Membrane type 1 (MT1) matrix metalloproteinase (MMP-14) is a membrane-tethered MMP considered to be a major mediator of pericellular proteolysis. MT1-MMP is regulated by a complex array of mechanisms, including processing and endocytosis that determine the pool of active proteases on the plasma membrane. Autocatalytic processing of active MT1-MMP generates an inactive membrane-tethered 44-kDa product (44-MT1) lacking the catalytic domain. This form preserves all other enzyme domains and is retained at the cell surface. Paradoxically, accumulation of the 44-kDa form has been associated with increased enzymatic activity. Here we report that expression of a recombinant 44-MT1 (Gly285–Val582) in HT1080 fibrosarcoma cells results in enhanced pro-MMP-2 activation, proliferation within a three-dimensional collagen I matrix, and tumor growth and lung metastasis in mice. Stimulation of pro-MMP-2 activation and growth in collagen I was also observed in other cell systems. Expression of 44-MT1 in HT1080 cells is associated with a delay in the rate of active MT1-MMP endocytosis resulting in higher levels of active enzyme at the cell surface. Consistently, deletion of the cytosolic domain obliterates the stimulatory effects of 44-MT1 on MT1-MMP activity. In contrast, deletion of the hinge turns the 44-MT1 form into a negative regulator of enzyme function in vitro and in vivo, suggesting a key role for the hinge region in the functional relationship between active and processed MT1-MMP. Together, these results suggest a novel role for the 44-kDa form of MT1-MMP generated during autocatalytic processing in maintaining the pool of active enzyme at the cell surface.

Membrane type 1 matrix metalloproteinase (MT1-MMP, MT1-MMP

1 is a type I-transmembrane protease and a major mediator of pericellular proteolysis. MT1-MMP is responsible for the proteolytic cleavage of multiple pericellular and membrane-associated substrates, including collagens and other extracellular matrix proteins, growth factors, growth factor receptors, cell adhesion proteins and their receptors, cytokines, protease inhibitors, and proteases, just to mention a few (1–5). MT1-MMP is also the major physiological activator of pro-MMP-2 (pro-gelatinase A) on the cell surface (6, 7), a process that further contributes to pericellular proteolysis. As a multifunctional protease, MT1-MMP elicits profound effects on cell behavior and has been implicated in the pathogenesis of various human diseases, including cancer (8–10), diabetes (11, 12), vascular (13, 14), and connective tissue diseases (2).

The importance of MT1-MMP for pericellular proteolysis demands a tight control of its catalytic activity at the cell surface. This is partly achieved by the action of endogenous protease inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site inhibiting catalysis (15). In addition, by virtue of being a membrane-anchored protease, MT1-MMP developed a unique set of regulatory mechanisms that control enzymatic activity independently of TIMPs. These processes include the targeting of active enzyme to specific plasma membrane locations, endocytosis, and autocatalytic processing (1, 16–19). Together, these distinct processes determine the level of active enzyme on the cell surface. However, how these processes are integrated to control the pool of active MT1-MMP is not understood.

The processing of MT1-MMP is a cell surface event in which the active enzyme is usually autocatalytically cleaved in trans to generate a major membrane-anchored product of ~44 kDa (also referred to as the 43- or 45-kDa species in some studies) and a soluble ~18-kDa inactive fragment of the catalytic domain (6, 20–25). The 44-kDa product of MT1-MMP is detected in cultured cells expressing natural MT1-MMP (20, 26–32) and has been found in platelets (33), human tumors extracts (34–36), and extracts of arthritic synovial tissues (37). MT1-MMP processing is stimulated by a variety of factors known to stimulate MT1-MMP expression, trafficking, and/or endocytosis, including phorbol ester (21, 24, 38, 39), concanavalin A (conA) (24, 40–45), bafilomycin A1 (46, 47),

scription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; DAPI, 4′,6-diamidino-2-phenylindole; EV, empty vector; conA, concanavalin A; TIMP, tissue inhibitor of metalloproteinase.
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cytochalasin D (40, 44), transforming growth factor-β1 (26), extracellular calcium (48), and extracellular matrix components (30, 43, 49–51). Expression of constitutively active Rac1 in HT1080 cells also promotes MT1-MMP processing (30). In addition, high levels of enzyme expression (36, 48, 52–54) and low levels of TIMP-2 relative to MT1-MMP (6, 21) are associated with enhanced MT1-MMP processing. Finally, agents such as conA and cytochalasin D, which are known to inhibit clathrin-dependent endocytosis (55, 56), promote MT1-MMP processing, which indicates that the rate of MT1-MMP internalization influences processing. Collectively, these findings suggest a relationship between processing and the level and activity of MT1-MMP at the cell surface.

Although MT1-MMP processing is thought to terminate activity on the cell surface, the structural characteristics of the remnant 44-kDa product suggest that it may play a more complex role in enzyme regulation. The 44-kDa species is composed of the hinge region, the hemopexin-like domain, and the complete anchoring apparatus with its cytosolic tail. Furthermore, the 44-kDa fragment is retained on the cell surface, and like active MT1-MMP it is also cleared from the cell surface by endocytosis (28, 48). Previous studies showed that expression of recombinant species of MT1-MMP lacking the catalytic domain, analogous to the 44-kDa fragment, inhibited MT1-MMP-dependent pro-MMP-2 activation (57–59), collagen degradation (60), in vitro tumor cell migration and invasion (61, 62), and in vivo tumor growth (62). These studies suggested that the 44-kDa species of MT1-MMP behaves as a dominant negative regulator of enzyme function. Paradoxically, and contrary to these observations, a plethora of studies have shown a positive correlation between enhanced processing of MT1-MMP and enzymatic activity (21, 29, 30, 36, 46, 52–54, 63–65). Given these conflicting observations, we set forth to investigate the functional relationship between active MT1-MMP and its processed 44-kDa form in various cellular settings and its effect on enzyme function in vitro and in vivo. We present evidence that the 44-kDa form of MT1-MMP positively influences the level and activity of surface MT1-MMP by regulating enzyme internalization. This positive effect of the processed form depends on the presence of the hinge region. We propose a model in which the 44-kDa form of MT1-MMP regulates the endocytosis of the active protease to preserve a viable level of MT1-MMP on the cell surface.

EXPERIMENTAL PROCEDURES

Cell Culture—Human fibrosarcoma HT1080 cells, human breast carcinoma MDA-MB-231 cells, human breast carcinoma T47D cells, and monkey kidney epithelial BS-C-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HT1080, MDA-MB-231, and BS-C-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. T47D cells were cultured in RPMI 1640 medium supplemented with 10% FBS, insulin, and antibiotics. Human glioma U87 cells were a gift from Dr. Bonnie Sloane (Wayne State University, Detroit, MI) and were cultured in minimum essential media supplemented with 10% FBS and antibiotics. Human breast carcinoma BT549 cells, a kind gift from Dr. Hyeong-Reh Choi Kim (Wayne State University, Detroit, MI), were cultured in DMEM/F-12 supplemented with 10% FBS and antibiotics. HeLa cells (CCL-2.2) were obtained from the ATCC and were cultured in Spinner medium (Quality Biologicals Inc., Gaithersburg, MD) supplemented with 5% horse serum and antibiotics.

