Molecular Biology of the Cell
Vol. 17, 5390–5399, December 2006

Cell Surface Collagenolysis Requires Homodimerization of the Membrane-bound Collagenase MT1-MMP

Yoshifumi Itoh,* Noriko Ito,* Hideaki Nagase,* Richard D. Evans,* Sarah A. Bird,* and Motoharu Seiki†

*Department of Matrix Biology, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, London W6 8LH, United Kingdom; and †Division of Cancer Cell Research, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan

Submitted August 23, 2006; Revised September 22, 2006; Accepted October 5, 2006
Monitoring Editor: Jean Schwarzbauer

Pericellular degradation of interstitial collagens is a crucial event for cells to migrate through the dense connective tissue matrices, where collagens exist as insoluble fibers. A key proteinase that participates in this process is considered to be membrane-type 1 matrix metalloproteinase (MT1-MMP or MMP-14), but little is known about the mechanism by which it cleaves the insoluble collagen. Here we report that homodimerization of MT1-MMP through its hemopexin (Hpx) domain is essential for cleaving type I collagen fibers at the cell surface. When dimerization was blocked by coexpressing either a membrane-bound or a soluble form of the Hpx domain, cell surface collagenolytic activity was inhibited in a dose-dependent manner. When MMP-13, a soluble collagenase active as a monomer in solution, was expressed as a membrane-anchored form on the cell surface, homodimerization was also required to cleave collagen. Our results introduce a new concept in that pericellular collagenolysis is regulated by correct molecular assembly of the membrane-anchored collagenase, thereby governing the directionality of the cell to migrate in tissue.

INTRODUCTION

Collagens are the most abundant extracellular matrix (ECM) component in the body. It consists of three polypeptide chains forming a triple-helical conformation (Ramachandran and Kartha, 1955). The interstitial collagens types I, II, and III further polymerize to form fibrils that serves as stabilizing scaffolds in ECM. During tissue remodelling, collagen degradation is an essential process in that the collagen is a structural frame work of tissues and a physical barrier for migrating cells (Cawston, 1996; Sternlicht and Werb, 2001; Visse and Nagase, 2003). Because of its triple helical structure, collagens are resistant to most proteinases at neutral pH. However, collagenases belonging to the matrix metalloproteinase (MMP) family can initiate degradation of triple helical collagen cleaving a single site about ¾ away from the N-termini.

Among the 23 members of human MMPs, there are at least five collagenases including MMP-1 (collagenase I), MMP-8 (collagenase 2), MMP-13 (collagenase 3), MMP-2 (gelatinase A), and MMP-14 (membrane type-1 MMP, MT1-MMP; Visse and Nagase, 2003). These MMPs consist of a propeptide, a catalytic domain, a hinge (or linker) region, and a hemopexin (Hpx) domain. In addition, MMP-2 has three repeats of fibronectin type II modules inserted in the catalytic domain, and MT1-MMP has a transmembrane and cytoplasmic domains at the C-terminus. The catalytic domain of these MMPs alone can cleave peptides or noncollagenous proteins, but collagenolytic activity requires the Hpx domain (Clark and Cawston, 1989; Murphy *et al.*, 1992; Knäuper *et al.*, 1993; Ohuchi *et al.*, 1997; Patterson *et al.*, 2001). The mechanism of action as to how collagenase cleave triple helical collagens together with the Hpx is not clear because the crystal structures of collagenases (Bode *et al.*, 1994; Borkakoti *et al.*, 1994) have indicated that the binding site of the catalytic domain is too narrow to accommodate the triple helical structure. Recent studies of Chung *et al.* (2004) demonstrated that collagenases interact with collagen and locally unwind the triple helical structure before they hydrolyze the peptide bonds of the three polypeptides chains. This action occurs cooperatively with the catalytic domain and the Hpx domain together. MMP-1, -2, -8, and -13 are secreted from the cells as soluble inactive zymogens (proMMPs) which will be activated in the tissue. Thus most collagenase studies were conducted with both collagen and proteinases in solution. However, MT1-MMP is a unique collagenase in that is activated intracellularly and anchored on the cell surface.

MT1-MMP is the only membrane-anchored collagenase. It is involved in many physiological and pathological events such as wound healing (Okada *et al.*, 1997), angiogenesis (Hiraoka *et al.*, 1998; Zhou *et al.*, 2000; Chun *et al.*, 2004), bone development (Holmbeck *et al.*, 1999), and cancer cell growth (Hotary *et al.*, 2003), invasion and metastasis (Sato *et al.*, 1994; Seiki, 1999). Besides cleaving collagens types I, II, and III, MT1-MMP degrades a range of extracellular macromolecules including fibronectin, laminin 1 and 5, vitronectin, fibrin, and aggrecan (d'Ortho *et al.*, 1997; Ohuchi *et al.*, 1997; Koshikawa *et al.*, 2000). Cell surface proteins such as trans-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–08–0740) on October 18, 2006.

Address correspondence to: Yoshifumi Itoh (y.itoh@imperial.ac.uk).

Abbreviations used: Hpx, hemopexin domain; MDCK, Madin-Darby canine kidney epithelial cells; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; NGFR, nerve growth factor receptor; TIMP, tissue inhibitor of metalloproteinase; PY, phosphotyrosine.

glutaminase (Belkin *et al.*, 2001), integrin α_V (Ratnikov *et al.*, 2002), CD44 (Kajita *et al.*, 2001), and syndecan 1 (Endo *et al.*, 2003). It also activates proMMP-2 (progelatinase A; Sato *et al.*, 1994) and proMMP-13 (procollagenase 3) on the cell surface and enhances pericellular matrix proteolysis. (Knäuper *et al.*, 1996). Although MT1-MMP has a wide variety of functions, its collagenolytic activity appears to be one of the most important functions in vivo. Deletion of the MT1-MMP gene in mice revealed a severe phenotype in bone development that was concluded to be due to inadequate collagen turnover (Holmbeck *et al.*, 1999). Its collagenase activity is also essential for branching morphogenesis of Madin-Darby canine kidney (MDCK) cells in a collagen gel (Kadono *et al.*, 1998), for cancer cells to grow within a three-dimensional (3D) collagen matrix (Hotary *et al.*, 2003), and for neovessel formation in a collagen-based matrix (Chun *et al.*, 2004).

We and others have previously shown that MT1-MMP forms a homodimer through its Hpx domain (Itoh *et al.*, 2001; Lehti *et al.*, 2002) and that this is essential for the activation of proMMP-2 on the cell surface. One of the two MT1-MMP molecules binds to its endogenous inhibitor, TIMP-2 to form an MT1-MMP-TIMP-2-proMMP-2 complex and the other MT1-MMP activates the proMMP-2 (Itoh and Seiki, 2006). MT1-MMP dimers form higher order complexes, but the functional significance of the oligomer is not known (Itoh *et al.*, 2001; Lehti *et al.*, 2002).

In the present work, we first addressed whether the homodimer formation of MT1-MMP on the cell surface is required for expression of its collagenolytic activity. This requirement for collagen degradation on the cell surface was also tested for MMP-13, a soluble collagenase, by expressing it as a membrane-bound form. Our data shows that dimer formation of membrane-anchored collagenases is essential to cleave insoluble collagen. Such molecular assembly may help to locate collagenolytic activity at the correct site of the cell to interact with collagen fibrils during cell migration in the tissue.

MATERIALS AND METHODS

Cell Culture and Transfection

COS7, HT1080, and MDCK cells were cultured in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% FBS and penicillin/streptomycin (BioWhittaker). For transfection, cells were cultured in six-well plates and were transfected with expression plasmids using FUGENE6 (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Antibodies

Mouse anti-FLAG M2 antibody and alkaline phosphatase–conjugated anti-(mouse IgG) antibodies were purchased from Sigma-Aldrich (Dorset, United Kingdom). Mouse anti-(phospho-tyrosine) antibody (PY-20) was purchased from ICN Biochemicals (Costa Mesa, CA), Alexa-488– and Alexa-568–conjugated anti-mouse and anti-(rabbit IgG) antibodies were from Molecular Probes (Cambridge, United Kingdom). Anti-human MT1-MMP catalytic domain antibody was generated in rabbit as described previously (Itoh *et al.*, 2001).

