MT-LOOP-dependent Localization of Membrane Type I Matrix Metalloproteinase (MT1-MMP) to the Cell Adhesion Complexes Promotes Cancer Cell Invasion*

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Background: MT1-MMP promotes cancer cell invasion. Results: Deletion of the MT-LOOP region of MT1-MMP or an antibody to the MT-LOOP inhibits cellular invasion.

Conclusion: MT-LOOP-dependent localization of MT1-MMP to the cell adhesion complex promotes cellular invasion.

Significance: Our work reveals a novel mechanism of MT1-MMP regulation during cellular invasion and identifies the MT-LOOP as a novel target region to develop specific inhibitors.

Localization of membrane type I matrix metalloproteinase (MT1-MMP) to the leading edge is thought to be a crucial step during cancer cell invasion. However, its mechanisms and functional impact on cellular invasion have not been clearly defined. In this report, we have identified the MT-LOOP, a loop region in the catalytic domain of MT1-MMP (163PYAYIREG170), as an essential region for MT1-MMP to promote cellular invasion. Deletion of the MT-LOOP effectively inhibited functions of MT1-MMP on the cell surface, including proMMP-2 activation, degradation of gelatin and collagen films, and cellular invasion into a collagen matrix. This is not due to loss of the catalytic function of MT1-MMP but due to inefficient localization of the enzyme to β1-integrin-rich cell adhesion complexes at the plasma membrane. We also found that an antibody that specifically recognizes the MT-LOOP region of MT1-MMP (LOOPAb) inhibited MT1-MMP functions, fully mimicking the phenotype of the MT-LOOP deletion mutant. We therefore propose that the MT-LOOP region is an interface for molecular interactions that mediate enzyme localization to cell adhesion complexes and regulate MT1-MMP functions. Our findings have revealed a novel mechanism regulating MT1-MMP during cellular invasion and have identified the MT-LOOP as a potential exosite target region to develop selective MT1-MMP inhibitors.

Cancer cell invasion is the initial step of metastasis and a complex process that involves the following three essential steps: cell adhesion to the extracellular matrix (ECM), proteolytic degradation of ECM molecules, and cytoskeletal reorganization. Invading cells have to orchestrate these events to efficiently invade into tissues.

A membrane-bound matrix metalloproteinase (MMP), MT1-MMP, plays a role in the ECM degradation step and has been highly implicated in the process of cellular invasion into tissues (1–4). It is the only MMP shown to directly promote cellular invasion into collagen-rich matrices (5), and elevation in its levels has been associated with many different cancers (2) and other tissue-destructive diseases, including rheumatoid arthritis (6) and atherosclerosis (7). MT1-MMP degrades various ECM components, including collagen types I, II, and III; fibronectin; vitronectin; laminins 1 and 5; and aggrecan core protein (8, 9). It also activates other MMPs on the cell surface, namely proMMP-2 (1) and proMMP-13 (10), which expand the repertoire of pericellular proteolytic activities. Unlike MT1-MMP, MMP-2 can degrade type IV collagen (8, 11), a major component of basement membranes. Therefore, proMMP-2 activation by MT1-MMP is thought to be crucial for epithelial cancer cells to degrade their own basement membrane to grow and invade (12). Furthermore, MT1-MMP enhances cellular motility by cleaving cell adhesion molecules, including CD44 (13), syndecan 1 (14), and the integrin αv chain (15). MT1-MMP has also been shown to activate ERK, which, in turn, increases MT1-MMP levels and, thus, promotes cellular invasion (16).

As MT1-MMP promotes cellular invasion by multiple pathways, its functions at the cell surface are tightly regulated on many levels, including gene expression, activation of the zymogen (17), inhibition by endogenous inhibitors (including tissue inhibitors of metalloproteinases (18), N-Tes (19), and reverse-inducing-cysteine-rich protein with kazal motifs (RECK) (20)), proteolytic processing (21), endocytosis (22, 23), recycling (24), lysosomal degradation (25), homodimerization (26), and palmitoylation (27). In addition, polarization of localization of MT1-MMP on the cell surface is another important regulatory mechanism that is responsible for concentrating the enzyme to invasion-associated membrane structures, such as lamellipodia (28), invadopodia (29), and focal adhesions (FAs) (30). The lamellipodium is a typical “leading edge” of cells migrating on a
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two-dimensional substratum. MT1-MMP has been shown to localize to lamellipodia through the association of its hemopexin (Hpx) domain with CD44 (31). MT1-MMP has also been shown to accumulate in invadopodia, invasive membrane structures formed by cancer cells at cell-substrate attachment sites (29, 32). MT1-MMP was found to be a key invadopodial enzyme responsible for matrix degradation, and its localization to this structure was shown to depend on the cytoplasmic (CP) domain of the enzyme (29). MT1-MMP has been reported recently to localize to and degrade the matrix at FAs (30). This localization was reported to be mediated by the interaction of the CP domain of MT1-MMP with the FAK-p130 Cas complex (30). Although the CP domain has been proposed to regulate MT1-MMP cell surface localization, there are also contradictory reports indicating that this domain is dispensable for MT1-MMP-mediated ECM degradation and cellular invasion (33–35).

