

# 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\*

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**Up-regulation of the collagenolytic membrane type-1 matrix metalloproteinase (MT1-MMP) leads to increased MMP2 (gelatinase A) activation and MT1-MMP autolysis. The autocatalytic degradation product is a cell surface 44-kDa fragment of MT1-MMP (Gly<sup>285</sup>-Val<sup>582</sup>) in which the ectodomain consists of only the linker, hemopexin C domain and the stalk segment found before the transmembrane sequence. In the collagenases, hemopexin C domain exosites bind native collagen, which is required for triple helicase activity during collagen cleavage. Here we investigated the collagen binding properties and the role of the hemopexin C domain of MT1-MMP and of the 44-kDa MT1-MMP ectodomain in collagenolysis. Recombinant proteins, MT1-LCD (Gly<sup>285</sup>-Cys<sup>508</sup>), consisting of the linker and the hemopexin C domain, and MT1-CD (Gly<sup>315</sup>-Cys<sup>508</sup>), which consists of the hemopexin C domain only, were found to bind native type I collagen but not gelatin. Functionally, MT1-LCD inhibited collagen-induced MMP2 activation in fibroblasts, suggesting that interactions between collagen and endogenous MT1-MMP directly stimulate the cellular activation of pro-MMP2. MT1-LCD, but not MT1-CD, also blocked the cleavage of native type I collagen by MT1-MMP *in vitro*, indicating an important role for the MT1-MMP linker region in triple helicase activity. Similarly, soluble MT1-LCD, but not MT1-CD or peptide analogs of the MT1-MMP linker, reduced the invasion of type I collagen matrices by MDA-MB-231 cells as did the expression of recombinant 44-kDa MT1-MMP on the cell surface. Together, these studies demonstrate that generation of the 44-kDa MT1-MMP autolysis product regulates collagenolytic activity and subsequent invasive potential, suggesting a novel feedback mechanism for the control of pericellular proteolysis.**

Type I collagen is the most abundant protein of the extracellular matrix and is an important structural component in blood vessels, skin, tendons, ligaments, and bone (1). Accordingly, the synthesis and degradation of type I collagen is tightly regulated. Disruptions in this homeostasis can lead to diseases such as pulmonary fibrosis, scleroderma, arthritis, and osteoporosis, which, if untreated, can result in loss of tissue function and integrity. In a number of cancer cells, the capacity to degrade type I collagen and invade through type I collagen matrices often correlates with metastatic potential (2), a characteristic that is as important for the local dissemination of tumor cells as type IV collagen degradation and basement membrane penetration is for metastasis (3). Despite the importance of maintaining correct collagen homeostasis in tissues, the proteases responsible for type I collagen degradation *in vivo* remain unclear. An intracellular pathway may play an important role in collagen degradation (4) that, in bone, utilizes the cysteine protease cathepsin K at low pH (5). Extracellularly, fibrillar type I collagen may be degraded at neutral pH by several matrix metalloproteinases (MMPs),<sup>1</sup> a 24-member family of zinc-dependent endopeptidases in humans (2). The major collagenolytic MMPs are the secreted collagenases, MMP1, MMP8, and MMP13 (6), and the cell surface membrane type 1 (MT1)-MMP (7, 8). MT1-MMP also activates collagenase-2 (MMP13) (9) and is the primary activator of MMP2 (10), a gelatinase that exhibits weak native type I collagenolytic activity (11–13).

MMPs share a common overall structure consisting of a propeptide, catalytic domain, linker (also called a hinge), and a hemopexin C domain (14). Whereas the majority of MMPs are secreted as latent zymogens, MT-MMPs, the largest subgroup of MMPs, are membrane-anchored by the presence of a type I transmembrane sequence and cytoplasmic tail (MT1-, MT2-, MT3-, and MT5-MMP) or by glycosylphosphatidylinositol linkage (MT4- and MT6-MMP) (14). MT1-MMP is activated intracellularly by proprotein convertase-dependent and -independent pathways (15, 16) and is expressed as an active protease on the surface of many normal and pathological cell types (10, 17). The importance of MT1-MMP is indicated by its requirement for the invasion of endothelial and cancer cells through type I collagen matrices (18–20). Moreover, mice deficient in MT1-

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<sup>1</sup> The abbreviations used are: MMP, matrix metalloproteinase; MT, membrane type; CD, C domain; LCD, C domain with linker; sMT1-MMP, soluble MT1-MMP; TIMP, tissue inhibitor of metalloproteinases; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; ConA, concanavalin A; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; Mca, 7-methoxycoumarin-4-yl)acetyl.

MMP developed severe aberrations in type I collagen-abundant tissues, such as bone and skin, and the mice exhibited arthritis and scleroderma (21, 22). In humans, homoallelic loss-of-function mutations in the *MMP2* gene result in excessive bone resorption and arthritis (23). This condition resembles the phenotype of the MT1-MMP knockout mouse, supporting the close functional connection of MMP2 and MT1-MMP in regulating pericellular collagen homeostasis in mice and humans.

Native type I collagen consists of two  $\alpha 1(I)$  chains and one  $\alpha 2(I)$  chain interwound in a right-handed triple helix that is resistant to cleavage by most proteinases at neutral pH with the exception of the MMP collagenases (14). Because the active site of collagenolytic MMPs can only accommodate a single  $\alpha$ -chain, cleavage of the three  $\alpha$ -chains occurs sequentially at the single collagenase-susceptible site, Gly<sup>775</sup>-Ile/Leu<sup>776</sup>, to generate  $\frac{3}{4}$  and  $\frac{1}{4}$  collagen fragments. To achieve this, the collagen helix must be initially unwound by a triple helicase mechanism in order to expose the scissile bonds. This critical step requires the presence of collagen-binding exosites (14), in addition to elements within the active site (24–26). In MMP1, MMP8, and MMP13, the hemopexin C domain supports binding to collagen and is required for native collagen cleavage (27–32). Deletion or mutation of the MMP8 linker also reduces collagenolysis (33, 34). Furthermore, synthetic peptide analogs of the MMP1 linker bound collagen and inhibited collagen cleavage (35). Interestingly, the 35-amino acid residue linker of MT1-MMP is twice the length of other collagenase linkers (18 residues); however, the significance of this and its role in collagen cleavage have yet to be examined.

The regulation of MT1-MMP activity, MMP2 activation and pericellular type I collagen levels is complex. In a variety of cells, stimulation by fibrillar type I collagen has been shown to increase the cell surface expression of MT1-MMP and induce the cellular activation of pro-MMP2 (36–42). This response is in part dependent on  $\beta_1$  integrin clustering and signaling (40, 42, 43) and is potentially self-regulating, since type I collagen is susceptible to MT1-MMP and MMP2 proteolysis (14, 44). Concentration of MT1-MMP by overexpression (45, 46) or clustering interactions (47–50) favors MMP2 activation and collagenolysis (50). Concomitant with increased MT1-MMP expression and MMP2 activation is the autocatalytic processing of MT1-MMP at Gly<sup>284</sup>-Gly<sup>285</sup> to shed the catalytic domain from the hemopexin C domain, which is retained on the cell membrane (40, 46, 51). Hence, the ectodomain of the residual 44-kDa MT1-MMP fragment (Gly<sup>285</sup>-Val<sup>582</sup>) on the cell surface consists of the linker, hemopexin C domain, and stalk segment only (see Fig. 1A, *ii*) and thus is catalytically inactive. The significance of the 44-kDa MT1-MMP *in vivo* is not clear. In addition to being present following cell binding to type I collagen, the 44-kDa MT1-MMP has also been detected on the surface of tumor cells (40, 51). During MMP2 activation, TIMP-2-free MT1-MMP must be in close proximity to a trimeric complex of MT1-MMP·TIMP-2·pro-MMP2 in order to activate the bound pro-MMP2 (52). The mechanisms of MT1-MMP oligomerization are not clear. The recombinant hemopexin C domain of MT1-MMP did not form oligomers in solution or modulate MMP2 activation when added to cells (47). Recent reports using transmembrane MT1-MMP chimera and deletion mutants have suggested that the hemopexin C domain can mediate homophilic complex formation of cellular MT1-MMP for efficient MMP2 activation (48, 49). Expression of a transmembrane-tethered MT1-MMP hemopexin C domain lacking the linker, termed PEX (Thr<sup>313</sup>-Val<sup>582</sup>), in HT1080 cells inhibited MT1-MMP oligomerization, the cellular activation of pro-MMP2, and Matrigel invasion (48), a function previously at-

tributed to MMP2 proteolytic activity against type IV collagen (53).