Construction of MT1-MMP Mutants

FLAG (DYKDDDDK)-tagged MT1-MMP (MT1F) was constructed as previously described (Itoh *et al.*, 1999) and subcloned into pSG5 (Stratagene, La Jolla, CA). A FLAG tag was inserted at the end of the propeptide (between Arg^{111} and Tyr^{112}), and properly activated enzyme will have the FLAG tag at its N-terminus and thus can be recognized by anti-FLAG M1 antibody (Itoh *et al.*, 1999). MT1F-ΔCat is a FLAG-tagged mutant MT1-MMP in which the region of Tyr^{112} to Pro^{312} was deleted. MT1F-ΔCatΔTM is a FLAG-tagged mutant MT1-MMP in which the region of Tyr^{112} to Gly^{288} and also Ala^{536} to Val^{582} were deleted. MT1-ΔHpx is a Hpx domain deletion mutant of MT1-MMP without a FLAG tag where Phe^{336}-Gly^{535} was deleted. MT1-ΔCat is the same as MT1F-ΔCat but does not have a FLAG tag. MT13F is a chimera mutant of human MMP-13 and MT1-F in which Tyr^{112} to Cys^{508} in MT1F was replaced with Tyr^{104} to Cys^{471} of MMP-13. MT13F-HPXMT1 is a chimera mutant in which Tyr^{112} to Gly^{284} in MT1-F was replaced with Tyr^{104} to Cys^{471} of MMP-13. These mutants were generated by the PCR extension method as described by Ho *et al.* (1989). Chimera mutants of the ectodomain of MT1-F, MT13-F, and MT13F-HPXMT1 and transmembrane/cytoplasmic domain of NGFR (MT1-F/NGFR, MT13F/NGFR, and MT13F-HPXMT1/NGFR, respectively) were also generated by PCR and subcloned into pSG5. The mutant is derived from sequences corresponding to Met^{1} to Asp^{515} of MT1-MMP and Glu^{384} to Gly^{790} of NGFR. The other chimera mutants were also generated at the corresponding sites. All the PCR-generated fragments were confirmed by DNA sequencing and subcloned into the pSG5 vector. MT1F-ΔCat and MT1F-ΔCatΔTM inserts were also subcloned into pCEP4 vector (Invitrogen, Paisley, United Kingdom) to establish stable MDCK cell lines.

Western Blotting and Zymography

Western blotting was carried out as described previously (Itoh *et al.*, 1998). Total cell lysates were prepared by addition of 1× SDS-PAGE loading buffer containing 2-mercaptethanol to cells in the culture plate and subsequent boiling for 20 min. Zymography was carried out as described previously (Itoh *et al.*, 1999).

In Situ Collagen Degradation Assay

Six-well culture plates were coated with a thin layer of chilled neutralized Vitrogen collagen (Nutacon, Leimuiden, Netherland) at 2.7 mg/ml in 1× RPMI medium (typically 100 μl/well) and incubated for 60 min at 37°C for fiber formation, and COS7 cells (4×10^5/well) were then seeded on the film. Eighteen hours later, cells were transfected with the expression plasmids in the growth medium (10% FBS/DMEM) using FUGENE6 according to the manufacturer's instruction. The following day, culture medium was changed to serum-free DMEM, and cells were cultured for a further 3 d at 37°C. The remaining collagen film was exposed by removing cells using repeated treatment with PBS containing 0.5 mg/ml trypsin and 1 mM EDTA. The collagen film was then fixed with 3% paraformaldehyde for 20 min at room temperature (RT). Collagen was visualized by staining with Coomassie brilliant blue R250, and the images were captured by a CCD camera-equipped microscope (Nikon TE-2000; Melville, NY) with a 20× objective lens. Degraded areas were visualized as a white, unstained, noncollagen-containing zone. In this assay, stained collagen was trypsin-resistant, suggesting that they were intact fibrillar collagen.

In Situ Gelatin Degradation Assay

Four-well chamber slides (Nunc, Napierville, IL) were coated with Alexa-488–conjugated gelatin (F-gelatin) prepared with an Alexa-488–labeling kit (Molecular Probes) as described previously (Itoh *et al.*, 2001). Transfected COS7 cells were cultured in the chamber slides for 16 h. Cells were then fixed with 3% paraformaldehyde in PBS and analyzed using Ultraview confocal microscopy (PerkinElmer Life and Analytical Sciences, Monza, Italy). The degraded area was visualized as a dark, nonfluorescent zone.

Expression and Purification of Recombinant MT1-MMP Hemopexin Domain

The cDNA fragment encoding Asn317-Gly511 was generated by PCR. The sequence encoding Met was included in the 5'end and 6xHis at 3'end, followed by a termination codon. The sequence of the PCR fragment was confirmed by DNA sequencing and subcloned into the pET3a *Escherichia coli* expression vector (Stratagene). BL21(DE3)pLysS cells (Stratagene) were transformed with the constructs, and protein expression was induced by 0.4 mM IPTG. Proteins were purified from inclusion bodies and folded as described previously (Itoh *et al.*, 2001) according to the method of Huang *et al.* (1996). Formation of disulfide bonds was confirmed by subjecting the purified protein to SDS-PAGE under reducing and nonreducing conditions.

Indirect Immunofluorescence Staining

To localize cell surface MT1-F, MT13-F, or MT13F-HPXMT1, transfected COS7 cells cultured on four-well Permanox slide chambers (Nalge Nunc) coated with collagen film or four-well glass slide chambers (Nalge Nunc) coated with F-gelatin were fixed with 3% paraformaldehyde in PBS. After blocking with 5% goat serum and 3% bovine serum albumin in TBS for 1 h at RT, cells were incubated with an anti-FLAG M1 antibody (5 μg/ml) at RT for 2 h without permeabilizing cells. $CaCl_2$, 1 mM, was included throughout the procedure of washing and incubation for the staining with the anti-FLAG M1 antibody. Alexa-488–conjugated goat anti-mouse IgG was used to visualize the antigen signal. Note that anti-FLAG M1 antibody can only recognize FLAG tag at the N-terminus of molecule (Itoh *et al.*, 1999); thus only active forms of the enzyme can be stained with this procedure. The signals were analyzed by Ultraview confocal microscopy (PerkinElmer).

Immunoprecipitation

Transfected COS7 cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 1% deoxycolate, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NaN_3) containing protease inhibitor cocktail (Sigma) and 10 μM GM6001. Three 35-mm dishes per constructs were used. FLAG-tagged enzymes were immu-

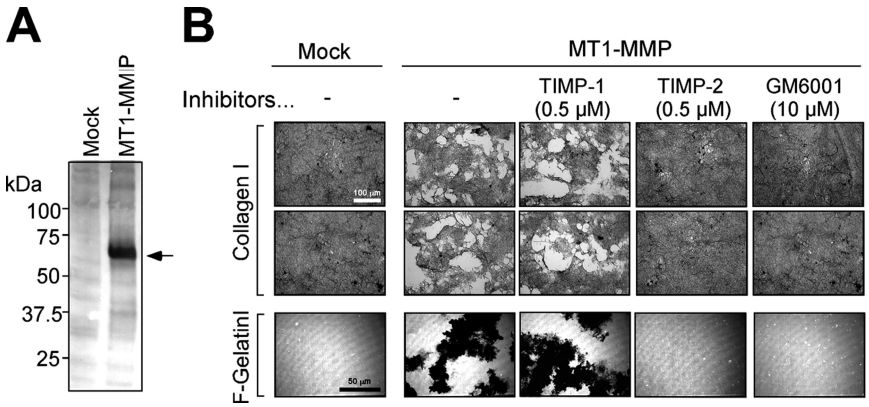

Figure 1. Degradation of solid-phase collagen by membrane-bound MT1-MMP. (A) COS7 cells were transfected with an expression plasmid for wild-type human MT1-MMP or empty vector (Mock), and the expression of MT1-MMP was analyzed in cell lysates by Western blotting using anti-human MT1-MMP catalytic domain antibody. (B) In situ solid-phase collagen degradation assay (top panel, collagen I) and solid-phase gelatinase assay (bottom panel, F-gelatin) were carried out as described in *Materials and Methods.* Cells were cultured in the presence or absence of TIMP-1 (0.5 μM), TIMP-2 (0.5 μM), or GM6001 (10 μM) for 3 d, and collagenolysis was detected as clear zones and gelatinolysis as dark zones. Bars, 100 μm (top) and 50 μm (bottom).

noprecipitated from the lysate using anti-FLAG M2-conjugated agarose beads (Sigma, 100 μl, 50% gel suspension) by reacting at 4°C for 2 h with gentle rotation. Enzymes bound to the beads were eluted by FLAG peptide (Sigma) in TBS (200 μg/ml, 100 μl) without any inhibitors.