In this work, we have identified a crucial region of MT1-MMP that mediates the degradation of the underlying matrix and cellular invasion. Using a series of domain deletion mutants of MT1-MMP, we discovered that deletion of the MT-LOOP region, an eight-amino-acid loop in the catalytic domain, significantly inhibited MT1-MMP activities, including degradation of underlying matrices, proMMP-2 activation, and cellular invasion. Our data indicate that this is attributed to an inefficient association of the MT-LOOP deletion mutant with β1 integrin-containing cell adhesion complexes. Furthermore, we have demonstrated that a specific MT-LOOP-binding antibody inhibited MT1-MMP activities, fully mimicking the phenotype of the MT-LOOP deletion mutant. Our data reveal a novel mechanism for MT1-MMP regulation during cellular invasion through the MT-LOOP region of the enzyme.

MATERIALS AND METHODS

Cell Culture—COS-7, HT-1080, A431, and HeLa cells were cultured in DMEM (Lonza, Verviers, Belgium) supplemented with 10% FBS (Life Technologies, Paisley, UK) and penicillin/streptomycin (PAA, Pasching, Austria). For transfection, cells were cultured in six-well plates and transfected with expression plasmids using TransIT-2020 Transfection Reagent (Mirus, Madison, USA), according to the manufacturer’s instructions.

Antibodies—Primary antibodies were purchased from the following sources: Mouse anti-FLAG M1 and M2 antibodies from Sigma-Aldrich (Dorset, UK); mouse anti-MT1-MMP catalytic domain (clone LEM-2/15.8); rabbit anti-MT1-MMP hinge region (AB6004), and mouse anti-human integrin β1 (clone 12G10) antibodies from Millipore (Watford, UK); rabbit monoclonal antibody (clone Y113) to paxillin from Abcam (Cambridge, UK); and rabbit anti-MT1-MMP antibody (LOOP, clone EP1264Y) and the corresponding isotype control (iso-IgG) from Epitomics (Burlingame, CA). Before addition to cell cultures, LOOP and iso-IgG were dialyzed against 1 liter of PBS buffer. For the enzyme assay, the antibodies were repurified using immunoprecipitation kit Dynabeads protein G (Life Technologies, Paisley, UK) and dialyzed against TNC buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl2, and 0.02% NaN3). Secondary antibodies were purchased from the following sources: anti-mouse and anti-goat alkaline phosphatase-linked secondary antibody from Sigma-Aldrich (Dorset, UK); and anti-rabbit alkaline phosphatase-linked secondary antibody from Promega (Southampton, UK). Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-mouse and anti-rabbit antibodies and Alexa Fluor 488-conjugated phalloidin were purchased from Invitrogen, Acti-stain 670 phalloidin from was from Cytoskeleton (Denver, CO), and DAPI was purchased from Sigma-Aldrich.

Construction of MT-MMP Expression Plasmids—FLAG epitope (DYKDDDDK)-tagged MT1-MMP (MT1F) and its mutants were constructed as described previously (33, 36) and subcloned into the pSG5 vector (Invitrogen). ΔLOOP was generated by the deletion of Pro163-Gly170 in MT1F by PCR extension methods. MT1-RFP was generated as described previously (37). ΔLOOP-RFP was a mutant of MT1-RFP lacking Pro163-Gly170. FLAG-tagged MT2-, MT3-, and MT5-MMP were generated as described previously (38).

Western Blotting and Zymography—Western blotting was carried out as described previously (26). Cell lysates were prepared by lysing cells directly in SDS-PAGE loading buffer containing 2-mercaptoethanol. Gelatin zymography was carried out as described previously (26).

Fluorescently Labeled Gelatin (F-gelatin) Film Degradation Assay—Glass coverslips (18 mm in diameter) were coated with Alexa Fluor 488-conjugated gelatin as described previously (26). Cells were seeded onto F-gelatin-coated coverslips and cultured for the indicated time (1 h if MT1-MMP was overexpressed or 17 h if degradation by endogenous MT1-MMP was assessed). After the incubation, cells were fixed with 3% paraformaldehyde in TBS for 15 min and immunostained. Samples were imaged using an Ultraview confocal microscopy (PerkinElmer Life Sciences, Cambridge, UK). Degraded areas were visualized as dark, non-fluorescence zones.

Collagen Film Degradation Assay—A collagen film degradation assay was carried out as described previously (36). Briefly, COS-7 cells (4 × 105) expressing MT1F or ΔLOOP were seeded on six-well culture plates coated with a thin layer of fibrillar collagen (PureCol; concentration, 3.0 mg/ml). After 62 h of incubation, cells were removed by trypsinization, and plates were fixed with 3% paraformaldehyde in TBS for 20 min and stained with Coomassie Brilliant Blue R250. Images were captured with a charge-coupled device camera-equipped microscope (Nikon TE2000-E). Degraded areas were visualized as white, unstained, non-collagen-containing zones. When HT-1080 cells were used in experiments, cells were incubated on collagen film for 72 h. Where indicated, iso-IgG or LOOPAb were added to the medium at a final concentration of 100 nM.

Transwell Collagen Invasion Assay—The assay was carried out as described previously (39). Briefly, 8 μm polyethylene terephthalate track-etched membrane culture transwells were inserted into wells of a 12-well plate and coated with 50 μl of neutralized Cellmatrix type I-A collagen (Nitta Gelatin Inc., Osaka, Japan) at a concentration of 3.0 mg/ml. Coated chambers were then incubated for 1 h at 37 °C to set the collagen. 1 × 104 HT-1080 cells or 3 × 104 HeLa cells expressing exogenous MT1-MMP or its mutants were seeded into the collagen-coated chamber in serum-free medium. 10% FBS-containing medium was added to the bottom well, and cells were cultured...
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for 17 h. Nuclei of cells that had migrated through the membrane were visualized by DAPI staining. Fluorescent images of the whole membrane surface were captured by a semiautomated Nikon TE2000-E microscope equipped with a CCD camera, and the number of nuclei were counted using Volocity software (PerkinElmer Life Sciences). Statistical significance was evaluated with analyses of variance, followed by Bonferroni’s multiple comparison test.