Recombinant Vaccinia Viruses—The production of the recombinant vaccinia virus (vTF7-3) expressing bacteriophage T7 RNA polymerase has been described (66). Recombinant vaccinia viruses expressing human pro-MMP-2, TIMP-2, or TIMP-1 were obtained by homologous recombination, as described previously (66).

Recombinant Proteins and Antibodies—Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as described previously (67). The rabbit polyclonal antibody (pAb) 437, raised against residues 437–454 of the hemopexin-like domain of human MT1-MMP was described previously (6). The rabbit pAb 198 raised against a synthetic peptide including residues 160–174 of the catalytic domain of human MT1-MMP was a generous gift from Dr. Q. X. Amy Sang (Florida State University) (68). The LEM2/15 monoclonal antibody (mAb), a generous gift from Dr. A. Arroyo (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain), was raised against a synthetic peptide comprising residues 218–233 of the catalytic domain of human MT1-MMP (69). The rabbit pAb to the cytosolic domain of MT1-MMP (RP2-MMP-14) was purchased from Triple Point Biologics, Inc. (Forest Grove, OR). The rabbit pAb 815 against the hinge domain of MT1-MMP was purchased from Chemicon (Temecula, CA). The mAb to the human transferrin receptor was purchased from Zymed Laboratories Inc. The mAb to β-actin was purchased from Sigma.

MT1-MMP cDNA Constructs—To express the 44-kDa form of MT1-MMP (referred to as 44-MT1 in Fig. 1A), the entire catalytic domain of MT1-MMP (Tyr112 to Gly285) was deleted by mutagenic PCR from pro-MT1-MMP using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA), specific primers, and wild-type pro-MT1-MMP cDNA as the template. This procedure generated a cDNA fragment in which the furin-recognition motif ending at Arg111 was positioned immediately upstream of Gly285 at the beginning of the hinge region. This cDNA fragment was cloned into the pcDNA3.1 (Invitrogen) expression vector for stable transfection, the pTF7-EMCV-1 vector for transient expression in the vaccinia expression system (66), and the pIND vector for inducible expression in response to ponasterone A (Invitrogen). In addition, we generated a series of cDNA constructs to express 44-MT1 lacking the cytosolic tail (ending at Arg563) and referred to as 44ACT; Fig. 1A) and 44-MT1 lacking the hinge region (residues 285–315 and referred to as 44A; Fig. 1A). These cDNA constructs were inserted into the pcDNA3.1 expression vector. The correct sequence of all the cDNA constructs was verified by DNA sequencing of both strands.

Stable Transfections—Cells (HT1080, MDA-MB-231, U87, and T47D) were stably transfected with empty pcDNA3.1 vector (referred to as EV cells) or pcDNA3.1/44-MT1 vector (referred to as 44-MT1 cells) using the Effectene transfection
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reagent (Qiagen, Valencia, CA) as described by the manufacturer. T47D breast carcinoma cells were also transfected with wild-type MT1-MMP in pcDNA3.1 vector. Geneticin® (G418 sulfate, Invitrogen)-resistant pooled populations were selected using conventional approaches. HT1080 and U87 cells were also transfected with 44ΔCT and 44ΔH in pcDNA3.1, and both clones and pooled populations were obtained by Geneticin® selection. For most studies, both pooled populations and representative single clones were used. For in vivo studies, two representative clones with each construct, which varied in detected amounts of recombinant protein expression, were pooled (pooled clones).

Inducible Expression System—BT549 breast carcinoma cells were double-transfected with the pVgRXR vector (Invitrogen), which expresses the heterodimeric ecdyson receptor and the retinoid X receptor, and the pIND-inducible expression vector that expresses a modified ecdyson-reaction element upstream of a minimal heat shock promoter and a multiple cloning site. In the presence of ponasterone A, heterodimeric receptors binding to a modified ecdyson-response element induce expression of the 44-MT1 cDNA that was previously inserted into the pIND-inducible expression plasmid (Invitrogen).

Infection-Transfection—BS-C-1 cells seeded in 6-well plates were infected with 1 plaque-forming unit/cell of VTF7-3 vaccinia virus for 45 min in DMEM containing 2.5% FBS. The media were removed, and the infected cells were co-transfected with pTF7-EMCV-1 vector expressing wild-type MT1-MMP alone or 44-MT1 at 1:1 and 1:10 molar ratios (MT1-MMP:44-MT1) or with empty vector alone, up to a total of 3 mMli sample buffer without reducing agents and heating. In some experiments, the stably transfected cells were treated with 10 μg/ml of concanavalin A (conA, Sigma) overnight in serum-free media. In other experiments, HT1080 transfecants suspended in complete media were seeded in 12-well plates, which were uncoated or previously coated with 1.5 mg/well of rat tail collagen I (Trevigen, Gaithersburg, MD). Six h later, the media were aspirated and replaced with 0.3 ml/well serum-free media followed by an overnight incubation. Pro-MMP-2 activation was monitored in the media by gelatin zymography, as described previously (70).

Cell Surface Biotinylation—Cells in 6-well plates were cooled down for 5 min, rinsed with cold phosphate-buffered saline (PBS), pH 7.4, containing 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-CM), and then biotinylated with 0.5 mg/ml EZ-link sulfo-NHS-biotin (Pierce) for 30 min at 4 °C, as described previously (24). As a control, a parallel plate of cells received PBS without biotin. Biotin was quenched with 50 mM NH4Cl in PBS-CM for 10 min at 4 °C. The cells were lysed with 0.2 ml/well of lysis buffer and centrifuged (13,000 rpm, 20 min), and the supernatant was incubated with 50 μl of streptavidin beads (Pierce) overnight at 4 °C. The beads were washed four times with harvest buffer (0.5% SDS, 60 mM Tris/HCl, pH 7.5, 2 mM EDTA) supplemented with 2.5% Triton X-100 (final concentration). The bound biotinylated proteins were eluted with reducing Laemmli SDS sample buffer, boiled, and resolved by 10% SDS-PAGE, followed by transfer to a nitrocellulose membrane. The biotinylated MT1-MMP forms were detected with the pAb 437, pAb 815, or mAbLEM2/15 MT1-MMP antibody. The blot was reprobed with transferrin receptor antibodies as a control for loading.

Semiquantitative RT-PCR—Total RNA of HT1080 cells was extracted with RNeasy® mini kit (Qiagen, Valencia, CA). RT-PCR was performed with 1 μg of each total RNA sample using SuperScript™ III reverse transcriptase (Invitrogen) and subsequently HotStar Taq® master mix kit (Qiagen) following the manufacturer’s instructions. The sequences of the specific primers (IDT, Coralville, IA) for human TIMP-2 used are as follows: forward, 5′-GAA ACG ACA TTT ATG GCA ACC-3′; reverse, 5′-GCT GGA CCA GTC GAA ACC-3′. Thirty cycles of PCR were performed. The housekeeping gene GAPDH was also amplified and used as an internal control. The sequences of the human GAPDH primers (IDT) are as follows: forward, 5′-CCA CCC ATG GCA AAT TTC ATG GCA-3′; reverse, 5′-GCT GGA CCA GTC GAA ACC-3′. The amplified fragments were resolved by 1% agarose gels and detected by ethidium bromide staining.

TIMP-2 ELISA—Cells in 6-well plates were incubated at 37 °C with serum-free media. Forty eight hours later, the media were collected, and the cells were rinsed twice with PBS, solubilized with cold lysis buffer, and centrifuged. The conditioned media were collected and clarified by centrifugation and concentrated with a Microcon YM-10 centrifugal filter device. Concentrated media and the lysate samples were analyzed for TIMP-2 protein by ELISA (QIA40, Oncogene Research Products, San Diego) as described by the manufacturer’s instructions.