3D Culture of MDCK Cells for Branching Tube Formation

MDCK cells were transfected with pCEP4 constructs, and transfectants were selected by treatment of 800 μg/ml hygromycin B (PAA Laboratories, Karlsruhe, Germany). A population of hygromycin-resistant cells were trypsinized and suspended in the neutralized type-I collagen (2 mg/ml, Cellmatrix type 1-A, Nitta Gelatin, Osaka, Japan) at 2×10^4/ml. Cells were cultured in the presence or absence of 50 ng/ml hepatocyte growth factor (HGF, PeproTech EC, London, United Kingdom). Bright-field images of the cells were taken by CCD-equipped microscope using 10× objective lens at the 7th day.

RESULTS

Degradation of Solid-Phase Type I Collagen by the Membrane-anchored MT1-MMP

Because MT1-MMP is membrane-anchored, correct orientation of the enzyme against a 3000-Å long collagen molecule to cleave only at one site can be restricted compared with soluble collagenases. We therefore investigated the mechanism that facilitate its collagenolytic activity.

When cultured on a collagen film, cells expressing membrane-anchored MT1-MMP (Figure 1A) degraded solid-phase collagen, producing visible holes in the film (Figure 1B, top panel). The COS7 cells used in this study do not produce proMMP-2 or proMMP-13 that degrade type I collagen when activated by MT1-MMP (data not shown). Thus, this collagenolytic activity is most likely due to MT1-MMP. This was further confirmed by transfection dependency, and inhibition by TIMP-2 (0.5 μM), but not by TIMP-1 (0.5 μM). These cells also degraded fluorescence-labeled gelatin (F-gelatin, Figure 1B, bottom panel) that was also inhibited by TIMP-2 but not by TIMP-1. It was therefore concluded that collagenolytic activity and gelatinolytic detected in this system is a direct action of MT1-MMP.

Dimerization of MT1-MMP on the Cell Surface Is a Prerequisite To Recognize and Cleave Fibril Type I Collagen

Like other collagenases the Hpx domain is also required for MT1-MMP to express collagenolytic activity on the cell surface. Enzyme lacking the Hpx domain did not degrade the collagen film but did degrade F-gelatin (data not shown). The Hpx domain of MT1-MMP also participates in dimerization of the enzyme on the cell surface (Itoh *et al.*, 2001), which is required for proMMP-2 activation. We therefore examined whether the Hpx-dependent dimerization of MT1-MMP is required for the collagenolytic activity. This possibility was tested by coexpressing MT1-MMP and the catalytic domain-deletion mutant of MT1-MMP (MT1F-ΔCat) in COS 7 cells (Figure 2A), which express the Hpx domain on the cell surface. As shown previously (Itoh *et al.*, 2001) overexpression of MT1F-ΔCat on the cell surface effectively inhibited homodimerization of MT1-MMP, as evidenced from inhibition of activation of exogenously added proMMP-2 (Figure 2B; also see Figure 3C below). Under these conditions, the collagenolytic activity of MT1-MMP was also inhibited in MT1F-ΔCat expression–dependent manner (Figure 2C), whereas MT1F-ΔCat did not inhibit general catalytic activity of MT1-MMP or cell surface expression of the full-length MT1-MMP because these cells degraded fluorescence-labeled gelatin (F-gelatin) as effectively as cells expressing the full-length enzyme alone. These were unexpected results because collagenases are thought to act on collagen as a monomer. To further examine if the inhibitory activity of MT1F-ΔCat was truly the Hpx domain–dependent phenomenon, the effect of a soluble Hpx domain construct MT1-MMP (MT1F-ΔCatΔTM) was tested (Figure 3A). As shown in Figure 3B, the expression of MT1F-ΔCatΔTM also inhibited cell surface collagenolysis of MT1-MMP in a dose-dependent manner, although with a lesser efficacy (see Figure 2C for a comparison). As we described previously (Itoh *et al.*, 2001), a chimera consisting of the MT1-MMP ectodomain and the transmembrane/cytoplasmic domain of the nerve growth factor receptor (MT1F/NGFR) allowed us to monitor ectodomain-driven homodimer formation of MT1-MMP by analyzing autophosphorylation at tyrosine residues in the NGFR cytoplasmic domain. As shown in Figure 3C, MT1F/NGFR expressed in COS7 cells was strongly phosphorylated, indicating that the ectodomains of MT1-MMP do form a dimer (lane 1). When MT1F-ΔCatΔTM or MT1F-ΔCat was coexpressed, the level of phosphorylation was decreased in an expression level–dependent manner (lanes 2–7). Calculating relative intensity of the phosphotyrosine (PY) bands by calibrating with relative band intensity of the anti-FLAG bands, MT1F-ΔCatΔTM reduced PY signal by 46% and MT1F-ΔCat by 93% at the highest expression (lanes 4 and 7). These results indicate that both constructs prevent dimerization of MT1-MMP.

The Hpx domain inhibits collagenolysis by interfering with the dimerization of MT1-MMP not by binding to collagen. Tam *et al.* (2002) have reported that MT1-MMP binds to type I collagen through the Hpx domain for collagenolysis and that the soluble Hpx domain inhibits collagenolytic activity of MT1-MMP by competing for its binding to collagen. We therefore considered whether inhibition of collag-

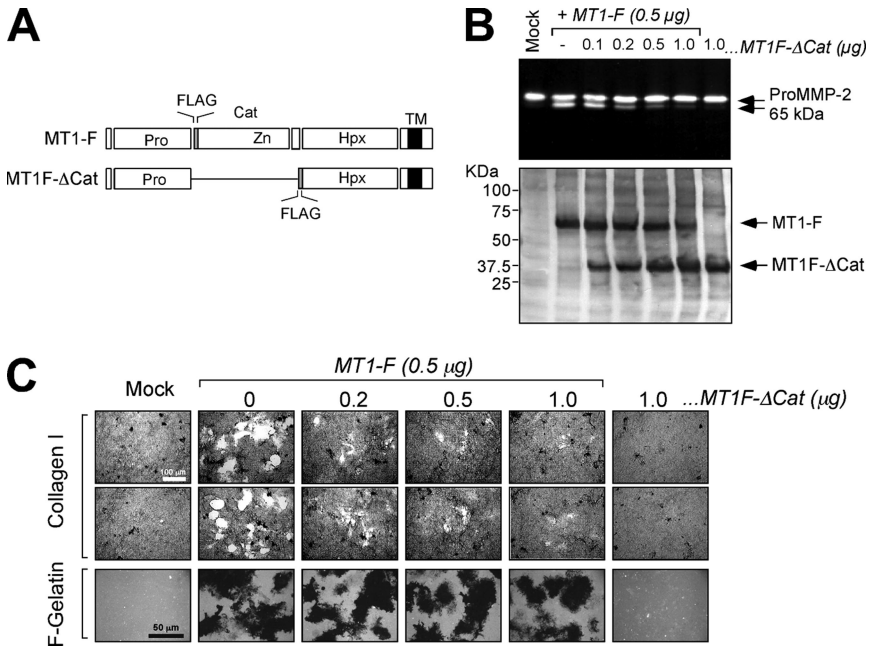

Figure 2. Effect of MT1F-ΔCat expression on cell surface collagenolytic activity of MT1-MMP. (A) Schematic representation of the constructs used in this experiment. Pro, propeptide; FLAG, FLAG tag (DYKDDDDK); Cat, catalytic domain; Hpx, hemopexin domain; TM, transmembrane domain; and Zn^{2+}, catalytic zinc ion. (B) COS 7 cells were transfected with expression plasmids for MT1-MMP-F and/or MT1F-ΔCat as indicated. Cells were then reacted with proMMP-2 in the serum-free culture medium for 18 h. ProMMP-2 activation in the media was analyzed by zymography (top panel), and cell lysates were analyzed for the expression of the proteins by Western blotting using anti-FLAG M2 antibody (bottom panel). (C) In situ solid-phase collagen degradation assay (top panel, collagen I) and solid-phase gelatinase assay (bottom panel, F-gelatin) were carried out using transfected COS7 cells as indicated. Bars, 100 μm (top) and 50 μm (bottom).

enolysis by the MT1F-ΔCatΔTM can be due to its direct binding to collagen. To test this, COS7 cells expressing MT1F-ΔCatΔTM mutant were cultured either on plastic or on a collagen film, and the level of the Hpx domain released into the culture medium from these cells was compared. If soluble Hpx domain has significant affinity to collagen to be able to inhibit MT1-MMP-collagen interaction, MT1F-ΔCatΔTM should be deposited to collagen matrix elsewhere on the bottom of culture, which would result in decreased levels in the culture medium when cells are on collagen

