Complex Pattern of Membrane Type 1 Matrix Metalloproteinase Shedding

REGULATION BY AUTOCATALYTIC CELL SURFACE INACTIVATION OF ACTIVE ENZYME*

Received for publication, January 22, 2002, and in revised form, April 15, 2002
Published, JBC Papers in Press, May 9, 2002, DOI 10.1074/jbc.M200655200

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Membrane type 1 matrix metalloproteinase (MT1-MMP) is a type I transmembrane MMP shown to play a critical role in normal development and in malignant processes. Emerging evidence indicates that MT1-MMP is regulated by a process of ectodomain shedding. Active MT1-MMP undergoes autocatalytic processing on the cell surface, leading to the formation of an inactive 44-kDa fragment and release of the entire catalytic domain. Analysis of the released MT1-MMP forms in various cell types revealed a complex pattern of shedding involving two major fragments of 50 and 18 kDa and two minor species of 56 and 31–35 kDa. Protease inhibitor studies and a catalytically inactive MT1-MMP mutant revealed both autocatalytic (18 kDa) and non-autocatalytic (56, 50, and 31–35 kDa) shedding mechanisms. Purification and sequencing of the 18-kDa fragment indicated that it extends from Tyr112 to Ala255. Structural and sequencing data indicate that shedding of the 18-kDa fragment is initiated at the Gly284-Gly285 site, followed by cleavage between the conserved Ala255 and Ile256 residues near the conserved methionine turn, a structural feature of the catalytic domain of all MMPs. Consistently, a recombinant 18-kDa fragment had no catalytic activity and did not bind TIMP-2. Thus, autocatalytic shedding evolved as a specific mechanism to terminate MT1-MMP activity on the cell surface by disrupting enzyme integrity at a vital structural site. In contrast, functional data suggest that the non-autocatalytic shedding generates soluble active MT1-MMP species capable of binding TIMP-2. These studies suggest that ectodomain shedding regulates the pericellular and extracellular activities of MT1-MMP through a delicate balance of active and inactive enzyme-soluble fragments.

* This work was supported in part by National Institutes of Health Grant CA-61986 and by United States Army Breast Cancer Program Grant DAMD17-99-1-9441 to R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: MT-MMP, membrane type matrix metalloproteinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; mAb, monoclonal antibody; pAb, polyclonal antibody; ConA, concanavalin A; TPA, 12-O-tetradecanoylphorbol-13-acetate.

26340 This paper is available on line at http://www.jbc.org
MT1-MMP in cells transfected to overexpress MT1-MMP (22). Other studies reported shedding of MT1-MMP from a breast carcinoma cell line after treatment with concanavalin A (ConA) (23–25), which was not inhibited by TIMP-2 (23); therefore, autocatalysis could not be involved. MT5-MMP sheds its catalytic domain in a process that appears to be mediated by a pro-convertase that removes the ectodomain intracellularly (26). Pre-mRNA splicing was reported to be involved in the generation of a soluble form of MT3-MMP, which retained catalytic activity and sensitivity to TIMP-2 inhibition (27). Thus, although different mechanisms of shedding may exist, collectively, these data suggest a unique property of MT-MMPs: the ability to generate soluble fragments by a process of ectodomain shedding, which may possess important functional consequences for pericellular proteolysis in normal and malignant processes. Here we have identified the major soluble forms of MT1-MMP and characterized the major autocatalytic fragment. We demonstrated that the autocatalytic shedding mechanism of MT1-MMP is likely to have evolved to terminate MT1-MMP-dependent proteolysis by hydrolyzing the enzyme at specific and vital sites.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The characteristics and culture conditions of nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) (28), human fibrosarcoma HT-1080 (CCL-121) (29), human fibroblasts HFL1 (CCL-153) (29), and human breast carcinoma MDA-MB-231 (HTB-26) (30) cells have been previously described. Human glioblastoma U-87 MG (HTB-14) cells, obtained from the American Tissue Culture Collection (ATCC), and immortalized homozygous Tempp2(−/−) mutant mice (31) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. The mAb LEM-2/15 directed against the catalytic domain of MT1-MMP (25) was a generous gift from Dr. A. Goldberg, Washington University, St. Louis, MO. A stable hygromycin-resistant clone designated Tempp2(−/−)-MT1 was selected for further studies.

**Recombinant Vaccinia Viruses**—The production of the recombinant vaccinia virus vTF7-3 expressing the human T7 RNA polymerase has been described (32). Recombinant vaccinia viruses expressing pro-MMP-2 (vT7-GEILA), MT1-MMP (vT7-MT1), TIMP-2 (vSC59-T2), or TIMP-1 (vT7-T1) were obtained by homologous recombination as previously described (17, 28, 33).