Phagokinetic Track Motility Assay—The assay was carried out as described previously (13, 27). Briefly, glass coverslips were coated with BSA, followed by colloidal gold. HT1080 cells transiently transfected with the expression plasmid for MT1F or ΔLOOP were cultured on the colloidal gold-coated glass coverslips and cultured for 18 h. At the end of the period, cells were fixed, and the cell-surface MT1F and ΔLOOP were stained with anti-FLAG M1 antibody in the presence of 1 mM CaCl₂. The area of phagokinetic track generated by FLAG-positive cells was analyzed by ImageJ software. Data are shown as mean ± S.D. (n = 50). Statistical significance was evaluated with analyses of variance, followed by Bonferroni’s multiple comparison test.

Surface Biotinylation and Subsequent Immunoprecipitation—
COS-7 cells transfected with a mock vector or plasmid DNAs encoding MT1F or ΔLOOP were subjected to surface biotinylation using sulfo-NHS-biotin (Thermo Scientific, Northumberland, UK) as described previously (26). Following surface biotinylation, cells were recovered in radioimmune precipitation assay buffer and subjected to affinity precipitation with streptavidin-conjugated Sepharose beads (Amersham Biosciences-Pharmacia, Little Chalfont, UK). Bound materials were eluted in SDS-PAGE loading buffer and subjected to Western blot analysis using anti-FLAG M2 antibody.

Expression and Purification of Recombinant MT1-Cat and MT1-CatΔLOOP—The cDNA fragment encoding the propeptide and the catalytic domain (Ser²⁴-Gly²⁸⁴) was generated by PCR and subcloned into pET3a Escherichia coli expression vector (Agilent Technologies, Wokingham, UK). MT1-CatΔLOOP was generated by deleting the MT-LOOP region (Pro¹⁶⁵-Gly¹⁶⁷) with a QuickChange site-directed mutagenesis kit (Agilent Technologies) according to the instructions of the manufacturer. BL21(DE3) cells (Agilent Technologies) were transformed with the constructs, and protein expression was induced by 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. Proteins were purified from inclusion bodies and folded as described previously (26). Purified enzymes were activated by treating with trypsin (0.1 μg/ml) for 1 h at 37 °C, followed by PMSF treatment. The enzyme concentrations were measured by titrating with a known amount of tissue inhibitors of metalloproteinase 2 as described previously (40).

Degradation of Gelatin by Recombinant MT1-MMP—Neutralized type I collagen (PureCol) was heat-denatured at 80 °C for 30 min and then diluted to 1 mg/ml in DMEM. MT1-Cat or MT1-CatΔLOOP was incubated with 80 μl of gelatin for 30 min at 37 °C at final enzyme concentrations of 0.01, 0.1, or 1.0 μg/ml. Degradation of gelatin was assessed by SDS-PAGE.

Degradation of Collagen by the Recombinant Soluble Whole Ectodomain of MT1-MMP—Neutralized guinea pig type I collagen (pepsin-extracted) at 1 mg/ml was reacted with 5 μg/ml soluble whole ectodomain of MT1-MMP in a buffer of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.05% Brij35 at 22 °C for 16 h in the presence or absence of different molar ratios of LOOPₐ₋ₐ or iso-IgG. Samples were then mixed with SDS loading buffer, boiled to stop the reaction, and analyzed by SDS-PAGE. Relative degradation with the control was analyzed by scanning the density of generated α1–3/4 bands by ImageJ software. The recombinant soluble whole ectodomain of MT1-MMP was provided by Prof. Gillian Murphy and Dr. Kenneth Boetkjaer at the University of Cambridge.

Enzyme Assay—Enzymic activity was measured using a quenched fluorescent substrate: Mca-Pro-Leu-Gly-Leu-Dap (Dnp)-Ala-Arg-NH₂, as described previously (40). Upon proteolytic cleavage by MT1-MMP, this substrate releases fluorescence with a peak emission at 393 nm.

Indirect Immunofluorescence Staining—Indirect immunofluorescence staining was carried out as described previously (26). Briefly, cells cultured on gelatin-coated coverslips were fixed with 3% paraformaldehyde in TBS and blocked with 5% goat serum and 3% bovine serum albumin in TBS. Cells were then incubated with primary antibodies (FLAG M1 (5 μg/ml), anti-MT1-Cat (1 μg/ml), or anti-MT1-hinge region (1 μg/ml) antibodies, as indicated). 1 mM CaCl₂ was included throughout the procedure of washing and incubation for staining with the anti-FLAG M1 antibody. Alexa Fluor 488- or Alexa Fluor 568-conjugated goat anti-mouse or anti-rabbit antibodies were used to visualize the antigen signal. To visualize F-actin, cells were incubated with Alexa Fluor 488 or Acti-stain 670 phalloidin in 0.1% Triton X-100 in TBS. Cell nuclei were visualized with DAPI. The fluorescent signals were analyzed by Ultraview confocal microscopy (PerkinElmer Life Sciences).