Considering that MT1-MMP is a collagenase, we hypothesized that exosites on the hemopexin C domain would bind to type I collagen and be essential for collagenolytic activity. Thus, the autolytically generated 44-kDa MT1-MMP ectodomain would be predicted to modulate pericellular collagenolysis on the membrane through dominant-negative interactions. Since native type I collagen stimulates MMP2 activation, we also hypothesized that collagen binding by the hemopexin C domain of MT1-MMP would modulate MMP2 activation with 44-kDa MT1-MMP opposing these effects *in vivo*. Experiments reported here demonstrate that collagen binding by the MT1-MMP hemopexin C domain is essential for collagenolytic activity and enhancement of MMP2 activation by MT1-MMP. Inhibition of this interaction either *in vitro* or on the cell surface inhibits collagen degradation. Together, these studies suggest a novel feedback mechanism through which generation of the 44-kDa MT1-MMP autolysis product regulates pericellular collagenolytic activity and subsequent invasive potential.

#### EXPERIMENTAL PROCEDURES

**Materials**—Rat tail type I collagen was prepared as previously described (54). Vitrogen<sup>®</sup> was purchased from Cohesion (Palo Alto, CA). Biotin-labeled type I collagen was prepared as previously described (55). Human placental type I collagen was purchased from Sigma. The triple helical nature of collagen was confirmed by the absence of trypsin sensitivity at an enzyme/substrate ratio of 1:10 over 3 h, 28 °C. The general hydroxamate inhibitor BB2116 was provided by British Biotech Pharmaceuticals (Oxford, UK). Hydroxamate inhibitor GM6001 and AB8102 (blocking antibody raised against the human MT1-MMP catalytic domain) were purchased from Chemicon (Temecula, CA). The polyclonal antibody RP1MMP-14 (raised against the MT1-MMP linker) was purchased from Triple Point Biologics (Portland, OR). The affinity-purified polyclonal antibodies  $\alpha$ MT1-CD and  $\alpha$ His<sub>6</sub> were described previously (47).

**Synthetic Peptides and Recombinant Proteins**—The MT1-MMP linker peptide analogs MT1-L18 (<sup>302</sup>RPSVPDKPKNPYGPNIC<sup>319</sup>) (University of Victoria, Victoria, Canada) and MT1-L35 (<sup>285</sup>GESGF-PTKMPQPRTTSRPSVPDKPKNPYGPNIC<sup>319</sup>) (Tufts University, Medford, MA) (Fig. 1A, *iii*) were synthesized and verified by mass spectrometry. Recombinant domains of human MT1-MMP and MMP2 were expressed in *Escherichia coli* as N-terminal His-tagged proteins. The MT1-MMP hemopexin C domain (CD) with or without the linker (L) (MT1-LCD, Gly<sup>285</sup>-Cys<sup>508</sup>; MT1-CD, Gly<sup>315</sup>-Cys<sup>508</sup>) (see Fig. 1A) and the MMP2 hemopexin C domain with the linker (MMP2-LCD, Gly<sup>446</sup>-Cys<sup>660</sup>) were prepared as previously described (47). Recombinant human MMP2-CBD (Val<sup>220</sup>-Gln<sup>393</sup>) (collagen binding domain consisting of three fibronectin type II modules) was prepared previously (54). Any bacterial endotoxins in purified recombinant protein preparations were removed by polymyxin B-agarose columns (Sigma). The fidelity of purified recombinant proteins was confirmed by electrospray ionization mass spectrometry (47) and N-terminal Edman sequencing of protein bands cut from the membrane of Western blots. Human soluble MT1-MMP, truncated C-terminal to the hemopexin C domain (sMT1-MMP), was kindly provided by British Biotech Pharmaceuticals. Recombinant human MMP2, TIMP-1, and TIMP-2 were expressed in a mammalian cell system and purified as previously described (56) or kindly provided by Dr. H. Nagase (Imperial College School of Medicine, London, UK).

**Electrophoretic Techniques**—Samples in reducing (65 mM dithiothreitol) or nonreducing sample buffer (125 mM Tris-HCl, pH 6.8, 2.0% SDS, 2.0 M urea, 0.05% bromophenol blue) were separated on 15% SDS-PAGE gels and analyzed by either silver nitrate staining or by Western blotting using  $\alpha$ MT1-CD and  $\alpha$ His<sub>6</sub> antibodies. ECL detection was performed according to the manufacturer's instructions (Amersham Biosciences). For zymographic analysis, samples were separated under nonreducing conditions on 10% SDS-PAGE gels co-polymerized with 0.5 mg/ml gelatin. Gels were washed for 30 min with 2.5% Triton X-100, rinsed with deionized water, and incubated with assay buffer (100 mM Tris, pH 8.0, 30 mM CaCl<sub>2</sub>, 0.05% Brij, 0.025% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) at 37 °C for 4 h before staining with Coomassie Brilliant Blue G250.

**Gel Filtration Chromatography**—Purified MT1-LCD (0.5 mg) was subjected to gel filtration chromatography on a Superdex 75 column equilibrated with PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl,

140 mM NaCl, pH 7.4) and run on an AKTA purifier (Amersham Biosciences). Protein elution was monitored at 215 nm. Molecular mass standards used were BSA (67 kDa), ovalbumin (43 kDa), chymotrypsin A (25 kDa), and ribonuclease A (13.7 kDa).

**Solid Phase Binding Assays**—Native and heat-denatured type I collagen (rat tail) (5  $\mu\text{g}/\text{ml}$ ) were diluted in 15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ , 0.02%  $\text{NaN}_3$ , pH 9.6 (100  $\mu\text{l}$ ), and coated onto 96-microwell plates (Falcon) overnight at 4 °C as described previously (54, 57). Wells coated with myoglobin served as a control for nonspecific binding. The coated wells were blocked with 1% BSA to which serially diluted recombinant proteins in PBS (100  $\mu\text{l}$  total volume) were added and incubated for 1 h at room temperature. After extensive washes, bound proteins were quantitated using affinity-purified polyclonal antibodies followed by incubation with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody. Substrate, *p*-nitrophenyl phosphate disodium (Sigma), was added to the wells, and color development was monitored at 405 nm in a Thermomax plate reader (Molecular Devices).

**Ligand Blot Assays**—Proteins (5  $\mu\text{g}$ ) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl were filtered onto an Immobilon-P® membrane (Millipore Corp.) by vacuum. Membranes were blocked with 1% BSA in PBS and incubated with biotin-labeled native type I collagen in PBS/Tween 20 for 1 h. Bound collagen was visualized using horseradish peroxidase (HRP)-conjugated streptavidin and ECL detection.

**Enzyme Assays**—Biotin-labeled type I collagen (0.025 pmol) was incubated with either sMT1-MMP or MMP2 in assay buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM  $\text{CaCl}_2$ , 3.8 mM  $\text{NaN}_3$ , 0.05% Brij) for 18 h at 28 °C. MMPs were activated with 2 mM 4-aminophenylmercuric acetate. Recombinant proteins and BB2116 in assay buffer were added to the reactions where indicated. Following digestion, samples were separated by 7.5% SDS-PAGE and analyzed by Western blotting using streptavidin-HRP and ECL detection.  $\alpha 1(\text{I})$  and  $\alpha 2(\text{I})$  chains were quantitated by scanning densitometry, and the percentage of native collagen cleavage was calculated as previously described (58). Cleavage of the quenched fluorescent substrate, Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH<sub>2</sub>, was performed as described previously (56). MT1-LCD and MT1-CD in assay buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM  $\text{CaCl}_2$ , 0.05% Brij) were added to the reaction where indicated.

**Transmembrane MT1-MMP and MT1-MMP Hemopexin C Domain Constructs**—The mammalian expression vector pCR3.1-Uni (Invitrogen) carrying human MT1-MMP cDNA was the generous gift of Dr. D. Pei (University of Minnesota). To express cell surface (c) transmembrane MT1-MMP hemopexin C domain, cMT1-CD ( $\Delta 112$ –315) and cMT1-LCD ( $\Delta 112$ –284), two-step overlapping PCR was used with T7 and reverse primer as external primers and either 5'-CGAAGGAAGC-GCCCAACATCTGTGACGGGAAC-3' and 5'-ACAGATGTTGGGGCG-CTTCCTTCGAACATTGGC-3' (cMT1-CD,  $\Delta 112$ –315) or 5'-CGAAGGAAGC-GCGGTGAGTCAGGTCTCCACC-3' and 5'-CCCTGACTACCGCGCTTCCTTCGAACATTGGCC-3' (cMT1-LCD,  $\Delta 112$ –284) as internal primer pairs. The catalytically inactive MT1-MMP (E240A) mutant construct was generated using 5'-GGTGGCTGTGCACGCGCTGG-GCCATGCC-3' and 5'-GGCATGGCCAGCGCGTGCACAGCCACC-3' (E240A) primers. Full-length constructs were synthesized by PCR with T7 and reverse primers, digested with *Hind*III and *Eco*RI, ligated back to pCR3.1-Uni vector, and fully sequenced.