**Recombinant Proteins, Synthetic MMP Inhibitors, and Antibodies**—Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HEK293 cells and purified to homogeneity, as previously described (34). Human TIMP-4 was a generous gift from Dr. C. Overall (University of British Columbia, Vancouver, Canada). A recombinant catalytic domain of human MT1-MMP (Tyr112 to Gly288) designated MT1-MMP cat was purchased from Calbiochem. Marimastat (BB-2516) was obtained from British Biotech (Annapolis, MD) (35). SB-3CT was produced and was purchased from Calbiochem. Marimastat (BB-2516) was obtained from Biogen. TIMP-2, TIMP-4, and TIMP-1 (0.5 mg/ml) were dissolved in PBS. TIMP-2, TIMP-4, and TIMP-1 (0.1 mg/ml) were dissolved in PBS and diluted with DMEM at 100 nM; marimastat (0.5 mg/ml); leupeptin (40 μg/ml); or E64 (10 μM) overnight, as described (20). TPA-treated HT1080 cells, as described above, received 100 ng/ml aprotinin or 100 nM human recombinant TIMP-2. The serum-free conditioned media were collected and processed for immunoblot analysis, as described below.

**Cloning, Expression, and Isolation of Recombinant MT1-MMP Mutants**—A catalytically inactive mutant of MT1-MMP was generated by replacing Glu246 with Ala (E246A-MT1) using the QuiikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A cytosolic domain (CD) deletion mutant (ΔCD-MT1) was constructed by introducing a termination codon at Arg86 by polymerase chain reaction (PCR) using specific primers and wild-type MT1-MMP cDNA as the template. The amplified E246A-MT1 and ΔCD-MT1 cDNA fragments were cloned into the pTF7EMCV-1 vaccinia expression vector using appropriate restriction sites to generate the respective expression vectors pTF7-E246A-MT1 and pTF7-ΔCD-MT1, as described (28, 33). The sequence of the inserts was verified by DNA sequencing. Expression of the MT1-MMP mutants was carried out in BS-C-1 cells by the infection/transfection procedure (28, 32, 33). Briefly, BS-C-1 cells were grown in 100-mm culture dishes to 80% confluence and infected with 5 plaque-forming units/cell vTF7-3 virus in infection medium for 45 min, as described (32). The cells were washed with phosphate-buffered saline and then transfected with 2 μg/dish pTF7-ΔCD-MT1 (wild type MT1-MMP), pTF7-E246A-MT1, or pTF7-ΔCD-MT1 DNA plasmids using Effectene transfection reagent (Qiagen, Valencia, CA), as described by the manufacturer. Control cells were infected but received no plasmid DNA. Four h after transfection, the cells were metabolically labeled (12 h, 37 °C) with 100 μCi/ml [35S]methionine. The media were collected, clarified by centrifugation, and concentrated (330 ml) to 30 ml. The concentrated media (30 ml) were infected to express MT1-MMP as described (17). Three h after infection, the culture was incubated at 37 °C with serum-free DMEM supplemented with 1% Triton X-100 (final concentration) followed by addition of either pAb 457 or pAb 160/Protein A-agarose beads. After a 12-h incubation period (4 °C), the beads with washed three times with PBS supplemented with 2.5% Triton X-100 (by volume) followed by one wash with collagenase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl2, and 0.02% Brij-35). The immunoprecipitates were eluted with Laemmli SDS sample buffer (40), boiled, and resolved by reducing 8–16% SDS-PAGE, followed by transfer to a nitrocellulose membrane. The biotinylated MT1-MMP forms were detected with streptavidin-horseradish peroxidase and ECL (Amersham). The biotinylated MT1-MMP forms were detected with streptavidin-horseradish peroxidase and ECL (Amersham). The biotinylated MT1-MMP forms were detected with streptavidin-horseradish peroxidase and ECL (Amersham).
150 mM NaCl, 0.1% Nonidet P-40, and 10% glycerol). The immunoprecipitated proteins were eluted with reducing sample buffer, boiled and subjected to 15% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie Blue R-250. As reference, an aliquot of the eluate was subjected to TIMP-2-affinity binding using immobilized TIMP-2 on an Affi-Gel 10 matrix, prepared as previously described (17). Briefly, the medium was concentrated (−80-fold), and −0.4 ml of the concentrated medium (0.3 μl final marimastat concentration) was incubated (12 h, 4 °C) with Affi-Gel 10-TIMP-2 matrix by continuous rotation. After a brief centrifugation, the supernatant containing the unbound proteins was collected. The bound proteins were eluted with reducing Laemmli sample buffer (40) at 10°C. The bound proteins were recovered by reducing 15% SDS-PAGE, followed by autoradiography or by immunoblot analysis.