In Situ Proximity Ligation Assay (PLA) —A proximity ligation assay was performed using a Duolink kit (Olink Bioscience, Uppsala, Sweden). In addition to the kit components, primary antibodies (rabbit anti-human MT1-MMP hinge region (AB6004) and mouse anti-human integrin β₁ (clone 12G10)) were used. 2 × 10⁵ COS-7 cells were seeded in a 6-well plate and transfected with a mock vector or expression plasmids for MT1F or ΔLOOP (0.2 μg DNA each). 24 h after transfection, 3 × 10⁴ cells were seeded on gelatin-coated coverslips in the presence of GM6001 (10 μM). After 2 h of incubation, cells were fixed and stained according to the instructions of the manufacturer. Images were captured using an Ultraview confocal microscope (PerkinElmer Life Sciences). Red dots in the image represent PLA signals, indicating MT1-MMP-β₁-integrin complexes.

RESULTS

The MT-LOOP Region Is Important for MT1-MMP-mediated Matrix Degradation and Cellular Invasion—MT1-MMP promotes cellular invasion by degrading the underlying ECM. We therefore investigated which domain of MT1-MMP is responsible for this activity. COS-7 cells transfected with full-length MT1-MMP (MT1F) or its domain deletion mutants (Fig. 1A) were subjected to a gelatin film degradation assay. Western blot analysis confirmed that all MT1-MMP mutants were expressed at similar levels (Fig. 1B). As shown in Fig. 1C, expression of MT1F resulted in efficient gelatin film degradation, and
deletion of the cytoplasmic domain (ΔCP) or the hemopexin domain (ΔHpx) or exchanging the transmembrane domain to the one derived from nerve growth factor receptor (NGFR\textsubscript{TM}) did not affect the capability of the MT1-MMP to degrade the gelatin film. In contrast, deletion of the MT-LOOP (\textsuperscript{163}PYAYIREG\textsubscript{170}), a characteristic loop region in the catalytic domain of MT1-MMP, markedly decreased gelatin film degradation, regardless of the presence or absence of the Hpx domain (Fig. 1C, ΔLOOP and ΔHpxΔLOOP). When COS-7 cells expressing similar cell surface levels of MT1F and ΔLOOP were compared, ΔLOOP was clearly inefficient in degrading the gelatin film (Fig. 1D). MT-LOOP deletion showed a consistent phenotype in different cancer cells, including fibrosarcoma (HT1080) and squamous carcinoma (A431) cells (Fig. 1E). ACP also degraded gelatin film efficiently in all cell types (Fig. 1, C and E).

We next examined the effect of MT-LOOP deletion on other functions of MT1-MMP, including proMMP-2 activation, collagen degradation, and collagen invasion. COS-7 cells were transfected with MT1F or ΔLOOP and incubated with exogenously added recombinant proMMP-2 in the media. In agreement with a previous report (41), we found that deletion of the MT-LOOP resulted in inefficient proMMP-2 activation on the cell surface (Fig. 2A). Next we analyzed collagen film degradation. Because COS-7 cells do not produce MMP-2, collagen degradation detected in this system is a direct activity of MT1-MMP against collagen. Our data indicate that the degradation of the collagen film was also inhibited by MT-LOOP deletion (Fig. 2B). We found that this phenotype of the MT-LOOP deletion mutant correlated with its reduced ability to promote collagen invasion as well. As shown in Fig. 2C, HeLa cells expressing MT1F effectively invaded in comparison to mock-transfected cells, and deletion of the MT-LOOP caused a significantly reduced invasion. Similar levels of MT1F and ΔLOOP were confirmed by Western blot analysis using the anti-FLAG M2 antibody (Fig. 2C, inset). In this experiment, we used HeLa cells because they do not express endogenous MT1-MMP and MMP-2.

MT-LOOP Deletion Does Not Affect Either Cell Surface Expression or Proteolytic Activity of MT1-MMP—MT-LOOP deletion caused attenuation of most MT1-MMP functions on the cell surface. To investigate the mechanism by which this occurred, we first examined whether the mutation affected cell surface levels of the enzyme using cell surface biotinylation. As shown in Fig. 3A, we found that cell surface levels of MT1F and ΔLOOP were comparable. Next, we examined whether deletion of the MT-LOOP affected enzyme localization to the cell-gelatin interface. COS-7 cells expressing MT1F or ΔLOOP were stained with anti-FLAG M1 antibody without permeabilization. In agreement, followed by imaging using confocal microscopy, and the ratio of MT1-MMP levels at the cell-gelatin interface versus total cell surface levels were analyzed. As shown in Fig. 3B, MT1F and ΔLOOP were present at comparable levels at the cell-gelatin interface. Because the MT-LOOP is located in the catalytic domain, it is possible that its deletion might have influenced the catalytic activity of the enzyme. To address this possibility, the recombinant catalytic domain of MT1-MMP (MT1-Cat) or the corresponding MT-LOOP deletion mutant (MT1-CatΔLOOP) were expressed and purified, and their pro-
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FIGURE 2. Deletion of the MT-LOOP inhibits MT1-MMP-mediated proMMP-2 activation, collagen degradation, and cellular invasion. A, COS-7 cells expressing MT1F, ∆LOOP, or a mock vector were incubated with exogenous proMMP-2 for 24 h. MMP-2 in the medium was analyzed by gelatin zymography (zymo, top panel), and expression levels of MT1-MMP mutants were analyzed by Western blotting using anti-MT1-MMP (LEM-2/15.8) primary antibody (bottom panel). Actin staining was used as a loading control. B, COS-7 cells expressing MT1F, ∆LOOP, or a mock vector were subjected to a collagen film degradation assay as described under “Materials and Methods.” Digested areas of collagen are shown as white areas against the gray collagen background. Protein expression of MT1-MMP mutants was confirmed by Western blot analysis using anti-FLAG M2 antibody (bottom right panel). C, HeLa cells expressing MT1F, ∆LOOP, or a mock vector were subjected to a transwell collagen invasion assay as described under “Materials and Methods.” Expression of MT1-MMP mutants in HeLa cells was confirmed by Western blot analysis using M2 anti-FLAG antibody (top left panel). *p < 0.05.