**Cell Culture and Stable Transfection**—Early passage human gingival fibroblasts were kindly provided by Dr. D. Brunette (University of British Columbia, Vancouver, Canada) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (Invitrogen). MDA-MB-231 breast carcinoma cells were kindly provided by Dr. V. G. Jordan (Northwestern University, Chicago, IL) and cultured in DMEM (Cellgro) supplemented with 10% fetal bovine serum (U.S. Bio-Technologies Inc.). MDA-MB-231 cells were transfected with MT1-MMP cDNA constructs using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Stable cell lines were clone-selected and maintained in medium containing 1 mg/ml G418 (Mediatech Inc.). For each line, five clones were pooled and used in the experiments.

**Transwell Invasion and Migration Assay**—MDA-MB-231 cell invasion and migration assays through type I collagen (human placental) were performed as described previously (42). Endotoxin-free recombinant proteins, linker peptide analogs and antibodies in PBS were added to the cell media with BSA or IgG as controls. GM6001 was added to the cells in  $\text{Me}_2\text{SO}$ .

**Collagen Gels**—To prepare collagen gels, 8 volumes of Vitrogen was neutralized with 1 volume of 10 $\times$  concentrated PBS and 1 volume of 0.1 M NaOH. Fibroblasts were detached with PBS containing 0.54 mM EDTA and 1.1 mM glucose and resuspended in a neutralized Vitrogen solution (2.0 mg/ml) containing 11.3% DMEM and 2.5% new born calf

serum. The cell/collagen solution (75  $\mu\text{l}$ ) was then transferred into 96-well tissue culture plates and incubated at 37 °C for 1 h to allow for collagen polymerization. Cells were supplemented with DMEM containing 2.5% newborn calf serum for 18 h. Collagen gels were then rinsed with DMEM, and cells were cultured under serum-free conditions with or without MT1-LCD (endotoxin-free, in PBS) for the duration of the experiment. Cell conditioned medium was replaced every 24 h and analyzed by gelatin zymography after 72 h.

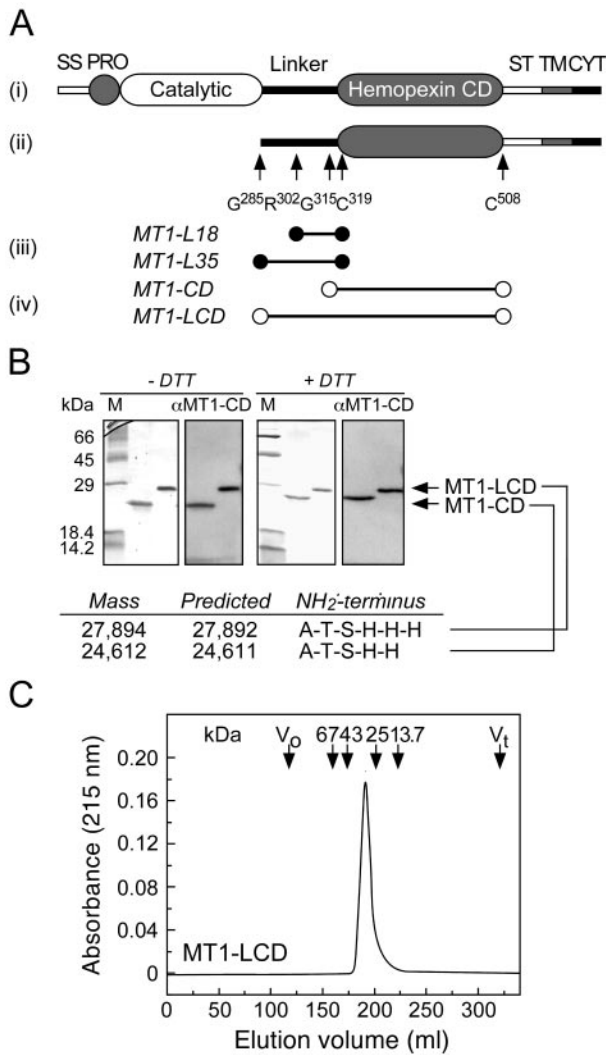
**Latex Beads**—Native and denatured type I collagen (100  $\mu\text{g}/\text{ml}$ ) were incubated with latex beads (1%) (Sigma) for 1 h at room temperature to allow for adsorption. The beads were then washed with PBS and blocked with 1% BSA for 1 h. Beads not absorbed with collagen served as a control. Blocked beads were rinsed with PBS and resuspended in DMEM at a concentration of 0.2% (v/v). Fibroblasts cultured in 96-well tissue culture plates were rinsed and incubated in serum-free medium for 1 h prior to incubation with the latex beads in DMEM (100  $\mu\text{l}$ ). Endotoxin-free MT1-LCD in PBS was added to the latex bead preparations where indicated. Cells were cultured for 24 h, after which the conditioned cell medium was analyzed by gelatin zymography.

## RESULTS

**Recombinant Protein Expression**—To characterize the hemopexin C domain of MT1-MMP and the ectodomain of 44-kDa MT1-MMP, two forms of the MT1-MMP hemopexin C domain were cloned and expressed in *E. coli*. MT1-LCD (Gly<sup>285</sup>–Cys<sup>508</sup>) corresponds to the N terminus of 44-kDa MT1-MMP and includes both the linker and the hemopexin C domain (Fig. 1A, *iv*). MT1-CD (Gly<sup>315</sup>–Cys<sup>508</sup>) consists of the hemopexin C domain only (Fig. 1A, *iv*). Yields of purified protein were typically ~20 mg from 3 liters of liquid culture. The identities of the purified proteins were confirmed by Western blotting with  $\alpha\text{MT1-CD}$  antibody (Fig. 1B) and  $\alpha\text{His}_6$  (data not shown). Non-reducing SDS-PAGE analysis demonstrated the absence of dimeric intermolecular disulfide cross-linked aggregates (Fig. 1B). Reducing SDS-PAGE and electrospray ionization mass spectrometry determination of the purified protein masses were consistent with the predicted masses. As shown in Fig. 1B, both MT1-LCD (27,894 Da) and MT1-CD (24,612 Da) were within 1–2 Da of the predicted mass after accounting for the removal of the N-terminal methionine and hydrogen atoms after disulfide bond formation. Edman sequencing also confirmed N-terminal methionine processing and the presence of the N-terminal His<sub>6</sub> tag (Fig. 1B). MT1-LCD did not form noncovalent multimeric complexes under native conditions in solution as shown by the elution of a single peak at 28 kDa corresponding to the monomeric form of MT1-LCD upon gel filtration chromatography (Fig. 1C).

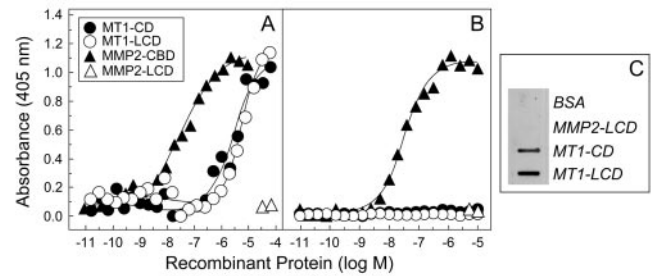
**Collagen Binding Properties of the MT1-MMP Hemopexin C Domain**—We first assessed the collagen binding properties of the MT1-MMP hemopexin C domain by performing solid phase binding assays with type I collagen, the preferred collagen substrate of MT1-MMP. As shown in Fig. 2A, binding of MT1-CD and MT1-LCD to native collagen films was similar, indicating that the linker had little apparent effect on collagen binding affinity. Unlike MMP2-CBD, both MT1-CD and MT1-LCD did not bind denatured collagen (Fig. 2B), confirming specificity. As a control, MMP2-LCD did not bind native or denatured type I collagen as shown previously (57). Binding of soluble native type I collagen to MT1-CD and MT1-LCD was confirmed by ligand blot analysis with MMP2-LCD and BSA serving as negative controls (Fig. 2C).