**Immunoprecipitation**—For immunoprecipitation of soluble MT1-MMP forms, serum-free media from 35S-labeled cells expressing recombinant or natural MT1-MMP were immunoprecipitated with pAb 160/Protein A-agarose beads under nondenaturing conditions as described (15). In some experiments, the 35S-labeled media were concentrated (10-fold) before immunoprecipitation. To immunoprecipitate denatured samples, the concentrated media were supplemented with 10% harvest buffer and boiled. The samples then received 2.5% Triton X-100 (final concentration) followed by addition of either pAb 437 or pAb 160 and Protein A-agarose beads as previously described (17). MT1-MMP-TIMP-2 complexes in 35S-labeled medium samples or in mixtures of recombinant MT1-MMP fragments and TIMP-2 were co-immunoprecipitated with mAb 101 to TIMP-2 under nondenaturing conditions as previously described (35). The immunoprecipitates were resolved by reducing 15% SDS-PAGE, followed by autoradiography or by immunoblot analysis.

**Gelatin Zymography and Immunoblot Analysis**—Gelatin zymography was performed using 10% or 15% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin as described (38). For immunoblot analysis, the serum-free conditioned media were collected, clarified by centrifugation, and concentrated (−80-fold) on a Centricon Plus-20 concentrator (Fisher, Itasca, IL). An aliquot was resolved by reducing 15% SDS-PAGE followed by transfer to nitrocellulose membrane. The membranes were incubated with the appropriate antibodies as described (38). The immunocomplexes were detected by ECL according to the manufacturer’s instructions (Pierce).

**Pro-MMP-2 Activation**—Purified human pro-MMP-2 (25 nm) in collagenase buffer was incubated (22 h, 37 °C) with either 5 nm MT1-MMPcat (Calbiochem) or recombinant 18- and 21-kDa MT1-MMP fragments or concentrated serum-free conditioned media from BS-C-1 cells expressing MT1-MMP or from BS-C-1 cells infected only with the T7 RNA polymerase-expressing virus (T7FP-3). As a positive control, pro-MMP-2 was activated with 1 mM p-aminophenylmercuric acetate for 1 h at 37 °C. Pro-MMP-2 activation was monitored by gelatin zymography.
were carried out either without stimulation or after stimulation with TPA or ConA, two agents known to induce MT1-MMP expression (10, 16, 29, 50). Surface biotinylation followed by immunoprecipitation with pAb 160 to the catactic domain or pAb 437 to the hemopexin-like domain demonstrated that untreated HT1080 cells display the 57-kDa species of MT1-MMP as the major enzyme form on the cell surface (Fig. 1A, lanes 1 and 2). Treatment with either TPA (Fig. 1A, lanes 3 and 4) or ConA (Fig. 1A, lanes 5 and 6) induced the appearance of a 44-kDa species on the cell surface, which was detected only with the pAb 437 (Fig. 1A, lanes 3 and 5), indicating that this species represents a membrane-inserted form lacking the catalytic domain, in agreement with our previous studies using a vaccinia expression system (17). Thus, both TPA and ConA promote the processing of natural MT1-MMP (57 kDa) into the inactive 44-kDa form.

We next examined the serum-free conditioned media of various cell lines (untreated or treated with TPA or ConA) and Timp-2 (−/−) mouse fibroblasts stable transfectant to express recombinant MT1-MMP for soluble MT1-MMP forms by immunoblot analysis (Fig. 1B) and immunoprecipitation (Fig. 1C). As shown in Fig. 1B, the medium of untreated HT1080 cells contains three proteins of 56, 50, and 18 kDa, which were recognized by a mAb to the catalytic domain of MT1-MMP (Fig. 1B, lane 7). TPA (Fig. 1B, lane 8) and ConA (Fig. 1B, lane 9) treatment of HT1080 cells enhanced the levels of these forms in the media and resulted in the appearance of an additional soluble MT1-MMP form of ~31–35 kDa, which was particularly evident with ConA (Fig. 1B, lane 9). Media of untreated HFL1 fibroblasts (Fig. 1B, lane 10) and U-87 glioblastoma cells (data not shown) showed presence of the 50-kDa species. Media of ConA-treated MDA-MB-231 contained mostly the 50-kDa form, as determined by immunoblot analysis (Fig. 1B, lane 13) or immunoprecipitation (Fig. 1C, lane 16). With the exception of the 18- and the 31–35-kDa species, the 56- and 50-kDa species were recognized by pAb 437 to the hemopexin-like domain, indicating that they comprise most of the ectodomain (data not shown).