teolytic activity against gelatin was examined. As shown in Fig. 3C, gelatinolytic activities, as well as patterns of degradation, were indistinguishable between MT1-Cat and MT1-Cat∆LOOP, indicating that MT-LOOP deletion does not influence the catalytic activity of MT1-MMP. We also analyzed the cell migration-promoting effect of ∆LOOP using a phagokinetic track motility assay in HT1080 cells. We reported previously that cell migration detected in this system is through CD44 shedding by MT1-MMP at lamellipodia (13). As shown in Fig. 3D, expression of MT1F or ∆LOOP similarly enhanced cell motility in comparison to mock-transfected cells. Taken together, the data suggest that the effect of MT-LOOP deletion is not due to altered secretion, catalytic activity, or misfolding but likely due to a more specific effect related to MT1-MMP functionality at the cell attachment sites.

MT-LOOP Deletion Affects Incorporation of MT1-MMP to β1-Integrin-containing Adhesion Complexes at ECM Attachment Sites—As shown in Fig. 3B, confocal microscopy analysis did not reveal any apparent differences in MT1F and ∆LOOP levels at the cell-matrix interface. However, degradation of the ECM only takes place when MT1-MMP is localized to the areas of the plasma membrane that come into direct contact with matrix molecules, such as integrin-rich adhesion complexes, and it is possible that the total levels of the enzymes at the basal surface may not reflect the level of matrix degradation. Therefore, we next examined the possibility that deletion of the MT-LOOP influenced localization of the enzyme to these distinct areas of the membrane. Because the resolution of the confocal microscopy is limited (the highest possible resolution is 200 nm in the x and y axes and 600 nm in the z axis), one cannot distinguish areas of plasma membrane that come into direct contact with the ECM and the areas in between. Therefore, we addressed this question using a Duolink in situ PLA, examining relative localization of MT1F and ∆LOOP to the integrin β1 subunit, a component of cell adhesion complexes. Because the PLA highlights two molecules within 40 nm of each other, it is possible to examine the presence of MT1-MMPs at the integrin-containing adhesion complexes. Transfected COS-7 cells seeded on the gelatin-coated coverslips were incubated with antibodies to MT1-MMP and β1-integrin and subjected to the PLA assay. As shown in Fig. 4A, top panel, COS-7 cells transfected with MT1F showed bright PLA signals at the basal side of the cells, indicating that MT1F is indeed localized close to β1-integrin. In contrast, cells expressing ∆LOOP showed significantly weaker PLA signals (Fig. 4A, top and bottom panels), suggesting that MT-LOOP deletion attenuated localization of MT1-MMP to β1-integrin-containing adhesion complexes.

In addition, we also found that overexpression of MT1F in COS-7 cells resulted in loss of focal adhesions and stress fiber formation, as shown in Fig. 4, B and C. In these experiments, we captured images of at least 50 individual cells/treatment and categorized them into positive, partially positive, or negative for FA formation (Fig. 4, C, right panel) to analyze the effect of each MT1-MMP mutant expression. Representative images are shown in Fig. 4B. In the mock-transfected cell population, 70% of cells were positive for FA, whereas 20% were negative. GM6001 treatment had only a minor effect, increasing positive cells to 80%. In contrast, 84% of cells expressing MT1F were completely negative for FA formation, and this phenomenon was effectively reversed by addition of GM6001, where 84% of cells were positive for FA (Fig. 4C). This suggests that the proteolytic action of MT1-MMP at FA sites is likely to be the cause of loss of FA. Interestingly, significant populations of cells expressing ∆LOOP were positive (34%) or partially positive (36%) for FA and stress fiber formation, supporting the notion that the MT-LOOP facilitates localization of MT1-MMP to integrin-containing cell adhesion complexes. Loss of FA and stress fiber formation were also observed in cells expressing the cytoplasmic domain deletion mutant (∆CP) (Fig. 4, B and C), suggesting that the cytoplasmic domain is not involved in the localization of MT1-MMP to FA sites.

Effect of MT-LOOP-binding Antibody (LOOPAb) on MT1-MMP Functions—On the basis of the data above, we hypothesized that the MT-LOOP may be an interface for molecular interactions and that it may regulate the incorporation of the enzyme into integrin-containing ECM adhesion complexes. If this is the case, it should be possible to mimic the phenotype of ∆LOOP by disrupting these interactions. To test this hypothesis, we utilized an antibody that binds to the MT-LOOP (LOOPAb). We confirmed that LOOPAb recognized MT1F, but not the ∆LOOP mutant, on Western blot analysis (Fig. 5B). In immunofluorescence staining, LOOPAb recognized only full-
length MT1-RFP but not ΔLOOP-RFP (Fig. 5C). We also confirmed that LOOPAb is specific to MT1-MMP and does not bind to other MT-MMPs, including MT2-, MT3-, and MT5-MMP, that possess a similar MT-LOOP in their catalytic domains (Fig. 5D).