**Collagen/MT1-MMP Hemopexin C Domain Interactions during Collagen-induced MMP2 Activation**—Physical clustering of MT1-MMP was previously shown to facilitate the pro-MMP2 activation reaction by increasing the proximity of catalytically active MT1-MMP to the trimeric activation complex (52). Due to the collagen binding properties of the MT1-MMP hemopexin C domain, we postulated that type I collagen may function as an *in vivo* mechanism to directly bind and concentrate cell surface MT1-MMP to facilitate the cellular activation of pro-

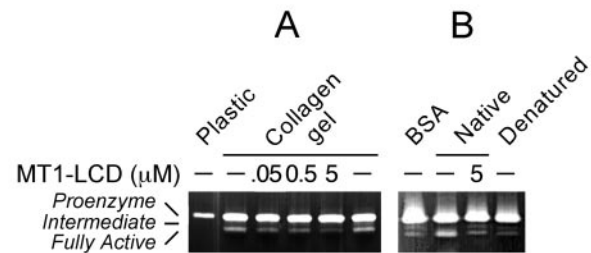


**FIG. 1. MT1-MMP hemopexin C domain constructs and linker peptide analogs.** *A*, the domain structures of pro-MT1-MMP (*i*) and 44-kDa MT1-MMP (*ii*) are shown in a linear diagram. The signal sequence (SS), propeptide domain (PRO), stalk segment (ST), transmembrane sequence (TM), and cytoplasmic tail (CYT) of MT1-MMP are indicated. Schematic representations of MT1-MMP linker peptide analogs MT1-L18 and MT1-L35 (*iii*) and hemopexin C domain constructs MT1-CD and MT1-LCD (*iv*) are shown with N- and C-terminal residues indicated. *B*, purified MT1-CD and MT1-LCD (0.1  $\mu$ g) were electrophoresed on SDS-PAGE (15%) gels under reducing (+DTT) and nonreducing (-DTT) conditions. Gels were analyzed by either silver staining or by Western blotting using  $\alpha$ MT1-CD antibody. Protein masses measured by electrospray ionization mass spectrometry (Mass), predicted masses, and N-terminal Edman sequence analysis are indicated. *C*, MT1-LCD was subjected to FPLC filtration chromatography on a Superdex 75 column, and elution was monitored at 215 nm. The elution volumes of the void volume ( $V_o$ ) and column volume ( $V_t$ ) and molecular weight standards are indicated.

MMP2. To test this, human gingival fibroblasts were cultured in three-dimensional type I collagen gels for 72 h to stimulate the activation of pro-MMP2. Soluble MT1-LCD was added to the cultures to compete with endogenous MT1-MMP for collagen binding. As shown in Fig. 3A, activation of pro-MMP2 in the cell cultures was reduced with increasing concentrations of MT1-LCD. Control cells cultured on plastic did not activate pro-MMP2. To confirm this response, latex beads coated with type I collagen were found to stimulate pro-MMP2 activation in fibroblasts cultured on plastic (Fig. 3B). Consistent with our observations of cells in collagen gels, induction of pro-MMP2 activation by native collagen-adsorbed beads was reduced by the presence of MT1-LCD to the levels seen with BSA-adsorbed



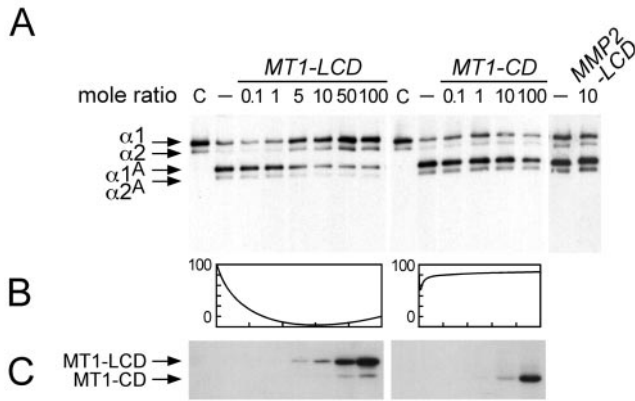
**FIG. 2. Type I collagen binding properties of MT1-MMP hemopexin C domain constructs.** Serial dilutions of MT1-CD and MT1-LCD were incubated in the same 96-microwell plates coated with native (*A*) or heat-denatured (65 °C for 1 h) (*B*) type I collagen (0.5  $\mu$ g/well) as described under "Experimental Procedures." MMP2-CBD and MMP2-LCD were included as positive (54) and negative controls (57), respectively. Bound recombinant domains were detected using  $\alpha$ -His<sub>6</sub> antibody. *C*, ligand blot assay. Immobilized MT1-CD and MT1-LCD and control proteins, BSA and MMP2-LCD (5  $\mu$ g each), were incubated with biotin-labeled type I collagen (0.1  $\mu$ g/ml) in PBS as described under "Experimental Procedures." Bound collagen was detected using streptavidin-HRP and ECL detection.



**FIG. 3. Recombinant MT1-MMP hemopexin C domain reduces collagen-induced activation of MMP2.** *A*, fibroblasts ( $1 \times 10^4$ ) grown on tissue culture plastic or within type I collagen gels (2.0 mg/ml) were incubated with MT1-LCD (0, 0.05–5  $\mu$ M) in DMEM for 72 h. MMP2 in the conditioned cell media was visualized by gelatin zymography. *B*, fibroblasts ( $1 \times 10^4$ ) grown on plastic were incubated with latex beads adsorbed with type I collagen (native or denatured) or BSA in DMEM for 24 h. Exogenous MT1-LCD (5  $\mu$ M) was added to the cultures where indicated. Conditioned cell media were analyzed by gelatin zymography. Lanes presented in both *A* and *B* are from the same zymogram.

beads (Fig. 3B). The requirement for fibrillar collagen was confirmed, since gelatin-adsorbed beads did not stimulate pro-MMP2 activation. In the absence of latex beads, the addition of soluble native collagen to fibroblasts cultured on plastic produced inconsistent and variable levels of activation (data not shown). Together, these results demonstrate that native type I fibrillar collagen interactions with the MT1-MMP hemopexin C domain in fibroblasts may concentrate cell surface MT1-MMP to stimulate the cellular activation of pro-MMP2.

**Effect of Exogenous MT1-MMP Hemopexin C Domain on Collagenolysis by sMT1-MMP and MMP2**—Studies of collagenases have shown that the hemopexin C domain is required to support binding to and cleavage of collagen (27–29, 31, 32, 59). To examine the role of the hemopexin C domain in MT1-MMP collagenolysis, recombinant hemopexin C domain constructs were incubated with sMT1-MMP and biotin-labeled type I collagen. Reactions were performed at 28 °C to maintain collagen triple helicity, as confirmed by the lack of collagen cleavage in the presence of trypsin even at a 1:10 enzyme/substrate molar ratio (data not shown). sMT1-MMP cleaved native type I collagen (Fig. 4A) and was inhibited by TIMP-2 and BB2116 (data not shown). As seen in Fig. 4A (left panel), the sMT1-MMP cleavage of native type I collagen was inhibited by the presence of MT1-LCD in a concentration-dependent manner. In contrast, neither MT1-CD (Fig. 4A, right panel) nor the control protein, MMP2-LCD, had any effect on cleavage. The percent-



**FIG. 4. Recombinant MT1-MMP hemopexin C domain reduces MT1-MMP collagenolysis.** *A*, biotin-labeled type I collagen was incubated in the absence (*C*) or presence of sMT1-MMP (1 pmol) for 18 h at 28 °C. Recombinant proteins, MT1-LCD, MT1-CD, and MMP2-LCD were added at the indicated molar equivalents relative to sMT1-MMP. Reactions were separated by SDS-PAGE (7.5%), followed by Western blotting using streptavidin-HRP. *B*, percentage of  $\alpha$ -chain cleavage was determined by densitometric analysis as described under “Experimental Procedures” and plotted against the amount of recombinant hemopexin C domain added. *C*, MT1-LCD and MT1-CD were detected in reaction samples by SDS-PAGE (15%) and Western blotting using the  $\alpha$ MT1-CD antibody.