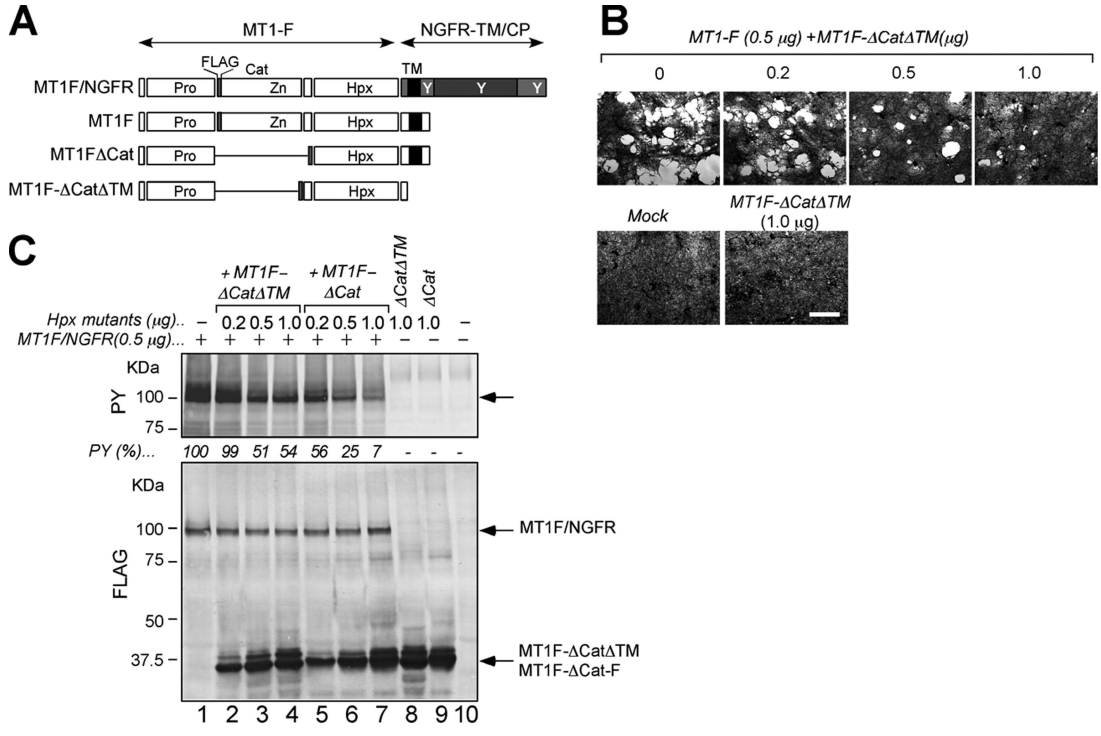

Figure 3. Effect of soluble Hpx domain expression on cell surface collagenolytic activity of MT1-MMP. (A) Schematic representation of the constructs used in this experiment. NGFR-TM/CP, transmembrane and cytoplasmic domain of nerve growth factor receptor; 6xHis, His-tag. (B) In situ solid-phase collagen degradation assay was carried out using transfected COS7 cells as indicated. Bar, 100 μm. (C) COS7 cells were transfected with expression plasmids for MT1F/NGFR, MT1F-ΔCat, and/or MT1F-ΔCatΔTM at the amount indicated. Cells were lysed and subjected to Western blotting analyses using anti-phosphor-tyrosine antibody (PY, top panel) and anti-FLAG M2 antibody (FLAG, bottom panel).

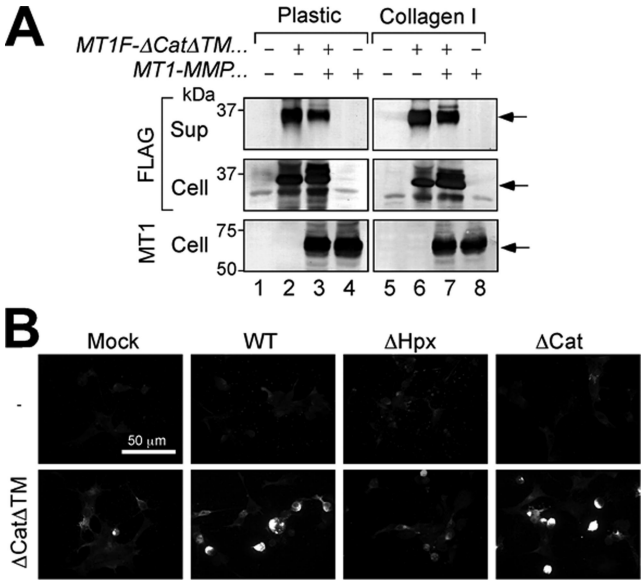

Figure 4. Soluble Hpx domain does not bind to solid-phase collagen I. (A) COS7 cells expressing MT1F-ΔCatΔTM and/or nontagged MT1-MMP were cultured either on plastic or collagen. Culture supernatants (Sup) and cell lysates (Cell) were then subjected to Western blotting analysis using anti-FLAG M2 antibody to detect the Hpx domain (FLAG, Sup and Cell) or anti-MT1-Cat antibody to detect full-length wild-type MT1-MMP (MT1, Cell). (B) COS7 cells were transfected with MT1F-ΔCatΔTM in the absence and presence of nontagged wild-type MT1-MMP (WT), Hpx domain–deleted MT1-MMP (ΔHpx), or catalytic domain–deleted MT1-MMP (ΔCat) were cultured on collagen film. Cells were fixed and stained with anti-FLAG M2 antibody without permeabilization. Bar, 50 μm.

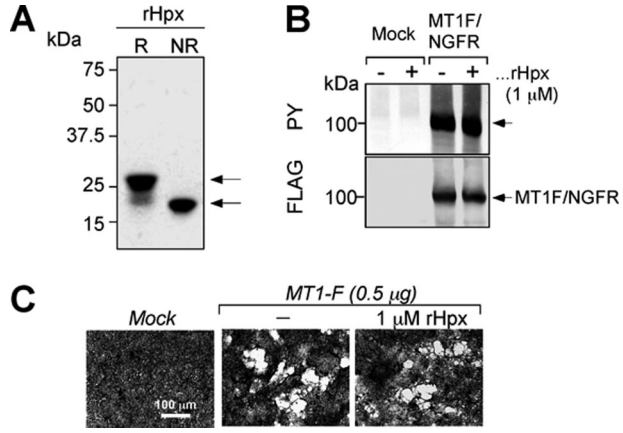

Figure 5. Exogenously added recombinant Hpx domain does not inhibit dimer formation and collagen degradation on the cell surface. (A) Purified recombinant Hpx domain (rHpx, 250 μg/ml) was analyzed by SDS-PAGE under reducing (R) and nonreducing (NR) conditions. Protein was stained with Coomassie brilliant blue R250. Note the shift of electrophoretic mobility upon reduction of the sample, indicating the disulfide bond was correctly formed. (B) Effect of rHpx on dimerization of MT1-MMP. COS7 cells were transfected with MT1F/NGFR plasmid or empty vector. After 24 h cells were treated with or without rHpx at 1 μM and were further cultured for 18 h. Cell lysates were then subjected to Western blotting analysis using anti-phosphor-tyrosine (PY-20, top) and anti-FLAG M2 (FLAG, bottom). (C) Effect of rHpx on cell surface collagenolytic activity. rHpx was added to culture medium of COS7 cells transfected with MT1-F plasmid at 1 μM during in situ collagenase assay of 4 d. Bar, 100 μm.

matrix. As shown in Figure 4A, similar levels of MT1F-ΔCatΔTM were found in the culture medium from cells cultured on plastic and collagen I (lanes 2 and 6, FLAG-Sup), suggesting that the soluble Hpx domain does not bind to the collagen film under these conditions. Coexpression of wild-type MT1-MMP (without a FLAG-tag) decreased the level of MT1F-ΔCatΔTM secreted into the culture medium and increased the level in the cell lysates (Figure 4A, lanes 3 and 7, FLAG-Sup and Cell). To further test whether there is any binding of the Hpx domain to the collagen matrix, MT1F-ΔCatΔTM was immunolocalized on cells cultured on a type I collagen gel without permeabilization. As shown in Figure 4B, a low level of the signal was found on the cell surface transfected with MT1F-ΔCatΔTM, but no signal was detected on the collagen matrix. When wild-type MT1-MMP was coexpressed with MT1F-ΔCatΔTM, the level of cell surface-associated soluble Hpx domain (MT1F-ΔCatΔTM) was significantly increased, but no signal was detected on collagen film (Figure 4B). A similar effect was also observed when the catalytic domain deletion mutant without a FLAG-tag (MT1-ΔCat) was coexpressed, but not with the Hpx domain deletion mutant (MT1-ΔHpx, Figure 4B), suggesting that soluble Hpx domain was bound to cell surface when the membrane-bound MT1-MMP or Hpx domain (MT1-ΔCat) is present, by interacting with its Hpx domain. These results indicate that soluble Hpx domain inhibits collagenolysis by preventing dimerization of MT1-MMPs through their Hpx domains on the cell surface.