We next tested whether binding of LOOPAb to MT1-MMP affects the catalytic activity of MT1-MMP using recombinant MT1-MMP (MT1-Cat) (Fig. 5E). MT1-Cat was incubated with LOOPAb or isotype-matched control IgG (iso-IgG) at a molar ratio of 1:0.5, 1:1, or 1:5. Because an IgG molecule has two antigen-binding sites, each antibody can bind up to two enzyme molecules, making the ratio of binding sites to the enzyme 1:1, 1:2, and 1:10. Our data indicate that neither antibody had a significant effect on the catalytic activity of the enzyme against a peptide substrate at all the ratios tested (Fig. 5E). We also examined whether LOOPAb has an effect on collagen degradation by MT1-CatΔLOOP and MT1-CatΔLOOP at 50 μg/ml were also analyzed by SDS-PAGE (right panel). Arrowheads indicate intact gelatin bands of α1 and α2 chains and β chains. Note that MT1-Cat and MT1-CatΔLOOP degraded gelatin in an almost identical manner. D, HT1080 cells transiently expressing MT1F, ΔLOOP, or empty vector (Mock) were subjected to a phagokinetic track motility assay as described under "Materials and Methods." Cell surface MT1-MMPs were stained with anti-FLAG M1 antibody.

Having characterized LOOPAb, we next examined its effect on MT1-MMP-mediated proMMP-2 activation. COS-7 cells expressing MT1-RFP or ΔLOOP-RFP were incubated with recombinant proMMP-2 in the presence or absence of 100 nM LOOPAb or iso-IgG (Fig. 6A, right panel). MT1-RFP efficiently activated proMMP-2 to the active form, and the addition of iso-IgG did not affect the processing. In contrast, the addition of LOOPAb decreased proMMP-2 activation by ~50%, which corresponded to the level of activation detected in ΔLOOP-RFP-expressing cells. As expected, the addition of neither LOOPAb nor iso-IgG to cells expressing ΔLOOP-RFP affected proMMP-2 activation. Comparable expression levels of MT1-
FIGURE 4. Deletion of the MT-LOOP abrogated efficient localization of MT1-MMP to cell adhesion complexes. A, localization of MT1F and ΔLOOP to β1-integrin-containing complexes at cell-matrix attachment sites was assessed by Duolink in situ PLA. Briefly, COS-7 cells transfected with MT1F, ΔLOOP, or a mock vector were seeded on gelatin film in the presence of 10 μM GM6001. After 2 h of incubation, cells were fixed and stained using rabbit anti-MT1-MMP antibody and anti-β1 integrin antibodies as described under "Materials and Methods." MT1-MMP/β1 integrin complexes are visualized as bright fluorescent dots under confocal microscopy. Cell surface levels of MT1F and ΔLOOP were also visualized by staining with secondary antibody to rabbit IgG. Each cell, the MFI of spots (MF1 Duolink) was divided by the MFI of cell surface staining of MT1-MMP (MF1 total). ***, p < 0.0001. Scale bar, 30 μm. B, COS-7 cells expressing MT1F, ΔLOOP, ΔCP, or a mock vector were seeded on gelatin film were stained for paxillin to visualize FAs. 1 h after seeding cells on gelatin film, 10 μM GM6001 was added to the media where indicated, and cells were incubated for an additional 2 h. Cells were fixed and stained for cell surface MT1-MMPs using anti-FLAG M1 antibody, paxillin, and actin. Areas of the cells within the yellow box were enlarged and are shown at the bottom. C, for each transfection and treatment, 50 cells were randomly imaged by confocal microscopy, and the presence of FAs in these cells was characterized as positive, partially positive, or negative, as shown in the right panel, and the population of cells in each category is shown in the graph (left panel). Note that the expression of MT1F and ΔCP effectively abrogated FA formation, whereas with expression of ΔLOOP, 70% of cells were still either positive or partially positive for FA formation.

FIGURE 5. Characterization of the anti-MT-LOOP antibody (LOOPAb). A, schematic of the MT1-MMP constructs used in this experiment. RFP, red fluorescent protein; SP, signal peptide; Pro, prodomain; Cat, catalytic domain; MT-LOOP, 163PYAYIREG170; L1, linker-1; L2, linker-2; Tm, transmembrane domain; CP, cytoplasmic domain.; Zn, zinc atom at the active site. B, COS-7 cells transfected with MT1F, ΔLOOP, or mock vector were analyzed by Western blotting using mouse monoclonal anti-Hapx domain antibody or LOOPAb. C, COS-7 cells expressing MT1-RFP and ΔLOOP-RFP were subjected to indirect immunofluorescence staining using LOOPAb. Images in the RFP channel and green channel (LOOPAb) are shown. For mock cells, DAPI staining is shown as an inset. D, COS-7 cells overexpressing FLAG-tagged MT1-, MT2-, MT3-, and MT5-MMP were subjected to Western blot analyses using LOOPAb or anti-FLAG M2 antibody. Sequences of the MT-LOOP region of the MT-MMP family are shown in the table (right panel). Amino acids identical to MT1-MMP are shown in boldface. E, effect of different doses of LOOPAb or iso-IgG on the enzymatic activity of the recombinant MT1-Cat domain of MT1-MMP was examined using a quenched fluorescent substrate. The activity of the MT1-Cat domain alone (Cont) is indicated as 100%. F, the effect of different doses of LOOPAb or iso-IgG on collagenolytic activity of recombinant soluble MT1-MMP was examined by reacting enzyme with type I collagen at 22 °C for 16 h. The generated a1–3/4 bands were scanned, and the relative degradation of collagen for each treatment against control without any antibody (Ab) was calculated and is indicated in percent.
RFP and ΔLOOP-RFP were confirmed by Western blot analysis (Fig. 6A, left panel).