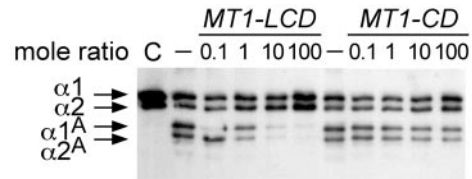
age of  $\alpha$ -chain cleavage for each reaction was quantitated by scanning densitometry and graphically plotted against the amount of MT1-LCD or MT1-CD added (Fig. 4*B*). The presence of hemopexin C domain proteins at the end of each reaction was confirmed by Western blot analysis (Fig. 4*C*). As a control, MT1-CD and MT1-LCD did not affect sMT1-MMP activity against the quenched fluorescent peptide, Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH<sub>2</sub> (Table I), demonstrating that inhibition by MT1-LCD is specific for triple helical substrates and that peptide bond cleavage by MT1-MMP does not require the hemopexin C domain. Due to the unique association between MT1-MMP and MMP2 *in vivo*, we assessed whether the MT1-MMP hemopexin C domain may affect MMP2 collagenolysis. Similar to that observed for MT1-MMP, MT1-LCD, but not MT1-CD, disrupted MMP2 cleavage of native type I collagen (Fig. 5).

**Collagen Binding Properties of MT1-MMP Linker Peptide Analogs and the Effect on Collagenolysis**—Although both MT1-MMP hemopexin C domain constructs share similar collagen binding properties, only MT1-LCD disrupted collagenolysis. Since this result indicated an important role for the linker in native collagen cleavage, we generated two synthetic peptide linker analogs to further study the effect of the MT1-MMP linker on MT1-MMP collagenolysis. From clustal alignments, we synthesized the peptide analog MT1-L18 (Arg<sup>302</sup>-Cys<sup>319</sup>), which corresponds to an 18-amino acid residue region of similarity possessed by the collagenolytic MMPs, MMP1, MMP2, MMP8, and MMP13 (Fig. 6*A*). MT1-L35 (Gly<sup>285</sup>-Cys<sup>319</sup>) encompasses the entire MT1-MMP linker and includes the unique 17-amino acid residue region that is N-terminal to the homologous 18-amino acid residue region (Fig. 6*A*). As shown in Fig. 6*B*, neither MT1-L18 nor MT1-L35 showed affinity for native (Fig. 6*B*, *i*) or denatured type I collagen (Fig. 6*B*, *ii*), indicating that the MT1-MMP linker alone does not contribute to collagen binding or that the collagen binding site spans the junction of the linker and hemopexin C domain. Similarly, both peptide analogs did not disrupt native type I collagen cleavage by sMT1-MMP, even at a 1000-fold molar excess (Fig. 6*C*). To determine whether either linker peptide sequence could confer regulatory activity on the MT1-CD polypeptide, MT1-L18 or MT1-L35 was added to the reaction mixture containing MT1-

TABLE I  
Quenched fluorescent peptide cleavage by sMT1-MMP

Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH<sub>2</sub> (0.1 nmol) was incubated with (control) or without (buffer) sMT1-MMP (0.035 pmol) for 1 h at 37 °C. MT1-LCD and MT1-CD were added to the reaction at a 100-fold molar excess to sMT1-MMP. RFU, relative fluorescence units.

	Buffer	Control	MT1-LCD	MT1-CD
Rate of cleavage (RFU $\times 10^{-3} \cdot s^{-1}$ )	0.05	6.7	7.0	7.0

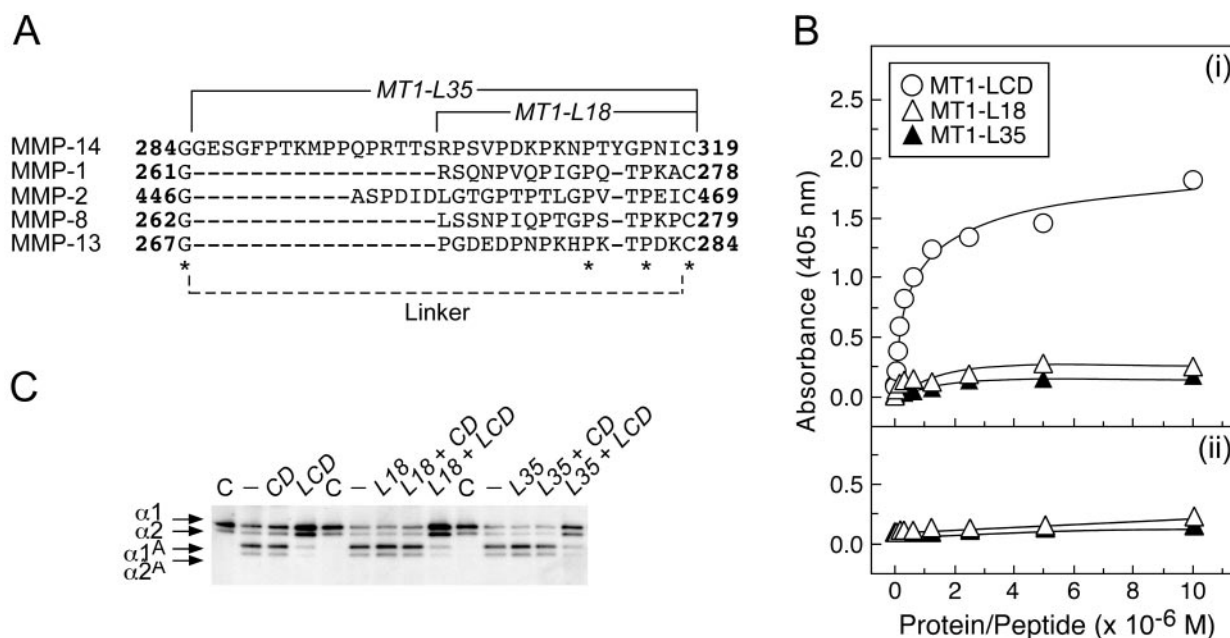


**FIG. 5. Recombinant MT1-MMP hemopexin C domain blocks MMP2 cleavage of native collagen.** *A*, biotin-labeled type I collagen was incubated in the absence (*C*) or presence of MMP2 (0.84 pmol) for 18 h at 28 °C. MT1-LCD and MT1-CD (0.1–100-fold molar equivalents) were added to the reactions where indicated. Following digestion, samples were separated by SDS-PAGE (7.5%), followed by Western blotting using streptavidin-HRP.

CD. As shown in Fig. 6*C*, no inhibition of collagenolysis was observed. In a second set of experiments, MT1-LCD inhibited collagen cleavage as previously observed (Fig. 4), regardless of whether MT1-L18 or MT1-L35 was added. Since the presence of the linker sequence and the hemopexin C domain together as separate polypeptides is not sufficient for disrupting cleavage, these data suggest that the ability of the MT1-LCD to inhibit collagenolysis is context- and/or conformation-specific.

**Cellular Invasion of Type I Collagen is Inhibited by a 44-kDa MT1-MMP Ectodomain Fragment**—Active MT1-MMP is efficiently processed to a 44-kDa ectodomain fragment containing the MT1-LCD sequence (Gly<sup>285</sup>-Cys<sup>508</sup>) that is retained on the cell membrane (40, 46, 51). Since the soluble MT1-LCD inhibits native collagen cleavage by sMT1-MMP, we hypothesized that 44-kDa MT1-MMP may also function in a similar manner at the cell surface to modulate the collagenolytic activity of transmembrane MT1-MMP. To test this hypothesis, we used MDA-MB-231 breast carcinoma cells, which express endogenous MT1-MMP in the absence of detectable levels of MMP2. Invasion of three-dimensional collagen gels overlaid onto a porous polycarbonate filter requires collagenolytic activity (42). In control experiments, MDA-MB-231 cellular invasive activity was inhibited by the hydroxamate inhibitor GM6001, indicating a requirement for metalloproteinase activity (Fig. 7*A*). TIMP-2 significantly reduced invasion ( $p < 0.05$ ), whereas TIMP-1 or the BSA control had no effect, confirming the dependence for MT-MMPs (60) in MDA-MB-231 cell invasion. A blocking antibody against the MT1-MMP active site (Fig. 7*A*, *anti-MT1*) also reduced invasion compared with IgG controls ( $p < 0.05$ ), identifying MT1-MMP as the critical protease in this process. Indeed, overexpression of MT1-MMP on MDA-MB-231 cells increased collagen invasion  $\sim 2.5$ -fold compared with vector transfectants ( $p < 0.05$ , Fig. 7*A*, *black bars*). Furthermore, expression of the inactive mutant, MT1-MMP (E240A), on MDA-MB-231 cells resulted in inhibition of invasion to below control values, suggesting that this species may function in a dominant negative manner.