Growth of Cells within a Three-dimensional Collagen I Matrix—Cells suspended in 50 μl of serum free-media were added to a solution of 3.0 mg/ml rat tail collagen I, which was previously neutralized with 0.1 N NaOH. The mixture of cells and collagen was poured into the wells (0.5 ml/well, 10⁴ cells/well for HT1080 and MDA-MB-231 cells, and 2 × 10⁴ cells/well for U87 cells) of a 12-well tissue culture plate in the absence of
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presence of recombinant 12.5 μg/ml TIMP-1 or 5 μg/ml TIMP-2, and the plates were incubated at 37 °C for 1 h to allow polymerization. Then each well received 3 ml of complete media (DMEM + 10% FBS + antibiotics and Geneticin®). After 11 days incubation for HT1080 cells and 14 days for MDA-MB-231 and U87 cells, the media were removed, and the collagen matrix was washed twice with PBS. Then 10 units of bacterial collagenase (Sigma) per 1 mg of collagen were added to the wells and incubated for 15 min at 37 °C. After the collagen gel was completely dissociated, complete media were added to stop collagenase activity, and the solution was centrifuged (1,000 rpm) for 5 min and washed twice with PBS. The cells were counted in the presence of trypan blue. Triplicate wells were used for each of the stable transfectant cell lines. Each experiment was performed in triplicate, and quantification values represent the means of three independent experiments ± S.D. Statistical significance was determined using the Student’s t test.

RNA Interference—Gene silencing of endogenous MT1-MMP in pooled populations of HT1080 cells transfected with 44-MT1 was performed using sequence-specific small interfering RNA (siRNAs), which were pre-designed by Ambion (Austin, TX). siRNAs used here to specifically target the human sequence of catalytic domain of MT1-MMP were as follows: forward, 5'-CGG AGA AUU UUG UGC UGC CTT-3'; reverse, 5'-GGC AGC ACA AAA UUC UCC GTG-3'. As a negative control, scramble and GAPDH siRNAs (Ambion) were used. Briefly, HT1080 cells were seeded in 12-well plates (8 × 10⁴ cells/well) in complete media. The next day, the cells were transiently transfected with several concentrations of the MT1-MMP and control siRNAs using Effectene. Twenty four h post-transfection, the media were diluted 2-fold with fresh serum-free media, and the cells were incubated an additional 24 h before analyses.

Indirect Immunofluorescence Microscopy—Cells grown on 22-mm² glass coverslips were rinsed with PBS and acid-washed (5 min on ice) with a buffer composed of 50 mM glycine-HCl, pH 3.0, and 150 mM NaCl to dissociate TIMPs bound to the cell surface. The cells were then washed several times with ice-cold PBS-CM, and nonspecific sites were blocked with 5% normal bovine serum albumin. Following repeated washes with ice-cold PBS-CM, the cells were incubated (1–2 h, 4 °C) with 73 g/ml pAb 198 to MT1-MMP in ice-cold PBS-CM for 1 h at 4 °C. After washing with PBS-CM, the cells were incubated (1 h, 4 °C) with a 1:50 dilution of FITC-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch) in PBS supplemented with 1% bovine serum albumin. Following repeated washes with ice-cold PBS-CM, the cells were fixed with ice-cold 4% paraformaldehyde in PBS, pH 7.4, washed with PBS supplemented with 100 mM glycine and incubated (10 min, room temperature) with DAPI (Molecular Probes, Eugene, OR) for nuclei staining. Finally, the coverslips were mounted with anti-fade reagent (Molecular Probes).

To detect total MT1-MMP (intracellular and surface), the cells were acid-washed as described above and then fixed with ice-cold 4% paraformaldehyde in PBS followed by permeabilization with 0.5% Triton X-100 in PBS at room temperature. The cells were then incubated with primary and secondary antibodies as described above with the exception that all of the incubations were performed at room temperature. The nuclei were stained with DAPI, and the coverslips were mounted with 50% glycerol in PBS. Negative controls included cells incubated only with secondary antibody or cells incubated with normal rabbit IgG as a primary antibody. These conditions produced no specific signals (data not shown).

The samples were examined and photographed using a Zeiss 60× oil-immersion objective with a Zeiss LSM-510 META laser scanning confocal microscope at the Microscopy and Imaging Resources Laboratory at Wayne State University School of Medicine. DAPI was excited with a 745 nm multiphoton laser, and FITC was excited with a 488 nm argon laser. To compare the intensity of the fluorescence signal on the cell surface among the various transfectants, all the parameters for brightness and contrast were held consistent during the experiment. Average intensity of fluorescence signal per cell (as identified by DAPI staining) was measured and analyzed using the Meta-morph™ software, version 6.3, revision 2 (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate, and quantification values represent the means of three independent experiments ± S.D. Statistical significance was determined using the Student’s t test.

Quantitative Fluorescence-based MT1-MMP Antibody Uptake Assay—This assay was performed as described (54, 71). Briefly, HT1080 transfectants (EV, 44-MT1, 44ΔCT, and 44ΔH) were seeded (7 × 10⁴ cells per well) on 22-mm² glass coverslips placed on 6-well plates. Cells were cooled down for 5 min on ice and acid-washed with 50 mM glycine-HCl, 0.1 M NaCl, pH 3, for 5 min on ice followed by several washes with ice-cold PBS-CM, as described above. The cells were then incubated with 73 μg/ml pAb 198 to MT1-MMP in ice-cold PBS-CM for 2 h at 4 °C. Negative controls included cells incubated with equal amount of normal rabbit IgG as a primary antibody or only with secondary antibody. These conditions produced no specific signals (data not shown). Endocytosis was then allowed to proceed by shifting the cells to 37 °C for 10 min in warm PBS-CM. Endocytosis was stopped by the addition of 0.01% NaN₃, pH 7.2, in PBS-CM until the end of experiment. Cells were washed several times with ice-cold PBS-CM containing 0.01% NaN₃, pH 7.2, and then incubated with normal donkey serum (5% in PBS-CM) for 1 h at 4 °C. Following several washes with ice-cold PBS-CM at 4 °C, the cells were incubated with FITC-conjugated anti-rabbit IgG antibody (1:50 dilution in ice-cold PBS-CM) for 1 h at 4 °C. After washing with ice-cold PBS-CM, the cells were fixed with ice-cold 4% paraformaldehyde for 20 min at 4 °C and washed with PBS supplemented with 100 mM glycine. The nuclei were stained with DAPI, and the coverslips were mounted with anti-fade reagent (Molecular Probes). In some experiments, the cells were seeded on collagen I-coated plates (50 μg collagen I/well) before the antibody uptake assay. Samples were examined and photographed with a Zeiss LSM-510 META laser scanning confocal microscope using a 60× oil-immersion objective. The intensity of the fluorescence signal on the cell surface was analyzed as described above. Each experiment was performed in triplicate, and quantification values represent the means of three independent
experiments ± S.D. Statistical significance was determined using the Student’s t test.