Metabolic labeling of TPA-treated HT1080 cells followed by immunoprecipitation with pAb 160 yielded the 56-, 50-, 31–35-, and 18-kDa species (Fig. 1C, lane 14), whereas the same procedure in Timp-2 (−/−) cells expressing MT1-MMP yielded mostly the 50- and 18-kDa species (Fig. 1C, lane 17). No signal was observed in samples precipitated with Protein A-agarose beads without antibody (Fig. 1C, lanes 15 and 18). Considering that the 18-kDa species contains only one methionine residue (based on sequencing data, as shown below), the results of the immunoprecipitation of the 35S-labeled media indicate that the 18-kDa species, compared with the other forms, exhibits a relatively higher specific activity and hence represents the major soluble form of MT1-MMP.
collected, concentrated (H11011 catalytic shedding of the latter species. The H9004 mutant shed the 50-kDa species and a H11011 lane 2 the 18-kDa fragment (Fig. 3, H9004 -35-, and 18-kDa species (Fig. 3, wild type MT1-MMP shed the 50-, 31 H9262 and 18-kDa fragment allowed its purification and characterization. N-ter- H11011 Soluble Form—The relatively higher amounts of the 18-kDa H388 fragment was as follows: none (lane 1); TIMP-2, 20 nM (lane 2); marimastat, 10°, 10°, 10°, and 1 μM (lanes 3, 4, 5, and 6, respectively); TIMP-1, 100 nM (lane 7); and aprotinin, 20 and 40 μg/ml (lanes 8 and 9, respectively). Inhibitor doses in C were as follows: TIMP-2, 0, 1, 10, and 100 nM (lanes 1, 2, 3, and 4, respectively); TIMP-4, 0, 1, 10, and 100 nM (lanes 5, 6, 7, and 8, respectively); marimastat, 0, 20, 100, and 500 nM (lanes 9, 10, 11, 12, and 13, respectively); and SB-3CT, 4, 20, and 100 nM (lanes 14, 15, and 16, respectively). Open arrows show the lane 31–35-kDa fragment.

2C, lanes 14–16), had no effect. Aprotinin (Fig. 2, A (lane 2) and B (lanes 8 and 9)) and leupeptin (40 μg/ml; data not shown), two serine protease inhibitors, and E64 (10 μM), an aspartic protease inhibitor (data not shown), had no effect on the shedding of the 18-kDa species. None of the inhibitors tested had a significant effect on the levels of the 50-kDa form and in fact, the levels of this species were somewhat increased in the presence of TIMP-2 and marimastat in BS-C-1 cells (Fig. 2B, lanes 2 and 6, respectively) but not in HT1080 cells (Fig. 2A, lane 3).

Interestingly, we also found that, both in HT1080 cells (Fig. 2A, lane 3) and in BS-C-1 cells expressing MT1-MMP (Fig. 2B, lanes 2–6), the ~31–35-kDa species accumulated in the presence of TIMP-2 and marimastat but not in the presence of aprotinin (Fig. 2, A (lane 2) and B (lanes 8 and 9)). The inhibitor profile studies suggested that shedding of the 18-kDa species is an autocatalytic event, whereas shedding of the 50-kDa species is not. To further investigate this process, we generated a catalytically inactive mutant of MT1-MMP by replacing Glu340 with Ala (E240A-α11-MT1). We also examined the role of the cytosolic domain of MT1-MMP in shedding. To this end, we constructed a truncated MT1-MMP lacking the cytosolic domain (ΔCD-MT1) by introducing a stop codon at Arg288. Wild type MT1-MMP and the E240A-α11-MT1 and ΔCD-α11-MT1 mutants were expressed in BS-C-1 cells using the infection/transfection procedure followed by metabolic labeling as described under “Experimental Procedures.” The 35S-labeled conditioned media were immunoprecipitated with pAb 160. As shown in Fig. 3, wild type MT1-MMP shed the 50-, 31-, 28-, and 18-kDa species (Fig. 3, lane 1). In contrast, the E240A-α11-MT1 catalytic mutant shed the 50-kDa species and a ~28-kDa form but not the 18-kDa fragment (Fig. 3, lane 2) consistent with the autocatalytic shedding of the latter species. The ΔCD-MT1 mutant showed a shedding pattern similar to that observed with the wild type enzyme (Fig. 3, lane 3).