To determine whether the MT1-LCD could inhibit cell-associated collagenolytic activity, cells were incubated with MT1-LCD, MT1-L35, or BSA. At low concentrations (4  $\mu$ M), the invasion of MT1-MMP transfected (Fig. 7*B*) or parental (data not shown) MDA-MB-231 cells was unaffected. MT1-L35 did not affect invasion at any concentration tested (data not



**Fig. 6. Characterization of MT1-MMP peptide linker analogs.** A, sequence alignment of collagenolytic MMP linkers using Megalign (DNASTAR Inc.) (Clustal method). Conserved residues are denoted with asterisks. Sequences of the peptide analogs of MT1-MMP are indicated. B, a 96-well plate was coated with either native (i) or denatured (ii) type I collagen (rat tail) (0.5  $\mu\text{g}/\text{well}$ ). Serial dilutions of MT1-LCD, MT1-L18, and MT1-L35 were added, and bound protein/peptide was detected using RP1MMP-14 antibody, which recognizes the linker. C, biotin-labeled type I collagen was incubated in the absence (C) or presence of sMT1-MMP (1 pmol) for 18 h at 28  $^{\circ}\text{C}$ . Molar excesses of MT1-MMP hemopexin C domain constructs (CD and LCD) (100-fold) and linker peptide analogs (L18 and L35) (1000-fold) were added to the reaction where indicated. Reactions were separated by SDS-PAGE (7.5%), followed by Western blotting using streptavidin-HRP.

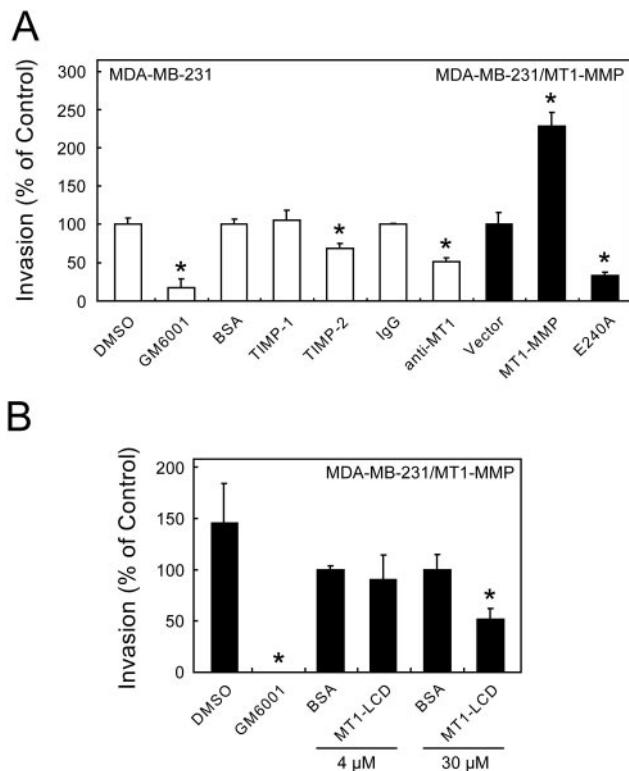
shown). However, since MT1-LCD binds native collagen, the effective concentration of free protein available to the cells may be reduced by binding to the collagen filters. Therefore, the highest concentration possible with these protein preparations (30  $\mu\text{M}$ ) was used to ensure saturation of binding sites within the collagen-coated filters and availability of free protein at the cell surface to interact with MT1-MMP. Under these conditions, collagen invasion was significantly reduced ( $p < 0.05$ ; Fig. 7B), demonstrating inhibition of cell-associated MT1-MMP collagenolytic activity and confirming the *in vitro* analysis of MT1-LCD inhibiting collagen cleavage.

Because autolysis of transmembrane MT1-MMP leads to the accumulation of a cell surface 44-kDa MT1-MMP ectodomain fragment containing the linker and hemopexin C domain but lacking the active site, the effect of this cell-associated product on cellular MT1-MMP-mediated collagenolysis was assessed. For this experiment, transmembrane constructs of MT1-LCD (Gly<sup>285</sup>-Val<sup>582</sup>, designated cMT1-LCD) and MT1-CD (Pro<sup>316</sup>-Val<sup>582</sup>, designated cMT1-CD) (Fig. 8A) were expressed in MDA-MB-231 cells, and type I collagen invasion was assessed relative to vector-transfected controls. Intracellular furin processing of these constructs at Arg<sup>111</sup> generates the 44-kDa MT1-MMP and the linker-deleted form thereof. Expression of cMT1-LCD significantly reduced invasion by 50% to levels similar to those seen with soluble MT1-LCD when compared with cells expressing cMT1-CD ( $p < 0.05$ ; Fig. 8B). This confirms the above results and the *in vitro* biochemical analyses and demonstrates the importance of the MT1-MMP linker-hemopexin C domain in native collagen cleavage by cellular MT1-MMP. In control experiments, migration of MDA-MB-231 cells toward type I collagen, a process independent of collagenase activity (42, 61), was unaffected by expression of cMT1-LCD or cMT1-CD (Fig. 8C). Since these data clearly demonstrate the ability of cMT1-LCD to modulate type I collagen cleavage by transmembrane MT1-MMP, our results suggest that a function of the endogenous MT1-MMP autolysis product, 44-kDa MT1-MMP, is to regulate pericellular collagenolytic activity.

#### DISCUSSION

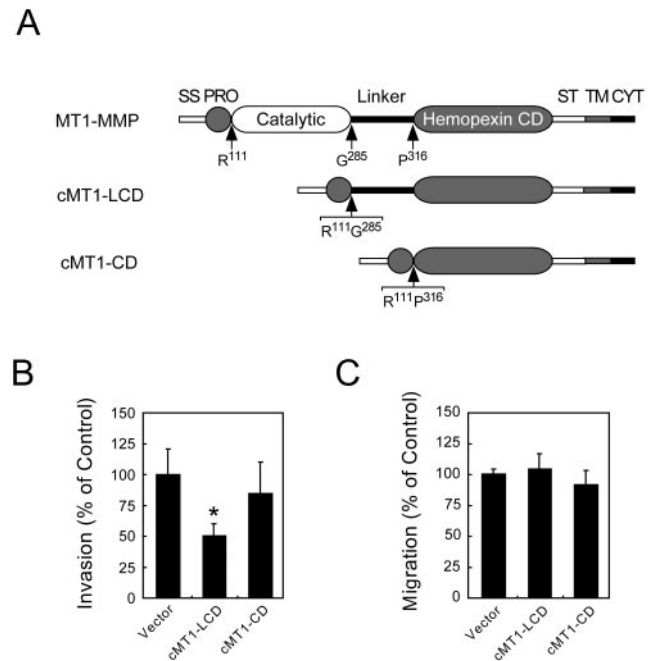
As an integral membrane protein, MT1-MMP appears suited for coordinating the homeostatic catabolism of pericellular type I collagen under the guide of the cell (62–65). MT1-MMP mediates collagen degradation directly by cleaving native collagen and, indirectly, by activating MMP13 (9) and the gelatinase and weak collagenase, MMP2 (11–13). Spatially and temporally, these two distinct activities of MT1-MMP regulate collagenolytic and gelatinolytic activities on the cell surface. Since MT1-MMP is a critical initiator and effector in the pericellular collagenolytic cascade, the regulation of its biological activity is very important in physiological and pathological collagen remodeling. The studies reported here have revealed the importance of the MT1-MMP hemopexin C domain and linker in the mechanism of collagen cleavage and demonstrated the role of collagen binding to MT1-MMP in stimulating MMP2 activation by cells. Moreover, these actions may be modulated in a dominant negative manner by the 44-kDa remnant form of MT1-MMP on the cell surface, revealing a novel regulatory function in proteolysis for an autolytic fragment of a protease.

The structure of collagen presents a challenge for proteolytic cleavage, as indicated by the low  $k_{\text{cat}}/K_m$  values for collagenases (66). Despite several studies from a number of laboratories, the triple helix mechanism remains enigmatic (14). Our use of recombinant domains and polypeptides to probe the exosite requirements of MT1-MMP for collagenolysis revealed similar domain requirements for triple helix activity as the secreted collagenases. The binding of the MT1-MMP hemopexin C domain, with or without the linker, to native collagen is consistent with previous reports for the collagenolytic MMPs (27, 30–32). The hemopexin C domain of MMP2, in contrast, does bind native collagen stably (57). Interestingly, the MT1-MMP hemopexin C domain does not bind denatured collagen. This suggests that, following cleavage, subsequent denaturation of the collagen would result in the release of MT1-MMP from the cleaved substrate facilitating turnover.