We also examined the possibility that soluble Hpx domain fragments can inhibit collagenolytic activity on the cell surface. For this purpose, we utilized *E. coli*–expressed recombinant Hpx domain (rHpx). The rHpx correctly formed a disulfide bond as evidenced from the difference in electrophoretic mobility under reducing (25 kDa) and nonreducing conditions (21 kDa; Figure 5A, left panel). The purified protein eluted from gel-permeation chromatography at 32 kDa, whereas similarly expressed recombinant Hpx domain of MMP-1 eluted around 17 kDa (data not shown). Because the MMP-1 Hpx domain showed a molecular weight on SDS-PAGE (24 and 20 kDa under reducing and nonreducing conditions, respectively; data not shown) that was similar to the rHpx of MT1-MMP, it was concluded that the rHpx of MT1-MMP is a dimer in solution. Addition of this rHpx to the culture at 1 μM could not inhibit homodimer formation of MT1-MMP, as monitored by MT1-F/NGFR (Figure 5B). This is most likely due to the fact that rHpx is already in dimer, and it is thus difficult to exchange with the dimer partner (Itoh *et al.*, 2001). Under these conditions, rHpx failed to inhibit collagenolytic activity of MT1-MMP on the cell surface (Figure 5C). Therefore, to inhibit dimerization and collagenolysis the Hpx domain needs to be coexpressed with the full-length enzyme in the cell where hetero complex of MT1-MMP and the Hpx domain can form during secretion before they form homo-dimers.

Degradation of Solid-Phase Collagens by Cell Surface–expressed Collagenase 3 (MMP-13) Requires Dimerization of the Enzyme

To examine whether or not the requirement of dimerization for cell surface collagenolysis is a unique feature of the membrane-bound MT1-MMP, we constructed two chimeras of collagenase 3 (MMP-13) and MT1-MMP: one chimera consists of the full-length MMP-13 and the TM and cytoplasmic domains of MT1-MMP and the other contains the Hpx of MT1-MMP after full-length MMP-13 (for nomencla-

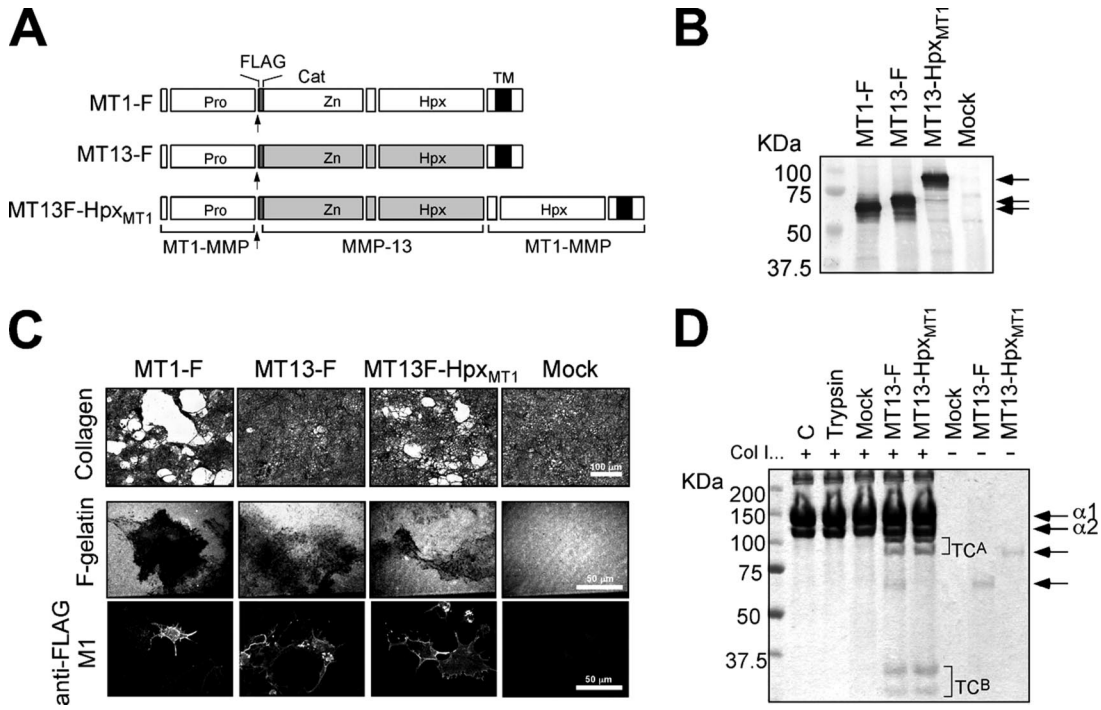

Figure 6. Cell surface-anchored MMP-13 also requires dimerization to digest solid-phase collagen. (A) Schematic representation of the constructs used in this experiment. (B) Expression of recombinant proteins in the lysate of COS7 cells transfected with expression plasmids for MT1F, MT13-F, MT13-Hpx$_{MT1}$, and empty vector (Mock). (C) Transfected COS7 cells were subjected to the in situ solid-phase collagen degradation assay (Collagen, top panel) and the solid-phase gelatinase assay (F-gelatin, middle). The cells on F-gelatin were further stained with anti-FLAG M1 antibody without permeabilization. Bars, 100 μm (top) and 50 μm (bottom). (D) MT13-F and MT13-Hpx$_{MT1}$ expressed in COS7 cells were partially purified by immunoprecipitation as described in *Materials and Methods.* The enzymes were reacted with type I collagen in solution (Col I, 1 mg/ml) at 20°C for 18 h. As a negative control, collagen was also reacted with trypsin at 1 μg/ml. Note that collagen was cleaved by both enzymes and generated [3/4] and [1/4] fragments (TC^A and TC^B, respectively).

ture of the constructs see Figure 6A). These chimeras contained the propeptide of MT1-MMP including RRKR ^{111} followed by a FLAG tag so that the chimeric enzyme can be processed intracellularly to an active form by furin or a related protein convertase and so that the activated enzyme can be detected by the anti-FLAG antibodies M1 and M2. The chimera MT13F consisting of full-length MMP-13, and the TM and cytoplasmic domain of MT1-MMP is expected to be monomeric on the cell surface, whereas the chimera MT13F-Hpx$_{MT1}$ containing the Hpx domain of MT1-MMP at the C-terminus of MMP-13 is expected to be dimeric. Transfection of these chimeras and MT1F into COS7 cells resulted in expression levels similar to those shown in Figure 6B. When these cells were cultured on type I collagen film, significant degradation of the film was observed with the cells expressing MT1F and MT13F-Hpx$_{MT1}$, whereas little or no degradation was found with MT13-F (Figure 6C, top panel). Both MT13F-Hpx$_{MT1}$ and MT13F were processed to an active form because they degraded F-gelatin on the cell surface to a similar extent (Figure 6C, middle panel). The enzyme on the cell surface was also recognized by anti-FLAG M1 antibody (Figure 6C, bottom panel). The M1 antibody only binds to the FLAG tag located at the N-terminus of the molecule and thus can only recognize the enzyme processed at the correct site upon activation (immediately downstream of RRKR^{111}), indicating that both chimeras were similarly activated during secretion. Lack of collagenolytic activity of MT13F on the cell surface was not due to loss of intrinsic collagenolytic activity, because partially purified MT13F and MT13F-Hpx$_{MT1}$ expressed in COS7 cells degraded type I collagen into typical [3/4] and [1/4] fragments to a similar extent in a test tube (Figure 6D), indicating that both chimeras were equally active collagenolytic enzymes in solution.