Structure and Characterization of the 18-kDa MT1-MMP Soluble Form—The relatively higher amounts of the 18-kDa fragment allowed its purification and characterization. N-termin al sequencing by Edman degradation revealed that the 18-kDa species displays Tyr112 in the N terminus, in agreement with the N terminus displayed by membrane-tethered active MT1-MMP (57 kDa) (9, 17). Mass spectrometry analysis of tryptic digests was performed to determine the C terminus of the 18-kDa fragment. As shown in the table of Fig. 4A (inset), a total of 32 peptides were isolated and their sequence determined. Three peptides demonstrated a C terminus ending at Ala255, indicating that the 18-kDa form extends from Tyr112 to Ala255 and therefore comprises most of the catalytic domain. Indeed, SDS-PAGE analysis demonstrated an ~3-kDa difference between the shed 18-kDa fragment (Fig. 4B, lane 2) and a commercially available recombinant catalytic domain of MT1-MMP (MT1-MMPcat), which is known to extend from Tyr112 to Gly258 (Fig. 4B, lane 1).

Considering that the 44-kDa membrane-tethered species of MT1-MMP starts at Gly258 (17), shedding of the 18-kDa fragment would require two cleavage events: one between Ala255 and...
and Ile256 and another between Gly284 and Gly285. To determine the relative location of these sites in the catalytic domain of MT1-MMP, we used an energy-minimized computational model of the ectodomain of human pro-MT1-MMP that was recently constructed in our laboratory. The only missing piece of structural information in this model pertained to the hinge between the catalytic and the hemopexin-like domains. The spatial location of the hemopexin-like domain in the computational model (data not shown) was based on that seen in the x-ray structure of MMP-2 (54). From this model, Fig. 5A shows only a view of the catalytic domain extending from Tyr112 to Ser287. In the event that the hinge actually would not dislocate the hemopexin-like domain in MT1-MMP, the Ala255-Ile256 bond is sheltered by the hemopexin-like domain, leaving the cleavage site Gly284-Gly285 as the only likely candidate for the first hydrolytic cleavage event. However, even if the hemopexin-like domain of MT1-MMP is dislocated away from the catalytic domain, leaving the surface regions shown in Fig. 5B fully exposed to the milieu, still the cleavage site Gly284-Gly285 is more readily accessible than is Ala255-Ile256 because of the nature of the secondary structures in the protein. Therefore, shedding of the 18-kDa fragment is likely to be initiated at the Gly284-Gly285 peptide bond, followed by a second cleavage at the Ala255-Ile256 site. Fig. 5C depicts a diagram of active MT1-MMP (Tyr112-Val1526), showing the two cleavages at the Gly284, Gly285 and Ala255-Ile256 sites leading to the formation of the inactive 44-kDa species, which has been isolated and characterized from plasma membranes (17), and the soluble 18 kDa species. To shed the 18 kDa species, this process would have also generated a ~21-kDa intermediate fragment (Fig. 5C, dashed bracket) extending from Tyr112 to Gly284. However, a soluble fragment of ~21 kDa, the putative precursor of the 18 kDa species, was not detected. Additionally, hydrolysis at the Ala255-Ile256 peptide bond predicted impaired catalytic activity of the 18-kDa species because of the proximity of this site to the conserved methionine residue (Met257) of the so-called methionine turn (55) and to the consensus sequence of the catalytic zinc ion binding site (Fig. 5C).