**FIG. 7. Invasion of MDA-MB-231 cells is MT1-MMP-dependent and is inhibited by recombinant MT1-MMP hemopexin C domain.** *A* and *B*, invasion of type I collagen. Cells ( $2.5 \times 10^5$ ) were seeded onto Transwell filters (8- $\mu$ m pore) coated with a type I collagen gel (20  $\mu$ g) and allowed to invade for 24 h as described under "Experimental Procedures." Noninvading cells were removed from the upper chamber with a cotton swab. Filters were then stained, and cells, adherent to the underside of the filter, were enumerated using an ocular micrometer. The average of triplicate experiments were normalized to corresponding controls (designated 100%) and are presented with S.D. value shown (\*,  $p < 0.05$ ). *A*, parental MDA-MB-231 cells (white) were allowed to invade in the presence of Me<sub>2</sub>SO (DMSO), GM6001 (10  $\mu$ M), BSA (10 nM), TIMP-1 (10 nM), TIMP-2 (10 nM), purified rabbit IgG (IgG; 10  $\mu$ g/ml), or AB8102 antibody (anti-MT1; 10  $\mu$ g/ml). MDA-MB-231 cells expressing MT1-MMP or MT1-MMP(E240A) (black) were also analyzed. Results are expressed as percentage of control invasion (versus BSA and Vector, as appropriate). *B*, MDA-MB-231 cells expressing MT1-MMP ( $2.5 \times 10^5$ ) were incubated with Me<sub>2</sub>SO, GM6001 (10  $\mu$ M), MT1-LCD, and BSA (4 and 30  $\mu$ M) and allowed to invade for 24 h. Results are expressed as percentage of control invasion (versus BSA).

Inhibition of sMT1-MMP collagen cleavage using MT1-MMP hemopexin C domain constructs required the presence of the linker, indicating that collagen binding, by the hemopexin C domain alone, is not sufficient to disrupt collagenolysis. This requirement was also observed in MMP2 collagenolysis, since MT1-LCD, but not MT1-CD, blocked MMP2 cleavage of native collagen. Protein engineering studies of MMP1 and MMP8 have previously shown a role for the linker in triple helix activity (33–35); however, our studies have revealed some unique features of the MT1-MMP linker. De Souza *et al.* (67) proposed that the MMP1 collagenase linker, due to its proline content, intercalates with the collagen triple helix, thereby displacing individual  $\alpha$ -chains for cleavage. We found that MT1-MMP linker peptide analogs of either the full-length 35-amino acid residue linker or the 18-amino acid residue region, corresponding to that found in the secreted collagenases, did not bind native or denatured type I collagen. These results indicate that the MT1-MMP linker may not bind or intercalate with the collagen triple helix as proposed for the MMP1 linker. Indeed, the low glycine content renders triple helix formation by these linkers impossible. Potentially, the full collagen bind-



**FIG. 8. Recombinant 44-kDa MT1-MMP inhibits MDA-MB-231 cell invasion.** *A*, linear diagram of MT1-MMP and deletion mutants, cMT1-LCD ( $\Delta 112$ –284) and cMT1-CD ( $\Delta 112$ –315). The signal sequence (SS), propeptide domain (PRO), stalk segment (ST), transmembrane sequence (TM), and cytoplasmic tail (CYT) are indicated. Invasion of type I collagen (*B*) and migration (*C*) of MDA-MB-231 cells expressing MT1-MMP deletion mutants, cMT1-LCD and cMT1-CD, were assessed. *B*, cells ( $2.5 \times 10^5$ ) were seeded onto Transwell filters (8- $\mu$ m pore) coated with a type I collagen gel (20  $\mu$ g) and allowed to invade for 24 h as described under "Experimental Procedures." *C*, cells ( $2.5 \times 10^5$ ) were seeded onto Transwell filters coated with a thin layer of collagen on the underside and incubated for 1.5 h to permit migration. In both assays, noninvading or nonmigrating cells were removed from the upper chamber with a cotton swab. Filters were then stained, and cells, adherent to the underside of the filter, were enumerated using an ocular micrometer. The averages of triplicate experiments were normalized to the vector control (designated 100%) and are presented with S.D. value as shown (\*,  $p < 0.05$ ).

ing exosite of the MT1-MMP hemopexin C domain that recognizes the  $3/4$ - $1/4$  collagen site may span the linker/hemopexin C domain junction, thereby accounting for the lack of collagen binding by the linker analogs alone and the ineffectiveness of MT1-CD in blocking collagenolysis. The MT1-MMP linker, when connected to the hemopexin C domain, may act as a specificity determinant directing binding of the protease to the  $3/4$ - $1/4$  collagen cleavage site. Thus, competition from MT1-LCD, but not MT1-CD, may block MT1-MMP from binding collagen here and so inhibit cleavage. Topographically, the MT1-MMP, and other collagenase linkers, may also correctly configure the catalytic domain relative to the hemopexin C domain for collagenolytic competence. Indeed, the MT1-MMP linker has predicted rigidity due to the presence of 9 proline residues, and we interpret the x-ray crystallographic structure of the MMP1 linker (68) to also indicate that the linker is not as flexible as generally thought. Hence, MT1-LCD binding of collagen may sterically disrupt the collagenolytic configuration of sMT1-MMP at the  $3/4$ - $1/4$  collagen cleavage site, thereby inhibiting cleavage.

The importance of MT1-MMP in collagen homeostasis is supported by the finding that fibrillar type I collagen induces cell surface expression of MT1-MMP and subsequent MMP2 activation through transcriptional and nontranscriptional pathways (36–41, 69). Induction of MT1-MMP transcription is dependent on  $\beta_1$  integrin receptors and actin cytoskeleton rearrangement (38, 43). Clustering of  $\beta_1$  integrins by collagen

ligation or antibody cross-linking induces *de novo* expression of MT1-MMP and subsequent MMP2 activation (40, 42). Interestingly, our data reveal that collagen may also assemble MT1-MMP on the cell surface via binding to the hemopexin C domain, thereby increasing the local concentration of MT1-MMP for collagenolysis and efficient MMP2 activation. In view of the demonstrated absence of oligomer formation by the MT1-LCD used here and previously reported (47), we interpret the reduction in collagen-induced MMP2 activation by MT1-LCD to be the result of competitive binding for collagen between the exogenous MT1-LCD and cell surface MT1-MMP, rather than competitively disrupting any MT1-MMP-MT1-MMP binding interactions. Indeed, this interaction between MT1-MMP and collagen may represent a biological mechanism similar to that observed with ConA, which clusters MT1-MMP on the cell surface during MMP2 activation (47). As originally shown, ConA increases the matrix-degradative phenotype of the cell through transcriptional and post-transcriptional regulation of MMP and TIMP genes that was reflected by extensive endogenous collagen degradation in the conditioned media and in biochemical assays (50). Cleavage of  $\beta_1$  integrin-ligated collagen also releases bound pro-MMP2, which can now enter the activation pathway, which otherwise is recalcitrant to activation (70). Hence, pericellular collagen has multiple effects in binding and regulating the activities of collagenolytic MMPs, representing an unusual relationship between a protease and cognate substrate that appears to contribute to the homeostatic maintenance of collagen levels.