To obtain further evidence of homodimerization of MT13F-Hpx$_{MT1}$, ectodomains of the two chimeras were fused with the transmembrane and cytoplasmic domain derived from NGFR (see the constructs in Figure 7A) to monitor ectodomain-driven complex formation as described in Figure 3. All the mutants were expressed in COS7 cells as detected by anti-FLAG M2 antibody (Figure 7B, bottom panel). When the same samples were analyzed with an anti-PY antibody, the strongest signal was detected with MT1F/NGFR. MT13F-Hpx$_{MT1}$/NGFR showed a relatively strong PY band, and the diffused weaker signal was detected with MT13F/NGFR (Figure 7B, top panel). Relative intensity of phosphorylated bands was calculated by calibrating with relative intensity of the bands detected with anti-FLAG antibody. The data indicate that MT13F-Hpx$_{MT1}$/NGFR shows about three times more PY signal than the one derived from MT13F/NGFR. These results confer that MT13-F does not form homodimers effectively, but that an addition of the Hpx domain of MT1-MMP to MMP-13 facilitates dimerization of the chimera. When MT13F-Hpx$_{MT1}$/NGFR or MT1F/NGFR was coexpressed with MT1F-ΔCat, the signal of phosphotyrosine was reduced in a manner dependent on the level of MT1F-ΔCat expression (Figure 7C). Furthermore, collagen degradation by both MT1-F and MT13F-Hpx$_{MT1}$ were effectively inhibited by the coexpression of MT1F-ΔCat and MT1F-ΔCatΔTM (Figure 7D), suggesting that dimerization through the Hpx domain of MT1-MMP is essential for MT13-Hpx$_{MT1}$ to degrade collagen on the cell surface.

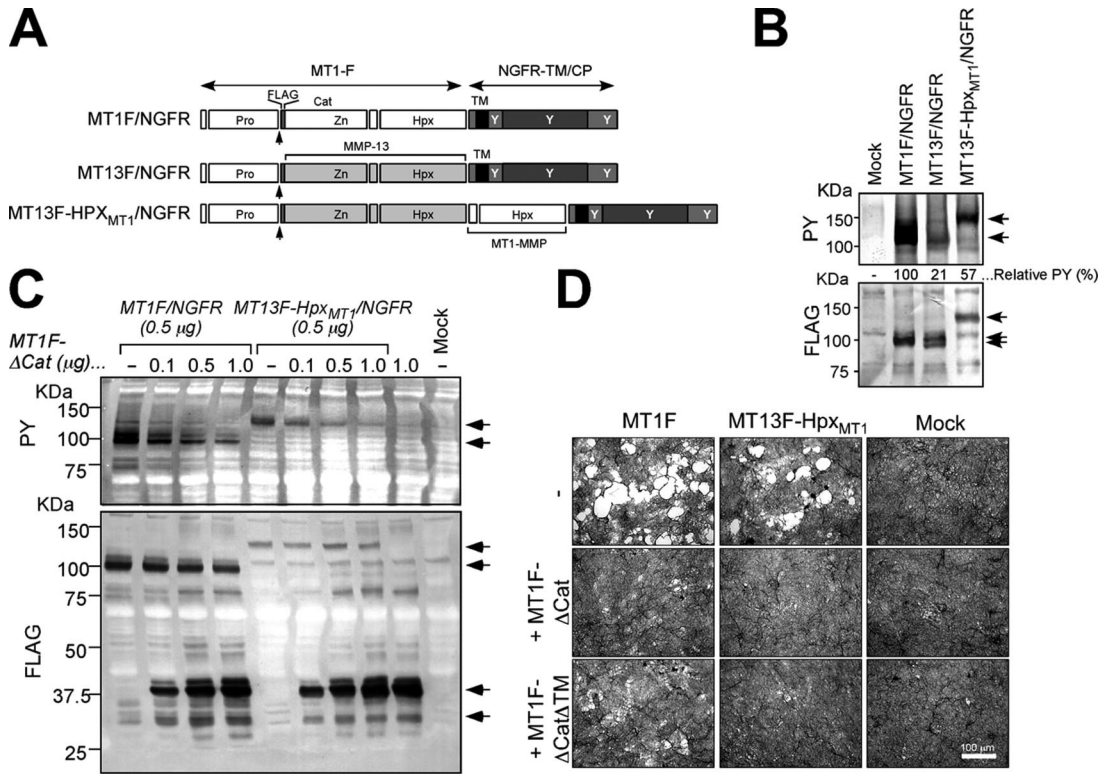

Figure 7. Homodimerization is necessary for cell-surface–tethered MMP-13 to degrade collagen on the cell surface. (A) Schematic representation of the constructs used in this experiment. (B) COS7 cells transfected with expression plasmids for MT1F/NGFR, MT13F/NGFR, MT13Hpx$_{MT1}$/NGFR, and empty vector were analyzed by Western blot using anti-phophotyrosine antibody (PY, top panel) and anti-FLAG M2 antibody (FLAG, bottom panel). The relative intensity of the bands detected by PY20 was analyzed by ImageJ, normalized by the relative intensity of the bands detected by anti-FLAG M2 antibody for each construct, as indicated. (C) COS7 cells transfected with expression plasmids for MT1F/NGFR and MT13Hpx$_{MT1}$/NGFR with or without the plasmid for MT1F-ΔCat were analyzed by Western blot using anti-phophotyrosine antibody (PY, top panel) and anti-FLAG M2 antibody (FLAG, bottom panel). (D) Transfected cells were subjected to in situ solid-phase collagenase assay. Bar, 100 μm.

Effect of MT1F-ΔCat and MT1F-ΔCatΔTM in Tube Formation of MDCK Cells in 3D Collagen Gels

We next tested whether endogenously expressed MT1-MMP also requires homodimerization to degrade collagen on the cell surface. For this purpose, we utilized an MDCK branching tubulogenesis model in 3D collagen gel because collagenolytic activity of MT1-MMP is essential in this process (Kadono *et al.*, 1998; Hotary *et al.*, 2000). MDCK cells stably expressing MT1F-ΔCat or MT1F-ΔCatΔTM (see Figure 8A) were tested for the ability to form branching tube structures. As shown in Figure 8B, mock cells form tube structures upon stimulation with hepatocyte growth factor (HGF). Formation of such structures was inhibited by addition of TIMP-2 (0.5 μM) or GM6001 (10 μM), but not with TIMP-1 (0.5 μM) as reported previously (data not shown), confirming that the process is MT1-MMP-dependent. When MT1F-ΔCat expressing cells were cultured in the presence of HGF, none of the cells were able to extend structures into collagen (Figure 8B, MT1F-ΔCat, +HGF). The effect of MT1F-ΔCatΔTM expression was not as strong as MT1F-ΔCat: some populations of the cells were completely blocked for tube formation, but some were still able to scatter around, al-

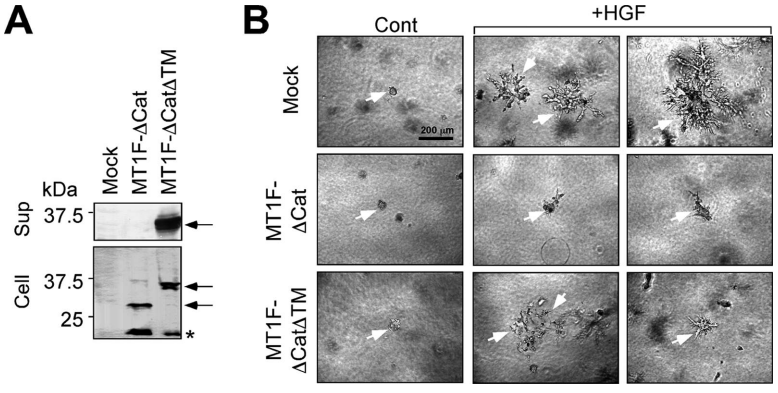

Figure 8. Expression of MT1F-ΔCat and MT1F-ΔCatΔTM inhibits tubule formation of MDCK cells in type I collagen gel. (A) MDCK cells stably transfected with the plasmids for MT1F-ΔCat, MT1F-ΔCatΔTM, or empty vector were analyzed by Western blot using anti-FLAG M2 antibody. Sup, culture supernatant accumulated for 24 h; Cell, cell lysates. Arrows indicate the bands of recombinant proteins. *Degradation products. (B) Transfected MDCK cells were cultured in type I collagen gel (2 mg/ml) with or without hepatocyte growth factor (50 ng/ml). Bright field images were captured on the 7th day of the culture. Bar, 200 μm.

though they did not form tube structures (Figure 8B, MT1F-ΔCatΔTM, +HGF). This may be due to the different expression level of each population of the cells and corresponds to the level of inhibition of collagenolytic activity on the cell surface (Figure 3). These results indicate that both MT1F-ΔCat and MT1F-ΔCatΔTM inhibit collagenolytic activity of endogenous MT1-MMP in MDCK cells and that dimer formation of MT1-MMP is crucial for the degradation of collagen gels during tube morphogenesis.