Reversible Biotinylation Assay—We followed the procedure of Wu et al. (72). To this end, we used HT1080 transfectants (EV and 44-MT1) and parental HT1080 cells seeded in 6-well plates. In the case of HT1080 parental cells, the cells were treated overnight at 37 °C with conA (20 μg/ml) in the presence or absence of 10 μΜ GM6001 (Chemicon) in serum-free media. The next day, the cells were rinsed and cooled down for 5 min with cold PBS-CM and then biotinylated with 0.5 mg/ml disulfide-cleavable EZ-link sulfo-NHS-SS-biotin (Pierce) for 30 min. Excess biotinylating reagent was quenched with 50 mM NH4Cl in PBS-CM for 10 min at 4 °C followed by two washes of the cells with cold PBS-CM. To initiate internalization, the wells received pre-warmed serum-free media (3 ml/well) and the plates were immediately incubated at 37 °C for various times (0–120 min). After each time point, the plates were cooled down in ice to halt internalization, and the media were aspirated and the cells washed with cold PBS-CM. Cell surface-bound biotinylating reagent was stripped by incubating the cells (20 min on ice, twice) in 2 ml/well of reducing solution (150 mM NaCl, 1 mM EDTA, 1% bovine serum albumin, 20 mM Tris, pH 8.6, supplemented with 40 mM glutathione). In each experiment, a plate of biotinylated cells was maintained at 4 °C for 0–120 min, as a control of total surface MT1-MMP. These plates labeled “Total” did not receive reducing solution; instead they received PBS-CM. After addition of the reducing solution (endocytosed MT1-MMP) or PBS-CM (total MT1-MMP), the cells were washed twice with cold PBS-CM and lysed with 0.2 ml/well of Nonidet P-40 lysis buffer. The lysates were centrifuged (13,000 rpm, 20 min), and the supernatants were collected. Protein concentration was then determined in each supernatant using the BCA method. An equal amount of protein from each lysate was then incubated (overnight at 4 °C) with 50 μl of immobilized neutravidin protein beads (Pierce) to pull down biotinylated proteins. The beads were washed four times with harvest buffer (0.5% SDS, 60 mM Tris/HCl, pH 7.5, 2 mM EDTA) supplemented with 2.5% Triton X-100 (final concentration). The bound biotinylated proteins were eluted with reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by reducing Laemmli SDS sample buffer, boiled, and resolved by diethyl pyrocarbonate water, Ambion), a positive control (Stratagene). PCR conditions included polymerase activation at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. Each assay included a negative control (diethyl pyrocarbonate water, Ambion), a positive control (human genomic DNA), a no-template control, and the experimental samples in duplicate. A quantitative measurement of mouse DNA was obtained through amplification of the mouse GAPDH genomic DNA sequence with mouse GAPDH primers (sense, 5′-AAC GAC CCC TTC ATT GAC-3′, and antisense, 5′-TCC ACG ACA TAC TCA GCA C-3′) using the same PCR conditions described for alu. The threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample using a Stratagene Mx 4000™ PCR machine. The alu signal was normalized against the relative quantity of mouse GAPDH and expressed as ΔCt = (Ctalu - CtGAPDH). The changes in alu signal relative to the total amount of mouse genomic DNA were expressed as ΔΔCt = ΔCtreatment − ΔCtcontrol. Relative changes in metastasis were then calculated as 2−ΔΔCt. Data processing and statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA).

Tumorigenicity and Experimental Metastasis Assays—Tumorigenicity assays were performed on 4–6-week-old male homozygous CB-17 SCID/SCID mice and female NCr nude mice (Taconic Farms, Germantown, NY) according to the Animal Welfare Regulations at Wayne State University. HT1080 cell transfectants (pool populations and pooled clones) were harvested and resuspended in serum-free DMEM. Each mouse was inoculated subcutaneously with 5 × 10⁶ cells in 100 μl of serum-free DMEM. The volume of the tumor was measured twice a week with a caliper, using the formula V = (LW²) × 0.4 (where V is the volume (mm³); L is the longest diameter (mm), and W is the shortest diameter (mm)). Data were analyzed for statistical significance by unpaired t test with Welch correction using the GraphPad InStat® version 3.0 (GraphPad Software, San Diego). The differences were considered to be statistically significant at p < 0.05. Tumor incidence represents the percentage of mice bearing a tumor larger than 100 mm³ at day 28. The mice were euthanized 4 weeks after tumor cell inoculation, and the tumors were harvested and frozen at −80 °C until use. Experimental metastasis assays were conducted in CB-17 SCID/SCID mice. Briefly, each mouse was inoculated in the tail vein with 1 × 10⁶ HT1080-EV or HT1080-44-MT1 cells (pooled populations) suspended in 100 μl of serum-free DMEM. Twelve days later the mice were sacrificed, and the lungs were harvested for assay of metastatic burden by real time PCR and counting of lung colonies in hematoxylin and eosin sections as described below.

Determination of Lung Metastatic Burden—Twelve days after tail vein cell inoculation, the mice were sacrificed and the lungs were harvested. Equal portions from each lung were snap-frozen in liquid nitrogen or formalin-fixed. Frozen lung tissues were used for quantitative analyses of human alu sequences by quantitative real time PCR, as described (73). Briefly, genomic DNA was extracted from harvested mouse lung tissues using the Puregene DNA purification kit (Genta Systems, Minneapolis, MN). The primers specific for the human alu sequences were as follows: sense, 5′-ACG CCT GTA ATC CCA GCA CTT-3′, and antisense, 5′-TCG CCC AGG CTG GAG TGC A-3′. Real time PCR was performed using the SYBR® Green QRT-PCR master mix kit according to the manufacturer’s protocol (Stratagene). PCR conditions included polymerase activation at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. Each assay included a negative control (diethyl pyrocarbonate water, Ambion), a positive control (human genomic DNA), a no-template control, and the experimental samples in duplicate. A quantitative measurement of mouse DNA was obtained through amplification of the mouse GAPDH genomic DNA sequence with mouse GAPDH primers (sense, 5′-AAC GAC CCC TTC ATT GAC-3′, and antisense, 5′-TCC ACG ACA TAC TCA GCA C-3′) using the same PCR conditions described for alu. The threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample using a Stratagene Mx 4000™ PCR machine. The alu signal was normalized against the relative quantity of mouse GAPDH and expressed as ΔCt = (Ctalu − CtGAPDH). The changes in alu signal relative to the total amount of mouse genomic DNA were expressed as ΔΔCt = ΔCtreatment − ΔCtcontrol. Relative changes in metastasis were then calculated as 2−ΔΔCt. Data processing and statistical analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA).

Formalin-fixed lung tissues were sectioned and processed for hematoxylin and eosin staining using conventional procedures. Lung colonies were visualized and counted in serial sections under a light microscope. At least three different slides from each tumor and five different tumors from each cell line were evaluated for the number of metastatic colonies.
Processed Form of MT1-MMP Positively Regulates Enzyme Function

RESULTS

Characterization of Transfectants—To investigate the relationship between active MT1-MMP (57 kDa) and its processed 44-kDa form in regulation of MT1-MMP activity, we engineered a cDNA construct that produces a 44-kDa recombinant protein extending from Gly285 to Val582 when expressed in cells (Fig. 1A). We also generated two deletion mutants lacking either the cytosolic tail (44ΔCT) or the entire hinge region (44ΔH), as described under “Experimental Procedures.” Lysates were resolved by 10% reducing SDS-PAGE followed by immunoblot analysis with anti-hemopexin-like domain pAb (Fig. 1B, panel i), anti-cytosolic domain pAb (Fig. 1C, panel ii), or anti-catalytic domain pAb (Fig. 1D, panel ii) followed by enhanced chemiluminescence. β-Actin is shown as a loading control. 57 kDa refers to active MT1-MMP. Arrow shows 44-MT1 (~44 kDa); black arrowhead, 44ΔCT (~40 kDa), and open arrowhead, 44ΔH (~38 kDa). The asterisk in D indicates a nonspecific band.

