-free media supplemented with purified pro-MMP-2 or proMMP-2 in fetal bovine serum (5% v/v) were added to cells. After the indicated time period, conditioned media were collected, cleared of cell debris by centrifugation and analyzed by SDS/PAGE impregnated with gelatin (1 mg/ml) as described (13,21). For immunoprecipitation and Western blotting, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% NP-40, protease inhibitors cocktail tablet Complete (Roche, Basel, Switzerland)). The lysates were centrifuged (14,000 × g, 20 min) to remove debris and the resulting supernatants were immunoprecipitated and blotted as described (28).

Immunostaining and Confocal Microscopy-- For internalization experiments, cells seeded on coverslips in 6-well plates were washed three times with PBS and shifted to 4°C. Anti-MT-MMP antibodies were added to the cells at 0.2 µg/ml for 2 h. Antibody was subsequently removed and cells were washed before being shifted to 37°C with prewarmed media for the indicated time. Cells were then fixed with 3.7% paraformaldehyde in PBS (pH7.4) for 30 minute at room temperature and blocked with PBS-Diluent (0.3% Triton X-100, 1% NDS, 1% BSA and 0.01% Sodium azide, pH 7.2) for one hour followed by staining with secondary antibodies (Molecular Probe, Eugene, OR). The coverslips were mounted by NO-FADE (10% glycerol in PBS, 0.1% p-phenylenediamine, pH 8.0). For colocalization experiments, cells were labeled with M2 anti-FLAG or anti-p230 antibodies as the primary antibodies after fixation, followed by Alex-595 conjugated secondary antibody (Molecular
Probe). Confocal microscopy was carried out in the Biomedical Image Processing laboratories (BIPL) at the University of Minnesota using a Bio-Rad MRC 1024 system attached to an Olympus microscope (Melville, NY) with a 60X-oil objective. The images were processed in Photoshop 7.0 (Adobe, San Jose, CA). Quantification is carried out in Openlab (Improvision, Coventry, UK). The statistic analysis was done with GraphPad Prism software program (San Diego, CA).
Results

Recycling Patterns for MT1-MMP and MT3-MMP: We have recently demonstrated that MT1-MMP and MT3-MMP differ greatly in their activities against type I collagen and proMMP-2 (25). It is not clear if they are regulated similarly by the cellular trafficking machinery, given the apparent divergence between their cytoplasmic domains (23). To determine the difference between these two enzymes, we extended our internalization experiments up to 90 min as described (23). Surprisingly, both MT1-MMP and MT3-MMP virtually followed the same pattern of recycling as shown in Fig. 1A. Both molecules were labeled on cell surface at 4°C (Fig. 1A, panels a and b). Upon shifting to 37°C to commence internalization, a lag period of 10 min was observed (Fig. 1A, panels c and d). In the next 20 min, most of the cell surface MT1-MMP and MT3-MMP were internalized (Fig. 1A, panels e and f). Between 50-70 min, the internalized molecules appeared to have recycled back to the cell surface (Fig. 1A, panels g, h, i, and j). Further incubations suggest that the recycled MT1-MMP and MT3-MMP are re-internalized again, resembling the steady state pattern observed for MT1-MMP and MT3-MMP (23,26) (Fig. 1A, panels k and l), suggesting that an equilibrium is achieved through the recycling of MT-MMPs between intracellular pools and the cell surface.