DISCUSSION

Collagen degradation by collagenases involves at least three steps: 1) binding of the collagenase to collagen; 2) unwinding of the triple-helical structure; and 3) cleavage of peptide bonds. A recent study by Chung *et al.* (2004) has demonstrated that unwinding of triple-helical collagen occurs locally around the cleavage site. Thus the interaction of the collagen molecule and collagenase for unwinding is expected to occur near or at the cleavage site. In contrast to the collagenolysis in solution, degradation of solid phase collagen on the cell surface by membrane-anchored MT1-MMP is spatially and temporally restricted because the enzyme cannot diffuse into the substrate by itself, and the half-life of the enzyme is <30 min on the cell surface because it is constantly internalized through clathrin- and caveolae-mediated pathways (Jiang *et al.*, 2001; Uekita *et al.*, 2001; Remacle *et al.*, 2003; Anilkumar *et al.*, 2005). Therefore, cell surface collagenolysis can be inefficient compared with that in solution. Here we have shown that homodimerization of MT1-MMP is one of the mechanisms that facilitates collagen degradation on the cell surface.

When dimerization of MT1-MMP was inhibited by coexpression of MT1F-ΔCat, cell surface–associated collagen degradation was effectively inhibited. Expression of MT1F-ΔCat does not inhibit its catalytic activity or cell surface expression of the full-length enzyme as observed with F-gelatin degradation (Figure 2). To inhibit dimer formation and collagenolytic activity of MT1-MMP on the cell surface, the Hpx domain does not have to be expressed as a membrane-anchored form (MT1F-ΔCat), because the soluble Hpx domain (MT1F-ΔCatΔTM) could also inhibit it, albeit less efficiently. In the latter case the MT1F-ΔCatΔTM needs to be coexpressed with full-length MT1-MMP. Addition of purified recombinant Hpx domain to the cells (Figure 5) or coculturing cells expressing MT1F-ΔCatΔTM with those expressing MT1F does not inhibit collagen degradation (data not shown). This is most likely because the Hpx domains form tight homodimers during synthesis or in the secretory pathway, and it is difficult to exchange the dimer partner with exogenously added Hpx domain, which is also in dimer form (Itoh *et al.*, 2001). It has recently been proposed that the Hpx domain of MT1-MMP binds to collagen I and that soluble Hpx domain can inhibit collagen degradation by the soluble form of recombinant MT1-MMP by competing for the binding to collagen (Tam *et al.*, 2002). However, in cell-based experiments, we were unable to confirm binding of Hpx domain to collagen I fibrils. The soluble Hpx domain (MT1F-ΔCatΔTM) was rather associated with the cell surface under the condition where MT1F-ΔCatΔTM inhibits collagenolytic activity of the full-length enzyme. This discrepancy may be due to the expression system of the recombinant Hpx domain and their folding, but we concluded that the Hpx domain of MT1-MMP does not have significant affinity to collagen I fibrils at least under the conditions where MT1F-ΔCatΔTM inhibits collagenolytic activity of full-length enzyme on the cell surface.

The exact reason why MT1-MMP needs to form a dimer to degrade collagen on the cell surface is not clear at present, but one possible explanation may be that the freedom of the ectodomains of the enzyme (catalytic domain, linker-1, and Hpx domain) on the cell surface is too restricted to carry out "unwinding" and "cutting" of triple helical collagen and requires collaboration of at least two molecules to cleave a single collagen molecule. Another possibility may be that dimeric, but not monomeric, form of MT1-MMP interacts with other cell surface molecules, which may help to present MT1-MMP to fibrillar collagen on the cell surface. Soluble enzyme can access the collagen cleavage site by diffusion, thus the orientation of the enzyme relative to the collagen molecule can be readily arranged. For immobilized fibrillar collagen to be cleaved by a membrane-anchored collagenase, correct positioning of the enzyme in relation to the cleavage site within long filamentous molecules is limited. Dimerization of the enzyme may therefore enhance the chances for the collagenase to act on collagen fibrils. Indeed when MMP-13 is expressed as a transmembrane enzyme, it failed to cleave solid-phase collagen on the cell surface. The cell surface MMP-13 fused with the Hpx domain of MT1-MMP regains the ability to cleave it. MT1-MMP forms not only dimers, but also oligomeric complexes (Itoh *et al.*, 2001; Lehti *et al.*, 2002). Oligomerization occurs in a concentration-dependent manner at least in vitro (Itoh *et al.*, 2001). At the present time, it is not known if a dimeric form is sufficient to degrade collagen or whether a higher order of complexes is required. It is quite possible that the collagenolytic efficiency would be increased by clusterization of dimeric MT1-MMP on the cell surface.

Collagen-degrading activity is considered to be one of the most important biological activities of MT1-MMP during development (Holmbeck *et al.*, 1999, 2004). MT1-MMP null mice showed dwarfism, osteopenia, and development of spontaneous arthritis and progressive fibrosis (Holmbeck *et al.*, 1999). These phenotypes are considered to be a result of inadequate collagen turnover. The collagenolytic activity of MT1-MMP is also important in tumor growth (Hotary *et al.*, 2003), neovessel formation (Chun *et al.*, 2004), and cancer invasion into a collagen-based matrix (Sabeh *et al.*, 2004). When cells are migrating through collagen, the barrier collagen must be removed by positioning the dimeric MT1-MMP at the leading edge. Because Rac1 small GTPases can regulate the dimerization (Itoh *et al.*, 2001), it is most likely that localization and dimerization of MT1-MMP are regulated according to reorganization of the cytoskeleton. As MT1-MMP is reportedly colocalized with β1 integrin (Ellerbroek *et al.*, 2001; Wolf *et al.*, 2003), there may be a regulatory link between integrin and MT1-MMP in order to sense a barrier matrix, to position the enzyme at the leading edge, and to facilitate the formation of a functional dimer.

MT1-MMP is essential during branching morphogenesis of MDCK cells in a 3D-collagen gel (Kadono *et al.*, 1998). Expression of either MT1F-ΔCat or MT1F-ΔCatΔTM inhibited the tube formation by these cells, but MT1F-ΔCatΔTM exhibited a weaker inhibitory phenotype than that of MT1F-ΔCat. The difference is most likely attributed to differences in their membrane anchoring because the membrane-anchored MT1F-ΔCat is likely to have a tighter interaction with wild-type enzyme. Nevertheless, our data suggest that dimerization is also required for endogenously expressed MT1-MMP. Recently we have also reported that expression of MT1F-ΔCat effectively inhibited gastric cancer progression (Nonaka *et al.*, 2005). It is not known whether this inhibitory activity was attributed to inhibition of collagenolysis, but it suggests that inhibition of dimerization of

MT1-MMP and/or protein interaction through the Hpx domain is an effective means of inhibiting the biological function of MT1-MMP. Human MT1F-ΔCat can inhibit zebra fish MT1-MMP (zMT1-MMPα and zMT1-MMPβ) activities in vitro and in vivo (Bai *et al.*, 2005). These studies suggest that MT1-MMP derived from human and zebra fish can form a complex through their Hpx domains and also that dimerization may be an evolutionally conserved regulatory mechanism for MT1-MMP at least from zebra fish to human.

Soluble collagenases and membrane-tethered collagenase (MT1-MMP) are utilized dependent on the situations in vivo. For collagenolysis in a broader area of tissues, soluble collagenases may function more readily. For cells to migrate in collagen-enriched tissues, it is more advantageous to utilize membrane-anchored MT1-MMP. Our study emphasizes that these collagenolytic processes are regulated differently. Soluble collagenases can be regulated by expression, activation of zymogens, and inhibition by endogenous inhibitors. In the case of membrane-anchored MT1-MMP, its expression and activation produce biochemically active MT1-MMP, but they are not sufficient to make the enzyme functionally active to degrade collagen and to participate cellular migration in the tissue. It further requires dimerization, localization to specific membrane structures, and association and coordination with cell adhesion molecules and other cellular signaling molecules. Because cell migration is a dynamic process that requires orchestration of many different molecules, MT1-MMP, as one of the cell migration machineries, is perhaps required to be regulated in a such multidimensional manner.

ACKNOWLEDGMENTS

We thank Rob Visse and Linda Troeberg for critical reading of the manuscript. This work was supported by Cancer Research United Kingdom project grant C1507/A5541 and the Wellcome Trust equipment grant, National Institutes of Health Grant AR40994, and the Arthritis Research Campaign core grant to the Kennedy Institute of Rheumatology.

REFERENCES

Anilkumar, N., Uekita, T., Couchman, J. R., Nagase, H., Seiki, M., and Itoh, Y. (2005). Palmitoylation at Cys574 is essential for MT1-MMP to promote cell migration. FASEB J. *19*, 1326–1328.