FIGURE 1. Recombinant 44-MT1 expression. A, a schematic diagram of 44-kDa MT1-MMP constructs and their domain organization. Wt MT1-MMP, wild-type MT1-MMP; 44-MT1, catalytic domain-deleted MT1-MMP (Gly285–Val582); 44ΔCT, catalytic and cytosolic domain-deleted MT1-MMP (Gly285–Arg563); 44ΔH, catalytic domain and hinge-deleted MT1-MMP (Pro316–Val582); SS, signal sequence; Pro, pro-domain; Cat, catalytic domain; HPX, hemopexin domain; TM, transmembrane domain; CT, cytosolic domain; S, disulfide bond; RRKR, furin-recognition site in the prodomain. B–D, immunoblot analyses of active (57-kDa) MT1-MMP and 44-kDa MT1-MMP forms in lysates of HT1080 transfectants (B, panel i, pooled population; panels ii and iii, clones, indicated by numbers after name), BT-549 cells treated with or without ponasterone A (Pon A) (C), and infected-transfected BS-C-1 cells with various ratios of wild-type MT1-MMP and 44-MT1 (D), as described under “Experimental Procedures.” Lysates were resolved by 10% reducing SDS-PAGE followed by immunoblot analysis with anti-hemopexin-like domain pAb 815 (B, panel i, and C and D), anti-hemopexin-like domain pAb 437 (B, panel ii), or anti-cytosolic domain pAb (B, panel iii) followed by enhanced chemiluminescence. β-Actin is shown as a loading control. 57 kDa refers to active MT1-MMP. Arrow shows 44-MT1 (~44 kDa); black arrowhead, 44ΔCT (~40 kDa), and open arrowhead, 44ΔH (~38 kDa). The asterisk in D indicates a nonspecific band.

Effect of 44-MT1 Forms on Pro-MMP-2 Activation—We first examined the effects of 44-MT1 forms on pro-MMP-2 activation, a major function of MT1-MMP (75). Unstimulated parental HT1080 and HT1080-EV cells showed no pro-MMP-2 activation (Fig. 2, A and B, respectively). In contrast, HT1080-44-MT1 and, to a much lesser extent, HT1080-44ΔCT cells exhib-
that observed in conA-stimulated HT1080-EV cells (Fig. 2B, 44ΔCT versus EV) suggesting a role for the cytosolic tail in this activity of 44-MT1.

Collagen I was shown to promote pro-MMP-2 activation in HT1080 cells (30). As reported, HT1080-EV cells on collagen I exhibited a partial but consistent activation of pro-MMP-2, mostly to the intermediate form, when compared with cells seeded on uncoated dishes (Fig. 2C). In contrast, full pro-MMP-2 activation was detected in HT1080-44-MT1 cells cultured on collagen I-coated dishes. Thus, 44-MT1 expression is associated with enhanced pro-MMP-2 activation even in the presence of collagen I.

Previous studies demonstrated that expression of an N-terminally tagged 44-kDa form inhibits MT1-MMP-mediated pro-MMP-2 activation (57, 59, 76). These species, as opposed to 44-MT1, lack significant parts of the hinge region, which may affect the experimental outcome (see "Discussion"). To address this possibility, we deleted the entire hinge (Gly285–Gly315) of 44-MT1 to generate 44ΔH (Fig. 1A) and expressed this form in HT1080 cells (Fig. 1B). As shown in Fig. 2B, two clones expressing 44ΔH did not exhibit constitutive (unstimulated) activation of pro-MMP-2. In fact, 44ΔH expression partially inhibited conA-induced pro-MMP-2 activation, and only the intermediate form of MMP-2 was detected, when compared with HT1080-EV cells (Fig. 2B). Thus, removal of the hinge region is associated with a negative effect of 44-MT1 on conA-stimulated pro-MMP-2 activation.

Together, these results show that 44-MT1 expression (extending into the constitutive full pro-MMP-2 activation in the absence of conA (Fig. 2B). ConA treatment induced a robust and almost complete activation of pro-MMP-2 in parental HT1080 and HT1080-EV cells, as reported (45) (Fig. 2, A and B). However, expression of 44-MT1 had no effect on conA-stimulated pro-MMP-2 activation when compared with untransfected HT1080-44-MT1 cells (Fig. 2B). Although expression of 44ΔCT in HT1080 cells resulted in unstimulated activation of pro-MMP-2 (Fig. 2B), 44ΔCT expression was associated with a reduced activation of pro-MMP-2 when compared with from Gly285 to Val315) can promote unstimulated pro-MMP-2 activation in HT1080 cells, and this effect is reduced by the absence of the hinge region.

To determine whether the constitutive stimulation of pro-MMP-2 activation observed in HT1080-44-MT1 cells is MT1-MMP-dependent, we used RNA interference to specifically knock down endogenous MT1-MMP expression without affecting the expression of 44-MT1. As shown in Fig. 2I, MT1-MMP siRNA significantly inhibited expression of MT1-MMP (57 kDa) in HT1080-44-MT1 cells, whereas expression of
44-MT1 expression was barely affected. Zymography of the media revealed that the MT1-MMP siRNA-transfected HT1080-44-MT1 cells lacked activation of pro-MMP-2 (Fig. 2I, upper panel). In contrast, constitutive pro-MMP-2 activation was evident in scrambled siRNA-transfected HT1080-44-MT1 cells. These results suggest that the enhanced activation of pro-MMP-2 by 44-MT1 is mediated by interactions between active and processed MT1-MMP. Consistent with this assertion, expression of 44-MT1 in T47D cells, which do not express endogenous MT1-MMP, did not result in pro-MMP-2 activation (Fig. 2H). However, active MMP-2 was readily detected in T47D cells transfected to express wild-type MT1-MMP, as expected (Fig. 2H).

Because activation of pro-MMP-2 depends on the level of TIMP-2 (6), we measured TIMP-2 mRNA and protein in the HT1080 transfectants. All of the transfectants exhibited similar levels of TIMP-2 mRNA (supplemental Fig. 1D). However, ELISA data showed a differential distribution of TIMP-2. In HT1080-44-MT1 and HT1080-44\Delta CT cells, TIMP-2 was mostly associated with the cell lysate, whereas in HT1080-EV and HT1080-44\Delta H cells most of the TIMP-2 was present in the conditioned media (supplemental Fig. 1C). The differential distribution of TIMP-2 observed in these transfectants correlated with their pattern of pro-MMP-2 activation (Fig. 2B). Because the cell association of TIMP-2 is mainly mediated by MT1-MMP, these results suggest that different levels of surface active MT1-MMP among the transfectants may explain pro-MMP-2 activation results, as is shown below.