Next, we tested the effect of LOOP<sub>Ab</sub> on MT1-MMP-mediated gelatin film degradation (Fig. 6B). In comparison to iso-IgG-treated MT1-RFP-expressing cells, the addition of LOOP<sub>Ab</sub> significantly decreased the degradation of the gelatin film to the level observed in the ΔLOOP-RFP sample. As expected, the addition of LOOP<sub>Ab</sub> to ΔLOOP-RFP-expressing cells had no effect on gelatin degradation. Similar results were obtained when the degradation of collagen film was examined (Fig. 6C). In contrast to non-treated cells or cells treated with iso-IgG, the addition of LOOP<sub>Ab</sub> significantly inhibited collagen film degradation. Next, the effect of LOOP<sub>Ab</sub> on MT1-MMP-mediated invasion assay was assessed by a transwell invasion assay. As shown in Fig. 6D, the addition of LOOP<sub>Ab</sub> to HeLa cells expressing MT1F almost completely inhibited cell invasion when compared with iso-IgG. Finally, the effect of LOOP<sub>Ab</sub> on formation of FA sites was examined. As shown in Fig. 6E, the addition of LOOP<sub>Ab</sub> and GM6001, but not iso-IgG, recovered FA formation.

We next examined the effect of LOOP<sub>Ab</sub> on endogenous MT1-MMP functions (Fig. 7). HT-1080 cells were stimulated with type I collagen to promote the activation of proMMP-2 by endogenous MT1-MMP (Fig. 7A). Zymography data revealed that LOOP<sub>Ab</sub>, but not iso-IgG, inhibited proMMP-2 activation. Comparable MT1-MMP expression between samples was confirmed by Western blot analysis (Fig. 7A, bottom panel). The addition of LOOP<sub>Ab</sub> also inhibited gelatin (Fig. 7B) and collagen film degradation (C) as well as collagen invasion (D). Taken together, these results indicate that incubation of MT1-MMP with LOOP<sub>Ab</sub> indeed mimics the phenotype of the ΔLOOP mutant and can efficiently inhibit MT1-MMP functions on the cell surface.

**DISCUSSION**

To promote cellular invasion, MT1-MMP needs to be localized to the leading edge of invading cells. However, it has been unclear which membrane structures represent the leading edge essential for cellular invasion. In this report, we found that integrin-rich cell adhesion complex can be one of the functional leading edges and that the MT-LOOP region of MT1-MMP is required for the enzyme to localize to this structure. Deletion of the MT-LOOP significantly reduced the ability of MT1-MMP to degrade gelatin and collagen matrices and to promote cellular invasion into collagen. We thus propose that MT-LOOP-dependent localization of MT1-MMP to the cell adhesion complexes is an essential step in cancer cell invasion (Fig. 8). At the basal side of cells, attachment of the plasma membrane to the ECM occurs through adhesion complexes (42). Our data revealed that MT-LOOP deletion did not affect the overall level of MT1-MMP at the basal side of cells but significantly reduced the association of the enzyme with β1-integrin (Fig. 8). Because integrins connect the ECM to the cellular cytoskeleton, it is possible that the MT-LOOP-dependent MT1-MMP localization is a part of the mechanisms to coordinate cell adhesion, cytoskeletal reorganization, and pericellular ECM degradation during the invasion process.
Although coimmunoprecipitation of MT1-MMP and β1-integrin has been reported previously (43, 44), we could not confirm this result (data not shown). However, knocking down β1-integrin did not inhibit MT1-MMP-dependent gelatin film degradation and did not affect the phenotype of the ΔLOOP mutant (data not shown), suggesting that β1-integrin is not the molecule in the adhesion complex responsible for interaction with the MT-LOOP region to drive MT1-MMP localization to FA sites. MT1-MMP has been shown recently to localize to FA sites through the interaction of its CP domain with a complex of focal adhesion kinase and p130Cas (30). This was concluded on the basis of data showing that deletion of the CP domain of MT1-MMP (ΔCP) diminished MT1-MMP-dependent gelatin film degradation at FA sites (30). Contrary to this report, data presented in this paper and previous reports (33–35) show that deletion of the CP domain does not inhibit the ability of MT1-MMP to degrade gelatin or collagen films or the ability to promote cellular invasion into a collagen matrix. Because the CP domain is essential for clathrin-dependent endocytosis of MT1-MMP (22, 23), the cell surface level of the ΔCP is higher than MT1F (23), and comparable or higher levels of gelatin film degradation can be observed compared with the full-length MT1-MMP (Fig. 1, C and E). It is unlikely that the effect of CP domain deletion is cell-type specific because the efficient degradation of gelatin film by ΔCP and decreased degradation by ΔLOOP were confirmed in different cell lines, including COS-7, HT1080, HeLa, and A431 cells (Fig. 1, C and E, and data not shown). Our data also revealed that expression of ΔCP disrupted FA and actin stress fibers as effectively as full-length MT1F, whereas around 70% of cells expressing ΔLOOP were
positive or partially positive for FA (Fig. 4C). Taking these observations together, we conclude that an MT-LOOP-dependent mechanism, but not a CP domain-dependent mechanism, plays a role in localization of MT1-MMP to FA sites.