Since internalized MT-MMPs may enter intracellular compartments where MT1-MMP or MT3-MMP dissociates from their respective antibodies, the observed findings in Fig. 1A may reflect the route of the dissociated antibody, rather than that of the two enzymes. To address this concern, we re-stained the cells underwent
internalization with rabbit anti-MT1-MMP antibody and found that the internalized
MT1-MMPs labeled with mouse anti-MT1-MMP antibody remained reactive with the
rabbit antibody (Fig. 1B, panel b), strongly suggesting that the internalized MT1-
MMP/antibody complex remains intact. Similar results were obtained for the MT3-
MMP/antibody complex (data not shown). Further quantification supports the
conclusion that both MT1-MMP and MT3-MMP undergo internalization and
recycling in a similarly fashion (Fig. 1C). To confirm whether the recycled MT1-
MMP are indeed re-exposed on cell surface, we labeled MT1-MMP on the cell
surface with FITC conjugated mouse monoclonal antibody (Fig. 1D, panels a-c),
allowed them to internalize for 30 min and stripped off any residual antibodies on
cell surface (Fig. 1D, panels g-l), and then incubated for a further 30 min to allow
the internalized MT1-MMP to resurface (Fig. 1D, panel j-l). Should the resurfaced
MT1-MMP molecules remain associated with the mouse antibodies, anti-mouse IgG
should be able to detect these resurfaced molecules. Indeed, we detected the
resurfaced mouse antibodies as shown in Fig. 1D, panel l. Similar results were
obtained when MT3-MMP recycling was analyzed in a similar fashion (data not
shown). Together, we conclude that both MT1-MMP and MT3-MMP recycles
through intracellular compartment.

Recycling of MT-MMPs through the trans-Golgi network: To understand the
route of MT1-MMP recycling, we traced the internalized MT1-MMP with markers for
various vesicles. Consistent with our previously conclusion that the internalized
MT1-MMP enters the endosomes (23), we observed that the newly internalized
MT1-MMPs co-localize with RUFY, a rab4-interacting protein associated with early
endosomes (29) (Fig. 2A). When most of the cell surface labels were internalized, a significant portion of the internalized MT1-MMP became co-localized with p230, a marker for trans-Golgi network (Fig. 2B, panel b). Interestingly, we also found that a majority portion of the internalized MT1-MMPs are co-localized with furin, a resident protein in the trans-Golgi network (Fig. 2C). Similar results were obtained with internalized MT3-MMP (Data not shown). Given these results, we conclude that MT1-MMP and MT3-MMP are internalized through the early endosomes, routed through the trans-Golgi network, where they are recycled back to the cell surface.

**Co-internalization of MT1-MMP and MT3-MMP:** The recycling patterns of MT1-MMP and MT3-MMP are very similar as shown in Fig. 1 and 2, suggesting that they may recycle through the same vesicles. To test this possibility, we co-transfected cells with both MT1-MMP and MT3-MMP, and performed internalization experiments with mouse anti-MT1-MMP antibody and rabbit anti-MT3-MMP antibodies as described in Fig. 1. A time course studies revealed that MT1-MMP and MT3-MMP are virtually co-localized throughout the recycling process. As shown in Fig. 3A, signals of internalized MT1-MMP and MT3-MMP overlapped in almost 100% of the vesicles observed. This observation is consistent with the recent report that MT1-MMP and MT3-MMP share similar motifs in their cytoplasmic domains for internalization (24). To rule out the possibility that the observed co-internalization is due to passive segregation of cell surface molecules in the same vesicles, we compared the internalization of MT1-MMP with that of transferrin. As shown in 3B, transferrin is internalized much faster than MT1-MMP with the
internalization process virtually completed in 5-10 min, compared to a time frame of 30-40 min for MT1-MMP. These data strongly suggest that MT1-MMP and MT3-MMP share the same internalization pathway, yet distinct from that employed by transferrin.