We next examined the effects of 44-MT1 on pro-MMP-2 activation using a ponasterone-inducible system in BT549 cells, which express endogenous MT1-MMP and pro-MMP-2, and in BS-C-1 cells infected-transfected to express MT1-MMP with or without 44-MT1. These expression systems allow functional evaluation of the gene of interest without issues of selection. Exposure of BT549 cells to ponasterone induced expression of 44-MT1 (Fig. 1C), which resulted in pro-MMP-2 activation (Fig. 2D). Likewise, co-expression of wild-type MT1-MMP with 44-MT1 in BS-C-1 cells also induced activation of pro-MMP-2, which was more evident at higher levels of 44-MT1 protein (Fig. 1D and 2E).

Treatment of MDA-MB-231-EV and U87-EV cells with conA induced pro-MMP-2 activation, as expected (Fig. 2F and G). However, expression of 44-MT1 in these cells had no effect on activation of pro-MMP-2 regardless of conA treatment (Fig. 2F and G). Furthermore, expression of 44\Delta CT or 44\Delta H in U87 cells had no effect on conA-stimulated pro-MMP-2 activation when compared with U87-EV cells (Fig. 2G).

44-MT1 Effect on Cell Growth within Three-dimensional Collagen I—MT1-MMP was described previously as conferring tumor cells with the ability to grow within a three-dimensional collagen I matrix (77). Therefore, we examined the effects of 44-MT1 expression on the proliferation of HT1080 and U87 cells within three-dimensional collagen I. As shown in Fig. 3, both HT108-44-MT1 and U87-44-MT1 cells exhibited enhanced growth within three-dimensional collagen I when compared with control cells. In contrast, 44-MT1 expression had no detectable effect on the rate of growth of MDA-MB-231 cells in three-dimensional collagen I (Fig. 3). Expression of 44\Delta CT or 44\Delta H in HT1080 or U87 cells had no significant effect on cell growth when compared with EV cells (Fig. 3), further demonstrating that both the cytosolic domain and the hinge region mediate the biological effect of 44-MT1. Next, we treated HT1080-44-MT1 cells growing within three-dimensional collagen I with either TIMP-2 or TIMP-1. As shown in Fig. 3, TIMP-2 but not TIMP-1 was associated with inhibited growth of HT1080-44-MT1 cells, consistent with this growth effect mediated by MT1-MMP. Thus, 44-MT1 expression promotes growth of two cell lines within a three-dimensional collagen I matrix. No differences in cell growth were detected when the HT1080 or U87 cell transfectants were cultured on uncoated dishes or on a two-dimensional collagen I matrix (data not shown).

Expression of 44-MT1 in HT1080 Cells Correlates with High Levels of Active MT1-MMP on the Cell Surface—The enhanced MT1-MMP activity associated with 44-MT1 expression, and the higher levels of cell-associated TIMP-2, raised the possibility that these effects were in part mediated by an increased level of surface MT1-MMP, which could not be resolved by conventional surface biotinylation. We therefore examined HT1080 transfectants for expression of surface-active MT1-MMP by indirect immunofluorescence. To detect only the endogenous active enzyme, we used pAb 198, which recognizes the catalytic domain of MT1-MMP (68) and does not react with 44-MT1. To increase the level of detection, the cells were briefly acid-washed to dissociate any potential TIMP bound to the active site of MT1-MMP. As shown in Fig. 4, nonpermeabilized HT1080-44-MT1 cells displayed prominent labeling of active MT1-MMP at the plasma membrane when compared with control cells or cells expressing 44\Delta CT or 44\Delta H. Quantification of microscopy data showed a 4-fold increase in active MT1-MMP in HT1080-44-MT1 cells when compared with HT1080-EV cells (Fig. 4A, surface). In contrast, no significant differences in surface MT1-MMP were found...
Enhanced surface expression of active MT1-MMP in HT1080-44-MT1 cells. A, HT1080 transfectants (clones) were immunostained at 4 °C with pAb 198 to the catalytic domain of MT1-MMP followed by an incubation with FITC-conjugated donkey anti-rabbit IgG antibody and fixed to detected surface MT1-MMP (Surface). For visualization of total (surface plus intracellular) MT1-MMP (Total), the cells were fixed, permeabilized, and then immunostained with pAb 198 followed by incubation with the secondary FITC-labeled antibody, as described under “Experimental Procedures.” The nuclei were stained with DAPI. Cells were examined by confocal microscopy and photographed under a 60× oil immersion objective. Insets show representative images of magnified single cells, bar = 10 μm. Pictures represent the result of at least three independent experiments. B, intensity of fluorescence signal per cell was quantified using the Metamorph™ software in at least three different fields for each transfectant. The results shown are mean ± S.D. of at least three fields; * , p < 0.05; Student’s t test; NS, not statistically significant relative to HT1080-EV cells. Similar results were obtained in three independent experiments.

Expression of 44-MT1 Delays the Internalization of MT1-MMP in HT1080 Cells—To examine whether the increase in surface MT1-MMP expression in HT1080-44-MT1 cells was because of a reduced rate of endocytosis, we monitored and quantified the percentage of active MT1-MMP remaining at the cell surface following antibody cross-linking mediated internalization. We compared the relative amounts of active MT1-MMP remaining at the cell surface after 10 min of incubation at 37 °C with that of cells maintained at 4 °C. Note that comparisons of fluorescence signal (remaining active MT1-MMP) were conducted within the same cell transfectant because the initial levels of surface MT1-MMP varied between EV and 44-MT1 cells, as shown in Figs. 4 and 5B. Fig. 5B shows the actual differences in signal between HT1080-EV and HT1080-44-MT1 cells at 0 min when imaged under identical contrast and brightness conditions. Fig. 5A shows confocal images of cells during the antibody uptake assay photographed using identical contrast and brightness conditions in each group, which reveals the difference in levels of surface MT1-MMP between 0 and 10 min within the same cell transfectant. Thus, the differences in signal (surface MT1-MMP) between control and 44-MT1-expressing cells are not seen in Fig. 5A. Quantitative analyses of surface fluorescence of confocal images (Fig. 5A) showed that nearly 15% of the MT1-MMP-bound antibody remained at the cell surface of HT1080-EV cells after 10 min of incubation at 37 °C when compared with cells maintained at 4 °C (Fig. 5C, p < 0.01). In contrast, ~40% of active MT1-MMP remained at the surface of HT1080-44-MT1 cells after 10 min at 37 °C when compared with the same cells maintained at 4 °C (Fig. 5C, not statistically significant relative to 4 °C). Under the same experimental conditions, HT1080-44ΔCT and HT1080-44ΔH cells had ~22% (p < 0.05) and ~10% (p < 0.01) of MT1-MMP remaining at the cell surface after 10 min at 37 °C, respectively, when compared with cells maintained at 4 °C (0 min). The relative amount of active MT1-MMP remaining on the surface of these cells did not differ from that remaining on HT1080-EV cells. Thus, expression of 44-MT1 in HT1080 cells is associated with a reduced internalization of active MT1-MMP. This effect is not seen with expression of 44ΔCT or 44ΔH and in HT1080-EV cells indicating that deletion of the cytosolic tail or the hinge region abolishes the ability of 44-MT1 to delay the internalization of active MT1-MMP.

Because collagen I was shown to regulate MT1-MMP internalization (54, 61), the antibody uptake assay was also conducted in HT1080 cell transfectants seeded on collagen I-coated coverslips, as described under “Experimental Procedures.” These data also showed a delayed internalization of active MT1-MMP in HT1080-44-MT1 cells when compared with control EV cells or cells expressing 44ΔCT or 44ΔH (supplemental Fig. 2). These results indicate that the process of MT1-MMP regulation by endocytosis is not influenced by the presence of collagen I.