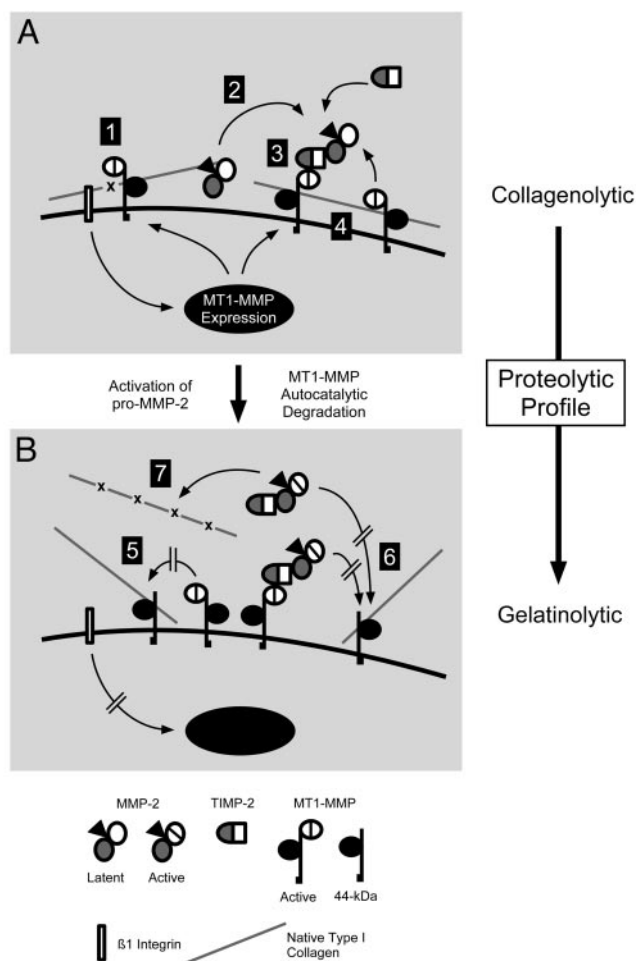
MT1-MMP activity on the cell surface is further regulated by endocytosis (71, 72), TIMP binding (45, 56, 73), and trimolecular complex formation (56, 74) as well as the autolytic shedding of the catalytic domain to yield 44-kDa MT1-MMP (46, 51, 75, 76). Currently, the role of 44-kDa MT1-MMP *in vivo* is not clear. It has been reported recently that the hemopexin C domain and the cytoplasmic tail of MT1-MMP mediate homophilic interactions that increase MMP2 activation (48, 49). Using HT1080 cells, Itoh *et al.* (48) found that expression of MT1-MMP PEX (Thr<sup>313</sup>-Val<sup>582</sup>), a truncated form of 44-kDa MT1-MMP that lacks most of the linker and hence is similar to cMT1-CD used here, reduced MMP2 activation and subsequent Matrigel invasion, presumed to be by disrupting the formation of oligomeric MT1-MMP complexes. PEX is unfortunately a confusing designation for the MT1-MMP hemopexin C domain, since PEX was already the name of a cell surface zinc metallopeptidase belonging to the neprilysin family (77, 78). As reported here and previously (47), we have found no evidence for oligomerization using MT1-LCD or MT1-CD, emphasizing the importance of cell membrane context or the stalk segment, transmembrane sequence, and cytoplasmic tail in these proposed complexes. Our recent data<sup>2</sup> indicate that the stalk segment also does not dimerize or drive oligomerization of 44-kDa MT1-MMP. Unlike the effects of MT1-LCD in disrupting the collagen-induced activation of MMP2 shown here, the inability of soluble MT1-LCD or MT1-CD to competitively block ConA-induced MMP2 activation in cells cultured on plastic reported previously (47) indicates the importance of cellular context for these effects and highlights the difference in collagen-mediated activation of MMP2, which is blocked by MT1-LCD, from activation induced by MT1-MMP overexpression or ConA, which is not.

In our previous studies of chemokine cleavage by MMP2, we found that MCP-3 and SDF-1 $\alpha$  binding to the hemopexin C domain markedly improved the catalytic efficiency of cleavage (79, 80). Notably, the addition of recombinant MMP2 he-

mopexin C domain to mixtures of chemokine and active MMP2 in enzyme assays could entirely block substrate cleavage (80). Therefore, the presence of the entire 35-amino acid residue linker and hemopexin C domain in the 44-kDa MT1-MMP ectodomain suggested to us that this autolytic product has the potential to antagonize the proteolytic activity of MT1-MMP in a dominant-negative manner by interacting with native collagen. Our data demonstrate that expression of cMT1-LCD (Gly<sup>285</sup>-Val<sup>582</sup>), representing the 44-kDa MT1-MMP in its entirety (46, 76), on MDA-MB-231 cells inhibits MT1-MMP-mediated type I collagen cleavage and cell invasion. The inhibitory effect of cMT1-LCD expression on cell invasion was confirmed by the addition of soluble MT1-LCD to MT1-MMP-transfected cells. Since MDA-MB-231 cells do not express MMP2, the effect of cMT1-LCD expression and MT1-LCD on collagenolysis and cell invasion is distinct from that reported previously (48) and discussed above. Nonetheless, the capacity of MT1-LCD to also block MMP2 native collagen cleavage may amplify the down-regulation of collagenolysis *in vivo* by blocking MMP2 in addition to MT1-MMP but sparing MMP2 gelatinolysis. Invasion was also inhibited with the expression of MT1-MMP (E240A), a dominant-negative mutant mimicking TIMP-2-inhibited MT1-MMP, further supporting the role of MT1-MMP in collagen invasion and of inactive MT1-MMP forms in competing for collagen binding and down-regulating collagen cleavage. Consistent with our biochemical analysis, neither the expression of cMT1-CD nor the addition of soluble MT1-CD (data not shown) affected cell invasion to a significant degree, confirming the importance of the MT1-MMP linker in context with the hemopexin C domain in collagenolysis. In view of these effects, we propose that 44-kDa MT1-MMP may reduce MMP2 activation by reducing MT1-MMP clustering mediated by pericellular collagen. Together, these results clearly reveal the 44-kDa MT1-MMP as a novel inhibitor of pericellular type I collagen cleavage by MT1-MMP and MMP2 activities. Our studies also demonstrate the feasibility of designing new MMP inhibitors that target the substrate rather than the protease (81). This new class of inhibitors may exert highly selective substrate-specific protease inhibition while sparing the cleavage of other substrates in the protease degradome. Similarly, targeting the protease exosite rather than the active site may also represent new avenues of substrate-specific inhibition to achieve levels of specificity not possible with active site inhibitors (81).

The degradation of pericellular type I collagen is revealed to be a dynamic self-regulated process. We have previously proposed models regarding the regulation of pericellular type I collagen levels upon  $\beta_1$  integrin stimulation of MT1-MMP and MMP2 activity (14, 40, 70). Our investigation into the role of the 44-kDa MT1-MMP ectodomain adds a new dimension to this homeostatic process. As modeled in Fig. 9, fibrillar type I collagen induces a  $\beta_1$  integrin-dependent increase in MT1-MMP expression on the cell surface, thus favoring an initial collagenolytic phase. Our data show that the collagen binding properties of the MT1-MMP hemopexin C domain are necessary for native collagen cleavage (Fig. 9A). As suggested previously (70), the release of collagen-bound pro-MMP2 from the cell surface following collagen cleavage by MT1-MMP allows pro-MMP2 reservoirs to be optimally activated temporally and spatially in relation to its substrate. Collagen binding by the MT1-MMP hemopexin C domain also potentiates MMP2 activation, most likely by concentrating MT1-MMP-TIMP-2-pro-MMP2 complexes with TIMP-free MT1-MMP (Fig. 9A). Furthermore, in the MMP2 activation process, MT1-MMP collagenolytic activities are suppressed by TIMP-2 binding to form the trimolecular pro-MMP2 complex and by MT1-MMP autolysis, converting the proteolytic signature of the cell from

<sup>2</sup> E. M. Tam and C. M. Overall, unpublished data.



**FIG. 9. Potential role of the 44-kDa MT1-MMP in pericellular collagen degradation.** A, collagenolytic cell profile. Upon collagen-induced engagement of  $\beta_1$  integrins and intracellular signaling, expression of MT1-MMP is up-regulated on the cell surface. Increased MT1-MMP expression promotes the cleavage of native collagen (1) and the release of collagen-bound pro-MMP2, which now enters into the activation pathway (2). The conversion from collagenolysis to gelatinolysis commences with the formation of the trimolecular complex (3), which reduces MT1-MMP collagenolytic activity, and the activation of pro-MMP2, which is enhanced by the collagen-mediated assembly of MT1-MMP (4). Collagen binding by TIMP-2-inhibited MT1-MMP in the trimolecular complex may also block collagen cleavage by uninhibited MT1-MMP. B, gelatinolytic cell profile. Following pro-MMP2 activation and MT1-MMP autolysis, the 44-kDa MT1-MMP accumulates on the cell surface, binds native collagen, and suppresses collagen degradation by inhibiting MT1-MMP (5) and MMP2 (6) collagenolysis but not MMP2 gelatinolysis (7). Stimulation of cell surface MT1-MMP expression is reduced due to the absence of native collagen and  $\beta_1$  integrin engagement which together allow collagen levels to increase.

collagenolytic to gelatinolytic. Following MT1-MMP autolytic shedding, our data show that the 44-kDa MT1-MMP continues to bind collagen, further reducing pericellular collagenolysis by MT1-MMP and MMP-2 (Fig. 9B). Overall, these intimately related and complex events allow for a conversion of proteolytic activity to take place on the cell surface. This shift from a collagenolytic to a gelatinolytic profile may be important for maintaining pericellular collagen levels. Thus, collagen is a unique substrate; by binding the proteases responsible for its cleavage, these interactions recruit and regulate collagenolytic and gelatinolytic activities in a homeostatic manner. Hence, the studies reported here reveal several new aspects in the biology of MT1-MMP as a consequence of native type I collagen binding by the hemopexin C domain. This also provides a novel explanation for the generation of MT1-MMP clusters on the cell

surface and adds a new layer of control to the complex regulation of focal proteolysis by MT1-MMP and MMP2.

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