Invadopodia are thought to be invasive membrane structures that cancer cells extend into the ECM (45). However, conclusive evidence supporting the role of invadopodia in cancer cell invasion has yet to be shown. MT1-MMP has been shown to be a major proteinase localized at invadopodia (46). Apart from MT1-MMP, invadopodia have been shown to be enriched in cortactin and actin (32), whereas their formation is regulated by cdc42 and neural Wiskott-Aldrich syndrome protein-dependent rearrangement of the actin cytoskeleton (47). MT1-MMP has been shown to be dispensable for the generation of invadopodia structures but to be essential for ECM degradation (32). The CP domain of MT1-MMP has been proposed to mediate enzyme localization to invadopodia (29). However, as discussed above, the CP domain is dispensable in the degradation of underlying ECM. Thus, involvement of the CP domain in invadopodial localization of MT1-MMP may need to be re-evaluated. β1-integrin has been shown to accumulate in invadopodia, where it docks proteases within these structures (48). In this study, we did not verify whether the cell lines we used formed invadopodia. Nevertheless, our data clearly indicate that MT-LOOP-dependent localization to the adhesion complexes is functionally significant in promoting cellular invasion.

During proMMP-2 activation, the MT-LOOP has been shown to interact with the fibronectin-like domain of proMMP-2, and the impaired activation of proMMP-2 by ΔLOOP mutant was due to loss of this interaction (41), indicating that the MT-LOOP is an interface of molecular interaction. Our data also suggest that the functional defects of the ΔLOOP mutant may result from a loss of MT-LOOP-mediated molecular interactions. This is supported by the effect of LOOPAb, where incubation with LOOPAb fully mimicked the phenotype of ΔLOOP. The addition of LOOPAb did not exceed the levels of inhibition observed for the ΔLOOP mutant, and the antibody did not inhibit the catalytic activity of MT1-MMP against gelatin or collagen. Together, our data suggest that the effect of LOOPAb is attributed to the inhibition of MT-LOOP-dependent molecular interactions on the cell surface. This led us to propose that the MT-LOOP is a potential exosite target region for the development of specific MT1-MMP inhibitors.

MT1-MMP is considered to be a therapeutic target for cancer (2) and rheumatoid arthritis (6). MT1-MMP promotes cancer cell invasion (1) and enables tumor stromal cells to help cancer cell growth (49), angiogenesis (50), invasion of inflammatory cells (51), and cartilage degradation by synovial pannus (6, 52). It is important to inhibit MT1-MMP in a specific manner, but small molecular weight inhibitors targeting the active site of the enzyme are difficult to make specific because 62 human metalloproteinases share similar topology of their active sites. To overcome this challenge, different attempts have been made to create selective inhibitors. One such attempts was to make an inhibitory antibody. It has been shown that DX-2400, a humanized MT1-MMP inhibitory antibody, was highly selective and inhibited cancer cell invasion, metastasis, angiogenesis, and tumor growth in vivo (53). Another strategy to make a specific inhibitor is by targeting the exosite(s) of the molecule. Functions of MT1-MMP on the cell surface require specific molecular interactions, including homodimerization through the Hpx domain for proMMP-2 activation and collagen degradation (26, 36) and interaction with TIMP-2 for proMMP-2 activation (54). Recently a small molecular weight compound was identified as an MT1-MMP dimerization inhibitor and was shown to significantly inhibit tumor growth in an animal model (55). Another example was a monoclonal antibody that prevents TIMP-2 binding. The 9E8 clone of this monoclonal antibody inhibited proMMP-2 activation but not collagen degradation (56), and such a function-specific inhibitor may avoid potential side effects. In this work, we showed that an antibody to the MT-LOOP effectively inhibited cancer cell invasion. Inhibition of MT-LOOP-dependent regulation could be developed as a realistic strategy to inhibit MT1-MMP-related diseases, including cancer and arthritis, in the future.

The MT-LOOP is a protruding loop structure in the catalytic domain of MT1-MMP and is located outside of the TIMP-2 interaction interface (57). This relatively distant location from the catalytic site was presumably the reason why MT-LOOP deletion or binding of LOOPAb did not affect the catalytic activity of the enzyme. Because the MT-LOOP bulges out from the surface of the molecule, this region may be readily accessible for molecular interactions with a partner molecule(s) on the cell surface. Among 23 MMPs, only transmembrane-type MT-MMPs (MT1-, MT2-, MT3-, and MT5-MMP) have the MT-LOOP insertion. The sequence of these loops differs among MT-MMPs, except that the Pro-Tyr at their N terminus are conserved (Fig. 5D). It is thus possible that each loop may interact with different proteins, which would determine the different biological functions of each MT-MMP. At present, it is not known which molecules bind to the MT-LOOP of MT1-MMP and mediate the localization of the enzyme to cell adhesion complexes. Identification of these molecules would provide a novel insight into the mechanism of MT-LOOP-dependent localization and cellular invasiveness in the future.

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