We also followed the internalization of MT1-MMP using a reversible biotinylation assay (72). This assay was performed using a cleavable, membrane-permeable biotin. After incubations at 37 °C, residual surface biotin was stripped with gluta-
thione, a membrane-impermeable reducing agent, and internalized biotinylated MT1-MMP was captured by avidin beads and detected by immunoblot analyses. Fig. 6A shows a representative blot depicting the endocytosed and total biotinylated active endogenous MT1-MMP and 44-MT1 form in the HT1080 transfecants. The data of Fig. 6A depict the densitometric analysis of internalized active MT1-MMP per cell was quantified using the Metamorph™ software in at least three different fields for each transfectant. Images at the indicated times within the same cell transfectant were photographed under same brightness and contrast conditions. Bars indicate S.D., Student’s t-test; **, p < 0.01; *, p < 0.05; NS, not statistically significant relative to 0 min. Similar results were obtained in three independent experiments.

FIGURE 5. Delayed endocytosis of active MT1-MMP in HT1080-44-MT1 cells. A–C, HT1080 cell transfectants (clones) were incubated on ice with pAb 198 against the catalytic domain of MT1-MMP. After the indicated times at 37 °C, the cells were incubated with FITC-labeled secondary antibodies and fixed, as described under “Experimental Procedures.” The nuclei were then stained with DAPI. Cells were analyzed by confocal microscopy and photographed. A, photographs show representative images for each transfectant using a 60× objective. Images at the indicated times within the same cell transfectant were photographed under same brightness and contrast conditions. Bar = 10 μm. B, representative images of HT1080-EV and HT1080-44-MT1 cells at 0 min were photographed under same brightness and contrast conditions to demonstrate the relative fluorescence intensity between these transfectants. C, remaining surface active MT1-MMP per cell was quantified using the Metamorph™ software in at least three different fields for each transfectant. Bars indicate S.D., Student’s t-test; **, p < 0.01; *, p < 0.05; NS, not statistically significant relative to 0 min. Similar results were obtained in three independent experiments.

Effect of 44-MT1 Forms on Tumor Growth and Metastasis—The HT1080 cell transfecants were inoculated subcutaneously in athymic mice, and tumor growth was monitored for 28 days. Initially we used pooled populations and SCID mice (Fig. 7, A and B). These studies showed that HT1080-44-MT1 cells formed significantly larger tumors than HT1080-EV cells (Fig. 7, A and B). In contrast, expression of 44ΔCT had no significant effect on tumor growth. Surprisingly, we found that 7/8 mice inoculated with HT1080-44ΔH cells showed no evidence of tumor formation (Fig. 7, A and B). We repeated this experiment using a mixture of two clones of each transfectant, which were inoculated in NCr mice to account for potential differences because of clonal variation and mouse strain. These studies essentially reproduced the previous results with the exception that the HT1080-44ΔCT clones grew faster than control clones (Fig. 7, C and D). Taken together, these results demonstrate that although 44-MT1 expression is associated with enhanced growth of HT1080 cells in mice, expression of 44ΔH is strongly correlated with decreased tumorigenic potential. These results suggest that the presence of the hinge regulates the activity of the processed form of MT1-MMP in vivo. We next examined the HT1080 transfectants, which were inoculated on ice with pAb 198 against the catalytic domain of MT1-MMP. After the indicated times at 37 °C, the cells were incubated with FITC-labeled secondary antibodies and fixed, as described under “Experimental Procedures.” The nuclei were then stained with DAPI. Cells were analyzed by confocal microscopy and photographed. A, photographs show representative images for each transfectant using a 60× objective. Images at the indicated times within the same cell transfectant were photographed under same brightness and contrast conditions. Bars indicate S.D., Student’s t-test; **, p < 0.01; *, p < 0.05; NS, not statistically significant relative to 0 min. Similar results were obtained in three independent experiments.

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**FIGURE 6. Endocytosis of active MT1-MMP in the presence of recombinant and natural 44-kDa species by reversible biotinylation assay.** 
A, surface proteins in HT1080-EV and HT1080-44-MT1 cells were labeled at 4 °C using sulfo-NHS-SS-biotin and allowed to internalize for 0–60 min at 37 °C. At the indicated times, the cells were treated with (+) or without (−) glutathione (40 mM) at 4 °C and lysed. Biotinylated proteins were recovered with streptavidin-agarose beads and endocytosed (glutathione-resistant). MT1-MMP was detected by immunoblotting with pAb 815 to the hinge region (upper panels). 57 kDa indicates the endogenous active MT1-MMP. Arrow shows 44-MT1 (−44 kDa). Blots were reprobed with an antibody to transferrin receptor (TrfR; −90 kDa, lower panel). B, biotinylated bands of internalized active MT1-MMP (+Glutath.) in HT1080-EV and HT1080-44-MT1 cells as shown in A were quantified by densitometry as described under “Experimental Procedures.” C, parental HT1080 cells were treated (16 h, 37 °C) with cAMP (20 μM/ml) in the presence or absence of 10 μM GM6001. Time-dependent endocytosis of MT1-MMP was then examined as described in A. D, biotinylated bands of internalized active MT1-MMP (+Glutath.) as shown in C (left panel) were quantified by densitometry, as described under “Experimental Procedures.” Data in A–D are representative results of at least three independent experiments with similar results.

tants for differences in their ability to form lung colonies after intravenous inoculation in SCID mice. Mice were sacrificed 12 days after cell inoculation and lungs harvested, and tumor burden was determined by a quantitative real-time PCR of genomic DNA using human *alu* sequences and mouse *GAPDH* as an internal control (73). As shown in Fig. 8A, the results of *alu* PCR indicated that mice inoculated with HT1080-44-MT1 cells contained significantly greater amounts of human genomic DNA in their lungs when compared with lungs of mice injected with HT1080-EV cells. As observed with the subcutaneous tumor assay, expression of 44ΔH also inhibited lung colonization after intravenous inoculation, as determined by *alu* PCR (Fig. 8A). These results were confirmed by counting tumor colonies in the lungs of mice (Fig. 8B). Thus, 44-MT1 expression in HT1080 cells is associated with enhanced tumor growth and lung colonization.

**DISCUSSION**

Once on the cell surface, the pool of active MT1-MMP is regulated by a complex array of processes, including autocatalytic degradation (78). This process leads to the cleavage of active MT1-MMP to an inactive 44-kDa membrane-tethered form lacking the catalytic domain but retaining key enzyme domains, and thus has the potential to directly and indirectly influence the function of neighboring active MT1-MMP molecules. Here we provide evidence that the product of MT1-MMP processing, the 44-kDa species, may regulate the amount of active enzyme at the cell surface and consequently influence enzymatic activity and cell behavior both *in vitro* and *in vivo*. Our data show that 44-MT1 expression is generally associated with enhanced pro-MMP-2 activation and growth in three-dimensional collagen in various cell types. It also correlates with enhanced tumor growth and lung colonization of HT1080 cells in immunodeficient mice. *In vitro*, the stimulatory action of 44-MT1 was dependent on the interactions between 44-MT1 and active MT1-MMP because inhibition of endogenous MT1-MMP expression or activity abolished the observed effects. Furthermore, in the absence of endogenous MT1-MMP expression, such as in T47D cells, 44-MT1 expression had no effects on pro-MMP-2 activation and growth in three-dimensional collagen. *In vivo*, however, the contribution of an MT1-MMP-independent effect of 44-MT1 on tumor growth and metastasis cannot be ruled out considering recent evidence showing
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44-MT1 expression. Consequently, our observations do not support the notion that homophilic interactions of active MT1-MMP are critical for enzymatic activity, if indeed a function of the 44-kDa form is to disrupt these interactions via its hemopexin-like domain and/or the cytosolic tail, as proposed earlier (57–59). In fact, our data show that the processed hemopexin-like domain and/or the cytosolic tail, as proposed previously (1), may not be essential for proper enzyme function.