**The Carboxyl motif DKV\textsubscript{582} in MT1-MMP is Required for its Recycling:** To begin to probe the cellular machinery regulating MT1-MMP recycling, we analyzed a panel of MT1-MMP mutants with c-terminal truncations. One of the mutants, MT1-MMP\textsubscript{Δ3} (Fig. 4A), revealed an interesting pattern of trafficking. Distinct from the pattern exhibited by wild type MT1-MMP shown in Fig. 1A (panels g and i), this mutant fails to recycle back to cell surface after an apparently normal internalization process (Fig. 4B). Further quantitative analysis confirmed that MT1-MMP\textsubscript{Δ3} follows the same kinetics as wild type MT1-MMP during internalization (Fig. 4C, 10’ and 30’), but remains intracellular when the wild type molecules recycle back to cell surface (Fig. 4C, 50’ and 70’). Similar results were obtained with MT3-MMP (data not shown). Together, these data suggest that the well conserved DKV\textsubscript{582} motif in MT1-MMP encodes a signal for recycling.
Discussion

In this report, we describe the recycling of MT1-MMP and MT3-MMP in MDCK cells in a coordinated fashion. Both molecules recycle every 60 min between plasma membrane and intracellular vesicles, mostly trans-Golgi and endosomal vesicles, in a pattern distinct from the transferrin receptor. These findings outline a basic trafficking map for the MT-MMPs which may be exploited for the development of drugs against MT-MMP dependent pathological conditions such as cancer metastasis. Furthermore, our data raise the possibility that MT-MMPs can be mobilized in a coordinated fashion to specialized areas of the cell membrane where focalized proteolysis is a prerequisite for many pathological and physiological conditions.

One concern about the experimental system described here is the bivalent nature of the antibodies used, which may bind to both the antigens (MT1-MMP or MT3-MMP) and the antibody receptors on cell surface, thus, altering the trafficking pattern. Fortunately, MDCK is a well characterized cell line utilized routinely for trafficking related studies (30,31). So far, no Fc receptors have been reported for MDCK cells and Fc receptor had to be transfected into MDCK cells when IgG transcytosis was investigated (30,31). Furthermore, Seiki and colleagues observed no difference between intact IgG and its Fab fragment in internalization and trafficking of MT1-MMP in several cell lines (24). Finally, the fact that both the wild type MT1-MMP and MT1-MMPΔ3 underwent distinct routes of trafficking as detected by the same antibodies in the same cells also suggests that the Fc moiety has no effect on MT1-MMP trafficking. In agreement, a report was published using
antibody labeling to demonstrate that MT1-MMP does recycle back to cell surface (32). Together, the data presented in this report reflect the trafficking pattern of MT-MMPs without the influence from any potential Fc-FcR interaction.

Recycling of MT-MMPs may be one of the strategies to mobilize their proteolytic activity rapidly in response to physiological and pathological signals. This mechanism may allow the cells to maintain a dynamic pool of MT-MMPs readily deployable to cell surface. Recycling may also enable the cells to redistribute cell surface molecules as described for endothelin-converting enzyme (33). Redistribution of the MT-MMPs may mobilize pre-existent molecules to cell surface locations or compartments undergoing active proteolysis, thus, affording the cells the capability to regulate proteolytic activity without altering the profile of gene expression. The observed co-recycling of MT1-MMP and MT3-MMP suggests that both enzymes could be coordinated at the cellular level through trafficking events to focus their distinct proteolytic activities on the ECM barriers encountered by cells such as metastasizing tumor cells. Indeed, both MT1-MMP and MT3-MMP have been localized together in malignant tumor specimens (34,35). Given the irreversible nature of proteolysis, a selective advantage may be achieved by redirecting MT-MMPs to focal area where the underlying ECM is hydrolyzed abnormally. The hydrolysis of ECM would certainly lead to alteration of the cell microenvironment which could confer growth advantages for the surrounding cells such that subsequent mutations and genetic alterations can be accumulated. Thus, therapeutic or preventive intervention may be designed against the trafficking pathway of MT-MMPs.
Finally, we defined a recycling deficient mutant in MT1-MMP, i.e., MT1-MMPΔ3. Urena and colleagues noted that the carboxyl terminal residue of MT1-MMP and TGFα determines their subcellular localization (36). However, the c-terminal mutant of MT1-MMP analyzed by these investigators failed to mature properly (36), in contrast to our finding that MT1-MMPΔ3 behaves just like the wild type molecule except its recycling to cell surface. More works are needed to reconcile our observation with that of Urena and colleagues. The cellular proteins interacting with this motif remains unknown. However, we have identified an adaptor protein with two PDZ motifs that can interact with the analogous EWV motif of MT5-MMP (Wang, et al, unpublished results). Interesting, through a phage display screen, the DKV^{582} motif of MT1-MMP was identified as a binding site for the PDZ domain of the nNOS protein (37). However, we have not been able to implicate nNOS in the trafficking of MT1-MMP (Jiang et al, unpublished results). Given the specific defect identified for MT1-MMPΔ3 in this report, we are currently searching for the cellular factor that can interact with the DKV^{582} motif of MT1-MMP and regulate its trafficking in cells.
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Footnote:

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The abbreviations used are: MMP, matrix metalloproteinase; MT, membrane-type; ECM, extracellular matrix; TM, transmembrane domain; PC, proprotein convertase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby Canine Kidney.
Figure legend:

Fig. 1. MT1 and MT3-MMP recycle back to cell surface after internalization in MDCK cells.

A. The internalization assay. MDCK cells transfected with MT1, 3-MMPs were labeled with corresponding antibodies at 4°C for 2 h and then shifted to 37°C. Cells fixed at designed time points, 0, 10, 30, 50, 70 and 90 min, were permeabilized and stained with Alexa-488 conjugated secondary antibody. Representative cell images for every slide were collected by confocal microscopy as described (23,26). B. The MT1-MMP/antibody complex remains intact after internalization. MDCK cells transfected with MT1 were labeled with a FITC conjugated mouse anti-MT1 antibody at 4°C for 2 h and then shifted to 37°C for 50 min for internalization (green, a). After fixation, cells were stained again with rabbit anti-MT1 antibody followed by Alexa-595 goat anti-rabbit secondary antibody (red, c). The merged penal (b) depicts the co-localization of internalized mouse anti-MT1-MMP antibody and the MT1-MMP protein. C. Quantitative analysis of the internalization assays. Five typical cells for each time point in the internalization assay (A) were recorded with three layers each. The total fluorescent intensity and the surface signal intensity for every recorded cell were measured in Openlab. The statistic analysis is done in GraphPad Prism software program. D. MDCK cells transfected with MT1 were labeled with FITC labeled monoclonal anti-MT1 antibody at 4°C for 2 h followed without (a) or with (d) stripping by Glycine (pH 2.5). Cells without stripping were allowed to internalized at 37°C for 30 min followed with stripping by Glycine (g) and
allowed further recycling at 37°C for 30 min (j). All these cells were relabeled with Alexa-595 conjugated anti-Mouse secondary antibodies (c, f, i, l, respectively) after fixation.

**Figure 2. Colocalization between MT1-MMP and various markers for recycling.**

**A.** MDCK cells transfected with MT1 and RUFY were labeled with rabbit anti-MT1 antibody (a) at 4°C for 2 h and allowed to internalize at 37°C. Cells were then fixed, permeabilized and co-stained with M2 antibody (c), followed by Alexa-488 and -595-conjugated secondary antibodies (a-c). The arrows indicate positive co-localization. **B.** MDCK cells transfected with MT1 were labeled with rabbit anti-MT1 antibody at 4°C for 2 h and allowed to internalize as described above. Cells were then fixed, permeabilized and co-stained with mouse anti-p230 antibody, followed by Alexa-488 and -595-conjugated secondary antibodies. **C.** MDCK cells transfected with MT1 were labeled with FITC labeled monoclonal anti-MT1 antibody (panel a) at 4°C for 2 h and allowed to internalize at 37°C for 30 min followed by fixation, permeabilization and co-stained with rabbit anti-Furin antibody (a-c).