Bai, S., Thummel, R., Godwin, A. R., Nagase, H., Itoh, Y., Li, L., Evans, R., McDermott, J., Seiki, M., and Sarras, M. P., Jr. (2005). Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. Matrix Biol. *24*, 247–260.

Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I., and Strongin, A. Y. (2001). Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. J. Biol. Chem. *276*, 18415–18422.

Bode, W., Reinemer, P., Huber, R., Kleine, T., Schnierer, S., and Tschesche, H. (1994). The X-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity. EMBO J. *13*, 1263–1269.

Borkakoti, N., Winkler, F. K., Williams, D. H., D'Arcy, A., Broadhurst, M. J., Brown, P. A., Johnson, W. H., and Murray, E. J. (1994). Structure of the catalytic domain of human fibroblast collagenase complexed with an inhibitor. Nat. Struct. Biol. *1*, 106–110.

Cawston, T. E. (1996). Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Pharmacol. Ther. *70*, 163–182.

Chun, T. H., Sabeh, F., Ota, I., Murphy, H., McDonagh, K. T., Holmbeck, K., Birkedal-Hansen, H., Allen, E. D., and Weiss, S. J. (2004). MT1-MMP-dependent neovessel formation within the confines of the three-dimensional extracellular matrix. J. Cell Biol. *167*, 757–767.

Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J. L., Fields, G. B., Visse, R., and Nagase, H. (2004). Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. EMBO J. *23*, 3020–3030.

Clark, I. M., and Cawston, T. E. (1989). Fragments of human fibroblast collagenase. Purification and characterization. Biochem. J. *263*, 201–206.

d'Ortho, M. P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith, B., Timpl, R., Zardi, L., and Murphy, G. (1997). Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. Eur. J. Biochem. *250*, 751–757.

Ellerbroek, S. M., Wu, Y. I., Overall, C. M., and Stack, M. S. (2001). Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. J. Biol. Chem. *276*, 24833–24842.

Endo, K., Takino, T., Miyamori, H., Kinsen, H., Yoshizaki, T., Furukawa, M., and Sato, H. (2003). Cleavage of syndecan-1 by membrane type matrix metalloproteinase-1 stimulates cell migration. J. Biol. Chem. *278*, 40764–40770.

Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J. (1998). Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell *95*, 365–377.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction [see comments]. Gene *77*, 51–59.

Holmbeck, K., *et al.* (1999). MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell *99*, 81–92.

Holmbeck, K., Bianco, P., Yamada, S., and Birkedal-Hansen, H. (2004). MT1-MMP: a tethered collagenase. J. Cell Physiol. *200*, 11–19.

Hotary, K., Allen, E., Punturieri, A., Yana, I., and Weiss, S. J. (2000). Regulation of cell invasion and morphogenesis in a three-dimensional type I collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3. J. Cell Biol. *149*, 1309–1323.

Hotary, K. B., Allen, E. D., Brooks, P. C., Datta, N. S., Long, M. W., and Weiss, S. J. (2003). Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. Cell *114*, 33–45.

Huang, W., Suzuki, K., Nagase, H., Arumugam, S., Van Doren, S. R., and Brew, K. (1996). Folding and characterization of the amino-terminal domain of human tissue inhibitor of metalloproteinases-1 (TIMP-1) expressed at high yield in *E. coli.* FEBS Lett. *384*, 155–161.

Itoh, Y., Ito, A., Iwata, K., Tanzawa, K., Mori, Y., and Nagase, H. (1998). Plasma Membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (Gelatinase A) activated on the cell surface. J. Biol. Chem. *273*, 24360–24367.

Itoh, Y., Kajita, M., Kinoh, H., Mori, H., Okada, A., and Seiki, M. (1999). Membrane Type 4 matrix metalloproteinase (MT4-MMP, MMP-17) is a glycosylphosphatidylinositol-anchored proteinase. J. Biol. Chem. *274*, 34260–34266.

Itoh, Y., and Seiki, M. (2006). MT1-MMP: a potent modifier of pericellular microenvironment. J. Cell Physiol. *206*, 1–8.

Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., Aoki, T., and Seiki, M. (2001). Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. EMBO J. *20*, 4782–4793.

Jiang, A., Lehti, K., Wang, X., Weiss, S.J., Keski-Oja, J., and Pei, D. (2001). Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. Proc. Natl. Acad. Sci. USA *98*, 13693–13698.

Kadono, Y., Shibahara, K., Namiki, M., Watanabe, Y., Seiki, M., and Sato, H. (1998). Membrane type 1-matrix metalloproteinase is involved in the formation of hepatocyte growth factor/scatter factor-induced branching tubules in madin-darby canine kidney epithelial cells. Biochem. Biophys. Res. Commun. *251*, 681–687.

Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., and Seiki, M. (2001). Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J. Cell Biol. *153*, 893–904.

Knäuper, V., Osthues, A., DeClerck, Y. A., Langley, K. E., Blaser, J., and Tschesche, H. (1993). Fragmentation of human polymorphonuclear-leucocyte collagenase. Biochem. J. *291*(Pt 3), 847–854.

Knäuper, V., Will, H., López-Otín, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G. (1996). Cellular mechanisms for human procollagenase 3 (mmp 13) activation: evidence that mt1 mmp (mmp 14) and gelatinase a (mmp 2) are able to generate active enzyme. J. Biol. Chem. *271*, 17124–17131.

Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000). Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J. Cell Biol. *148*, 615–624.

Lehti, K., Lohi, J., Juntunen, M. M., Pei, D., and Keski-Oja, J. (2002). Oligomerization through hemopexin and cytoplasmic domains regulates the activity

and turnover of membrane-type 1 matrix metalloproteinase. J. Biol. Chem. *277*, 8440–8448.

Murphy, G., Allan, J. A., Willenbrock, F., Cockett, M. I., O'Connell, J. P., and Docherty, A.J.P. (1992). The role of the C-terminal domain in collagenase and stromelysin specificity. J. Biol. Chem. *267*, 9612–9618.

Nonaka, T., Nishibashi, K., Itoh, Y., Yana, I., and Seiki, M. (2005). Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 in vivo. Mol. Cancer Ther. *4*, 1157–1166.

Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997). Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J. Biol. Chem. *272*, 2446–2451.

Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J. P., Rio, M. C., and Basset, P. (1997). Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. J. Cell Biol. *137*, 67–77.

Patterson, M. L., Atkinson, S. J., Knauper, V., and Murphy, G. (2001). Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. FEBS Lett. *503*, 158–162.

Ramachandran, G. N., and Kartha, G. (1955). Structure of collagen. Nature *176*, 593–595.

Ratnikov, B. I., Rozanov, D. V., Postnova, T. I., Baciu, P. G., Zhang, H., DiScipio, R. G., Chestukhina, G. G., Smith, J. W., Deryugina, E. I., and Strongin, A. Y. (2002). An alternative processing of integrin alpha. (v) subunit in tumor cells by membrane type-1 matrix metalloproteinase. J. Biol. Chem. *277*, 7377–7385.

Remacle, A., Murphy, G., and Roghi, C. (2003). Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. J. Cell Sci. *116*, 3905–3916.

Sabeh, F., *et al.* (2004). Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J. Cell Biol. *167*, 769–781.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature *370*, 61–65.

Seiki, M. (1999). Membrane-type matrix metalloproteinases. Apmis *107*, 137–143.

Sternlicht, M. D., and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. Annu. Rev. Cell Dev. Biol. *17*, 463–516.

Tam, E. M., Wu, Y. I., Butler, G. S., Stack, M. S., and Overall, C. M. (2002). Collagen binding properties of the membrane type-1 matrix metalloproteinase (MT1-MMP) hemopexin C domain. The ectodomain of the 44-kDa autocatalytic product of MT1-MMP inhibits cell invasion by disrupting native type I collagen cleavage. J. Biol. Chem. *277*, 39005–39014.

Uekita, T., Itoh, Y., Yana, I., Ohno, H., and Seiki, M. (2001). Cytoplasmic tail-dependent internalization of membrane-type 1 matrix metalloproteinase is important for its invasion-promoting activity. J. Cell Biol. *155*, 1345–1356.

Visse, R., and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ. Res. *92*, 827–839.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., Strongin, A. Y., Brocker, E. B., and Friedl, P. (2003). Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J. Cell Biol. *160*, 267–277.

Zhou, Z., Apte, S. S., Soininen, R., Cao, R., Baaklini, G. Y., Rauser, R. W., Wang, J., Cao, Y., and Tryggvason, K. (2000). Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. Proc. Natl. Acad. Sci. USA *97*, 4052–4057.