To gain insight into the biochemical properties of the 18-kDa (Tyr112-Ala255) fragment and the putative 21-kDa (Tyr112-Gly284) intermediate species, these proteins were expressed in bacteria and purified to homogeneity for further analyses. Fig. 6A shows the purity of the recombinant 18-kDa (Tyr112-Ala255) (lane 1) and 21-kDa (Tyr112-Gly284) (lane 2) proteins isolated. Activity assays demonstrated that, whereas the 21-kDa (Tyr112-Gly284) fragment exhibited gelatinolytic (Fig. 6B, lane 4) and pro-MMP-2-activating activities (Fig. 6C, lane 6), the 18-kDa (Tyr112-Ala255) fragment was catalytically inactive (Fig. 6, B (lane 3) and C (lane 5)). To obtain quantitative data, the recombinant fragments were examined for their ability to hydrolyze a fluorogenic peptide substrate as a function of time. MT1-MMPcat was used as a positive control. As summarized in Table 1, kcat and Km values of ~1 s-1 and 10 μM, respectively, were obtained yielding kcat/Km values of 104 M-1 s-1, which reflects the high reactivity of MT1-MMPcat and the 21-kDa (Tyr112-Gly284) enzymes toward the synthetic peptide substrate used. Moreover, indistinguishable values were obtained for these two MT1-MMP species. In contrast, no enzyme concentration dependence of the rate of substrate hydrolysis was detected with the 18-kDa (Tyr112-Ala255) fragment with concentrations up to 235 nM. In fact, the hydrolysis rate of the substrate in the presence of the enzyme was essentially indistinguishable from the background hydrolysis detected in buffer only. A comparable concentration of the 21-kDa (Tyr112-Gly284) species (54 nM) yielded an increase in fluorescence that rapidly exceeded the detection limit of the instrument used. Together, these studies indicate that autocatalytic processing of MT1-MMP at the Ala255-Ile256 site obliterates catalytic competence resulting in an inactive soluble form of 18 kDa.

Although the 18-kDa species of MT1-MMP is inactive, the other soluble species may maintain enzymatic activity. Unfortunately, the paucity of these enzyme species in the media precluded purification and characterization. Thus, to investigate the activity of the soluble MT1-MMP species, we used conditioned medium of BS-C-1 cells infected to express MT1-MMP and examined its ability to promote pro-MMP-2 activation after addition of exogenous recombinant pro-MMP-2. This cell expression system was chosen because it is devoid of MMP-2 (33) and because it releases the 50- and 18-kDa form of MT1-MMP into the media (Fig. 2, lane 3). As a control, we used conditioned media of BS-C-1 cells infected only with the T7 RNA polymerase-expressing vaccinia vTF7-3 virus (33). As shown in Fig. 7, the conditioned media derived from the MT1-MMP-expressing cells promoted the generation of the intermediate form of MMP-2 (Fig. 7, lane 6), consistent with the two-step model of surface activation of pro-MMP-2 by MT1-MMP, as previously proposed (53, 56). In contrast, no processing of pro-MMP-2 was observed with the control media (Fig. 7, lane 4), demonstrating the specificity of the reaction. Because the 18-kDa species is inactive, these studies suggest that MT1-MMP fragments other than the 18-kDa form, such as the 50-kDa species, are likely to be the enzyme species responsible in the media for the processing of pro-MMP-2.

**Interactions with TIMP-2**—We have previously shown that, on the cell surface, TIMP-2 binds to the active 57-kDa form of MT1-MMP but not the 44-kDa inactive species (17), indicating that binding is mostly mediated by the catalytic domain. Here
we examined the ability of the soluble MT1-MMP forms to bind TIMP-2. To this end, media of 35S-labeled BS-C-1 cells infected with mAb 101, only the 50-kDa species of MT1-MMP and some endogenously produced 35S-TIMP-2 were detected in the co-precipitate (Fig. 8A, lane 3). Indeed, BS-C-1 cells produce very low levels of endogenous TIMP-2 (20). No signal was detected in samples that received exogenous TIMP-1 and anti-TIMP-1 pAb (Fig. 8A, lane 4). Accordingly, the 50-kDa species could not be detected after immunoprecipitation with pAb 160 (Fig. 8A, lane 2). After addition of exogenous TIMP-2 and immunoprecipitation with mAb 101, only the 50-kDa species of MT1-MMP and some endogenously produced 35S-TIMP-2 were detected in the co-precipitate (Fig. 8A, lane 3). Indeed, BS-C-1 cells produce very low levels of endogenous TIMP-2 (20). No signal was detected in samples that received exogenous TIMP-1 and anti-TIMP-1 pAb (Fig. 8A, lane 4). Accordingly, the 50-kDa species could not
be detected with pAb 160 after TIMP-2 addition because of epitope occupancy in the enzyme-inhibitor complex as this pAb is directed to the catalytic domain (data not shown). These results indicate that TIMP-2, but not TIMP-1, binds to the soluble 50-kDa species via the catalytic domain, in agreement with the known TIMP-binding profile of MT1-MMP (53). In contrast, the soluble 18-kDa fragment cannot form a stable complex with TIMP-2.