**Fig. 3. Co-internalization of MT1-MMP and MT3-MMP.**

**A.** Co-internalization of MT1-MMP and MT3-MMP. MDCK cells transfected with MT1 (a and d) and MT3 (c and f) were labeled with mouse anti-MT1 and rabbit anti-MT3 antibodies at 4°C for 2 hours, then switched to 37°C for internalization process and then fixed at designed time point, 0 and 30 min. After permeabilization, the slides were stained with secondary antibodies as described above. MT1-MMP (green, a, d) and MT3 (red, c, f) were recorded by confocal microscopy. Merged panels (b, e) indicate the co-localization between MT1-MMP and MT3-MMP. **B.**
The trafficking of MT1-MMP is slower than that of transferrin. MDCK cells transfected with MT1 were labeled with Texas Red conjugated transferrin along with MT1-MMP antibody at 4°C for 2 hours, then switched to 37°C for internalization process and fixed at 0, 10 and 30 min. After incubation with secondary antibodies, MT1-MMP (green, a, d, g) and transferrin (red, c, f, i) were recorded by confocal microscopy and merged (b, e, h) to indicate the colocalization between MT1-MMP and transferrin.

**Fig. 4. Recycling of MT1-MMP is mediated by a C-terminal DKV$_{582}$ motif.**

**A.** MT1-MMP cytosolic domain. The domain structure of MT1-MMP and amino acid sequence of cytosolic domain for both wild-type and deletion mutant (MT1Δ3) were shown. S: signal peptide; Pro: prodomain; R: RXKR Furin motif; CAT: catalytic domain; H: hinge; Pexin: hemopexinlike domain; T: transmembrane domain; C: cytosolic domain. **B.** MT1Δ3 is defected in recycling. MDCK cells transfected with MT1Δ3 were labeled with MT1 antibodies at 4°C for 2 h and then shifted to 37°C. Cells fixed at designed time points, 0, 10, 30, 50, 70 and 90 min (a-f, respectively), were permeabilized and stained with Alexa-488 conjugated secondary antibody. Representative cell images for every slide were collected by confocal microscope. **C.** Quantification for the internalization assay. Five typical cells for each time point in the internalization assay (B) were recorded with three layers each. The total fluorescent intensity and the surface signal intensity for every recorded cell were measured in Openlab. The statistic analysis is done in GraphPad Prism software program.
Xing et al., Fig. 1.

MT1

0 min

a

10 min

b

c

30 min
d

e

50 min

f

70 min

MT3

g

h

i

j

k

90 min

l
Xing et al., Fig. 1.

B

50 min

Ms-Anti-MT1  Merged  Rb-Anti-MT1
Xing et al., Fig. 1.

MMP Internalization Kinetics

Labeled Surface MMP (%) vs. Time (min)

- MT1
- MT3

Error bars indicate standard deviation.
Xing et al., Fig. 2.

A

MT1-MMP  Merge  RUFY

B

MT1-MMP  Merge  p230

C

MT1-MMP  Merge  Furin
Xing et al., Fig. 3.

A

Ms-Anti-MT1  Merged  Rb-Anti-MT3

0 min

0 min

30 min

30 min

B

MT1  Merged  Transferrin

0 min

10 min

30 min
Xing et al., Fig. 4.

A

MT1-WT: \text{RRHGTPRRLLYCQRSLLDKV}

MT1-Δ3: \text{RRHGTPRRLLYCQRSSL}

B

0 min \hspace{1cm} 10 min \hspace{1cm} 30 min

0 min \hspace{1cm} 50 min \hspace{1cm} 70 min \hspace{1cm} 90 min

C

MT1WT and -Δ3 internalization kinetics

Labeled Surface MMP (%) vs. Time (min)
Co-recycling of MT1-MMP and MT3-MMP Through the trans-Golgi Network:
Identification of DKV582 as a Recycling Signal
Xing Wang, Dawei Ma, Jorma Keski-Oja and Duanqing Pei

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