Why do our results differ from those published earlier? The majority of the studies reporting a dominant negative effect of the 44-kDa form used recombinant 44-kDa-like proteins lacking most of the hinge region. In addition, these forms included either a FLAG or a 25-kDa glutathione S-transferase tag at the N terminus (57–59). The FLAG-tagged, hinge-deleted 44-kDa species was found to be processed to a smaller form (57), possibly because of the lack of O-glycosylation, as discussed below (25, 36). The glutathione S-transferase-tagged 44-kDa construct (59) lacked the signal sequence and the prodomain of MT1-MMP, which are considered to be essential for proper trafficking and membrane insertion. Thus, significant deletions within the hinge region and/or presence of N-terminal tags may affect the function of the recombinant 44-kDa protein and consequently its effects on active MT1-MMP. Consistent with this possibility, we found that deletion of the hinge in 44-MT1 was associated with inhibition of conA-stimulated pro-MMP-2 activation and tumorigenicity in HT1080 cells. Interestingly, 44ΔH had no effect on growth within the three-dimensional collagen I indicating a selective effect of this fragment on cell behavior. The fact that 44ΔH lacks the hinge region suggests that this domain may play a role in regulating the function of the 44-kDa processed form. Accumulating evidence suggests that the hinge of MT1-MMP plays an important role in enzyme activity and stability because of its high mobility and O-glycosylation, respectively (25, 36, 60, 81). Indeed, 44-MT1 is O-glycosylated, whereas 44ΔH is not (data not shown). Interestingly, a FLAG-tagged 44-kDa species lacking the hinge, which inhibited pro-MMP-2 activation in HT1080 cells, was found to be processed to a smaller form (57), possibly because of the lack of O-glycosylation. Because the hinge is the main target of autocatalytic processing (81), its presence in the 44-kDa form may also contribute to the stabilization of active MT1-MMP by interfering with processing, as proposed previously (59). On the other hand, significant deletions within the hinge region and/or presence of N-terminal tags may cause conformational or structural changes in the 44-kDa protein, which in turn may disrupt active MT1-MMP-44-kDa interactions leading to inhibition of enzyme function. Although the mechanism(s) by which these hinge-truncated 44-kDa species inhibit MT1-MMP activity is unclear, it is worth noting that under physiological processing conditions, the resultant major 44-kDa form would include the hinge region. We posit that this species should be expected to behave similarly to the recombinant 44-MT1 form used here. Regardless, our results suggest a novel role for the hinge in regulating the function of the 44-kDa form of MT1-MMP.

FIGURE 7. Effect of 44-kDa recombinant forms on tumor growth of HT1080 cells. A–D, HT1080 cell transfectants were inoculated subcutaneously into SCID (A and B) or Ncr (C and D) athymic mice on day 0 as described under “Experimental Procedures.” The animals were monitored for tumor formation every 3–4 days until day 28. Tumors were measured every 3–4 days, and tumor volume was calculated. Points represent mean of tumor volume for each group, and bars indicate S.E. Unpaired t test; *, p < 0.05. Similar results were obtained in three independent experiments. B and D, tumor incidence and volume of tumors at day 28 from data shown in A and C. * p values relative to HT1080-EV tumors. NS, not statistically significant relative to HT1080-EV tumors.
In addition to differences in the nature of the 44-kDa form as discussed above, careful analyses of previous data suggest that use of cell lines with low levels of endogenous MT1-MMP expression (59, 60, 62) with highly expressed recombinant 44-kDa proteins may also contribute to the described dominant negative effects.

Our data indicate that expression of 44-MT1 in HT1080 cells correlates with higher levels of surface MT1-MMP because of a slower rate of MT1-MMP endocytosis, as determined in two distinct assays. The relatively higher levels of surface-active MT1-MMP correlated with higher levels of TIMP-2 associated with the cell lysates of HT1080-44-MT1 cells (supplemental Fig. 1C). Together, these findings suggest that generation of the 44-kDa form by autocatalytic turnover serves to preserve a basal level of functional protease at the cell surface by regulating the rate of endocytosis of active MT1-MMP. Given that 44-MT1 maintains the cytosolic tail and thus possesses the same endocytic signal of the full-length protease, this process may be due to competitive interactions between the cytosolic tails of the MT1-MMP forms for components of the endocytic machinery (82). Indeed, expression of 44ΔCT had no effect on the rate of active MT1-MMP internalization. This suggests the possibility that components of the endocytic pathway, including those mediated by di-leucine motifs, as present in the cytosolic domain of MT1-MMP, are saturable and thus prone to competition within the same type of receptors, as established in previous studies with several transmembrane receptors (82–84). Competition for the endocytotic machinery between MT1-MMP species may be due to the accumulation of the 44-kDa form as a consequence of autocatalytic processing. Alternatively, preferential internalization of the processed form cannot be ruled out. At present, the data presented here do not allow us to discriminate between these possibilities. This will require determining the rate of internalization of each species alone and in combination. However, this study is complicated by the need to express comparable levels of each species in cells without endogenous MT1-MMP and to prevent processing of the wild-type enzyme, which further complicates this type of analyses. Nevertheless, our data strongly suggest that there is a direct relationship between processing and endocytosis which serves to maintain a viable level of protease at the cell surface.

Based on the results presented here, we propose a model for the regulation of active MT1-MMP by its processed form. Conditions that increase the level of active MT1-MMP at the cell surface augment the probability of autocatalytic processing (78). This leads to the formation of a membrane-anchored 44-kDa degradation product (Gly285–Val582) that lacks the entire catalytic domain. Processing decreases the amount of active protease, and the overall activity of MT1-MMP at the cell surface is reduced. The net amount of active MT1-MMP can be recovered by de novo synthesis, enhanced trafficking, and/or reduced turnover and endocytosis. MT1-MMP processing also leads to accumulation of the 44-kDa product. Consequently, the 44-kDa form may saturate the local endocytic pathway resulting in delayed internalization of active MT1-MMP, which would eventually lead to recovery and preservation of enzymatic activity. Although the precise mechanism(s) by which the 44-kDa species delays the internalization of active MT1-MMP needs to be elucidated, this model provides a plausible explanation to the positive association between high levels of processed MT1-MMP (43–45-kDa forms) and increased enzymatic activity reported in many studies. We also propose that the regulation of active MT1-MMP by its processed 44-kDa form is also analogous to the stabilization model of active MT1-MMP by TIMPs and synthetic MMP inhibitors, which postulates that under certain conditions these can also promote MT1-MMP activity by inhibiting autocatalytic processing (17, 85). The findings of this study should facilitate the re-evaluation of the contribution of MT1-MMP autocatalytic processing and its degradation product to enzyme function.

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