A TIMP-2 affinity binding procedure was carried out to assess whether the 31–35-kDa species could bind TIMP-2. To this end, we used conditioned medium from BS-C-1 cells that were infected to express MT1-MMP in the presence of 1 μM marimastat, which induces the appearance of these species, as shown in Fig. 2B. The media were subjected to TIMP-2 affinity binding using immobilized TIMP-2, and the bound and unbound fractions were analyzed by immunoblot analysis as described under "Experimental Procedures." As shown in Fig. 8B, the 31–35- and the 50-kDa species were detected in the bound fraction (Fig. 8B, lane 3), albeit a significant amount of these species remained in the unbound fraction (Fig. 8B, lane 2) when compared with the load (Fig. 8B, lane 1), suggesting that they exhibit a relatively low affinity for TIMP-2, under the experimental conditions. In contrast, the 18-kDa species did not bind to the TIMP-2 affinity matrix, in agreement with the immunoprecipitation data. Although these studies are not quantitative, the poor binding of the soluble MT1-MMP species to the immobilized TIMP-2 is unlikely to be the result of the presence of marimastat because the affinity matrix is saturated with TIMP-2 and the final concentration of marimastat was less than 0.3 μM.

We next examined the ability of the recombinant MT1-MMP fragments to bind TIMP-2 by co-immunoprecipitation. These studies demonstrated that only the 21-kDa (Tyr112–Gly284) species was able to form a stable complex with the inhibitor (Fig. 8C). The 21-kDa (Tyr112–Gly284) fragments were incubated (1 h) with TIMP-2 (1:1 molar ratio) in 50 μl of conditioned medium received 100 ng of unlabeled recombinant TIMP-2 (rT2, lane 2) or TIMP-1 (rT1, lane 4) followed by immunoprecipitation with anti-TIMP-2 (mAb 101) or anti-TIMP-1 pAb, respectively, and Protein G-agarose beads. The immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography. The asterisk indicates the lane with 14C-labeled molecular size standards. B, BS-C-1 cells were co-infected with vaccinia viruses to express MT1-MMP. After infection the cells were incubated overnight in serum-free DMEM supplemented with 1 μM marimastat to induce appearance of the 31–35-kDa species. The conditioned media were collected, concentrated (10–fold), and subjected to TIMP-2 affinity binding as described under "Experimental Procedures." The bound and bound MT1-MMP forms were resolved by reducing 15% SDS-PAGE followed by immunoblot analysis using the mAb LEM-2/15. Lane 1, sample before affinity binding; lane 2, bound fraction; lane 3, unbound fraction. C, the recombinant 15-kDa (Tyr112–Ala255) (lanes 1 and 2) and 21-kDa (Tyr112–Gly284) (lane 3) MT1-MMP fragments were incubated (1 h) with TIMP-2 (1:1 molar ratio) in 50 μl of collagenase buffer. The samples were subjected to immunoprecipitation with pAb 160 to MT1-MMP (lane 2) or mAb 101 to TIMP-2 (lanes 1 and 3). The immunoprecipitates were resolved by 15% SDS-PAGE followed by immunoblot (IB) analysis with the same antibodies.

**DISCUSSION**

The stability of active MT1-MMP on the cell surface is a complex process involving a balance between autocatalytic processing (17) and enzyme internalization (57, 58). Both processes can regulate the amount of active enzyme available for pericellular proteolysis and appear to be regulated in part by the presence of TIMPs. The major pathway of active MT1-MMP processing on the cell surface is an autocatalytic event that generates a 44-kDa membrane-anchored fragment starting at Gly285 and thus lacks the entire catalytic domain (17). This process may switch the proteolytic machinery from the cell surface to the pericellular space if the released fragments are competent enzymes or may obviate proteolysis on all fronts, if the soluble fragments are inactive and/or in a complex with TIMPs. To define the fate of the catalytic domain after processing, we set out to investigate the nature and properties of the MT1-MMP soluble fragments produced in various cellular systems. Here we have shown that media of cells expressing natural or recombinant MT1-MMP contain a complex profile of...
MT1-MMP species including two major species of 50- and 18 kDa and a series of minor fragments of 56, and 31–35 kDa, which are differentially regulated by TPA, ConA, and MMP inhibitors. With the exception of the 56-kDa species, which was retained in the pellet after ultracentrifugation and thus, may be associated with membrane fragments (52), all other MT1-MMP species were soluble and thus, represent true shedding. Shedding of the 50- and 18-kDa species occurred without external stimulation, indicating that they represent a normal process of MT1-MMP turnover under basal conditions. However, exposure of cells to either TPA or ConA, two nonphysiological agents known to induce MT1-MMP expression and pro-MMP-2 activation (29, 50, 59), resulted in increased levels of all the soluble forms in the media. Based on the protease inhibitor profile, both autocatalytic and non-autocatalytic processes are involved in MT1-MMP shedding. High affinity natural (TIMP-2 and TIMP-4, but not TIMP-1) and synthetic MMP inhibitors, known to stabilize active MT1-MMP on the cell surface by inhibiting the processing of MT1-MMP to the 44-kDa form (17, 19–21), inhibited shedding of the 18-kDa species. Additionally, the E240A-MT1 catalytic mutant enzyme, which cannot be processed to the 44-kDa species (22, 60), failed to shed this fragment. Thus, shedding of the 18-kDa species is the product of the autocatalytic processing of active MT1-MMP (57 kDa) on the cell surface, which yields a major inactive membrane-tethered species of 44 kDa (17). Consistently, TPA and ConA treatments, which promote MT1-MMP expression and processing on the cell surface, stimulate shedding of the 18-kDa fragment. The ability of cells to elicit autocatalytic shedding depends on the expression level of MT1-MMP on the cell surface and the levels and availability of TIMPs. High levels of TIMP-2 and/or presence of other TIMP-2-binding MMPs will alter the autocatalytic pathway by modifying TIMP-2 availability as shown in MDA-MB-231 cells, which, as opposed to HT1080 cells, do not produce MMP-2; therefore, the autocatalytic shedding (release of the 18-kDa species) is restricted.

A battery of protease inhibitors including metalloprotease, serine, and aspartic protease inhibitors failed to reduce the levels of the 50- and 31–35-kDa species. Additionally, these species were observed in the media of cells expressing the E240A-MT1 catalytic mutant. Thus, production of these soluble MT1-MMP fragments is a non-autocatalytic event. Interestingly, TPA and ConA, which promote autocatalytic processing, enhanced the levels of these species, suggesting an additional level of regulation by these agents (50). The identity of the protease(s) responsible for the non-autocatalytic shedding of MT1-MMP remains to be determined. Our evidence and previous evidence (23, 24) suggest that, in the case of the 50-kDa species, the protease(s) must cleave within the juxtamembrane (stem) region of MT1-MMP causing the release of the entire ectodomain. Consistently, the soluble 50-kDa species was able to form a complex with TIMP-2, in agreement with early studies (24). Whether cleavage at the stem region takes place at the cell surface or intracellularly, as shown with MT5-MMP (18), remains to be determined. However, it is unlikely that a furin-like enzyme is involved in this process because, in contrast to MT5-MMP, a specific furin-recognition motif was not found in the ectodomain of MT1-MMP (18). Based on the pattern of MT1-MMP forms present on the cell surface (the 57- and 44-kDa species) and the high levels of the 18-kDa species, as determined by the immunoprecipitation experiments, the non-autocatalytic pathway is likely to comprise a minor aspect of the shedding process. However, this process may produce functional enzyme fragments, such as the 50-kDa species (24), which would promote pro-MMP-2 processing, as demonstrated here with the conditioned media of BS-C-1 cells expressing MT1-MMP, and as shown previously in gelatin zymography assays (24). Thus, soluble ectodomain fragments with catalytic activity may extend MT1-MMP-dependent proteolysis beyond the cell surface environment by promoting the hydrolysis of a variety of substrates including extracellular matrix components (4). In addition, these fragments by binding TIMP-2 may deprive the membrane-tethered enzyme of inhibitor regulation.

An interesting observation of this study was the appearance of a 31–35-kDa soluble species that was induced either by ConA treatment or high levels of TIMP-2 or marimastat. The appearance of the 31–35-kDa species in the presence of TIMP-2 or marimastat correlated with a decrease in the levels of the 18-kDa species, suggesting the possibility that the formation of these species may be related. For example, it is possible that, in addition to the autocatalytic shedding, there is a non-MMP-dependent shedding mechanism that releases the 31–35-kDa fragment, which in turn is processed to the 18-kDa species via a metalloprotease-dependent process, as suggested by the accumulation of the 31–35-kDa species in the presence of TIMP-2 or marimastat. Alternatively, TIMP-2 binding to the 31–35-kDa species may prevent a non-metalloprotease from accessing the Ala255-Ile256 site, thus resulting in accumulation. Indeed, the 31–35-kDa species binds TIMP-2, albeit with an apparent low affinity. Another possibility is that the accumulation of the 31–35-kDa species in the presence of TIMP-2 or marimastat represents shedding of MT1-MMP catalytic domain-inhibitor complexes. Stabilization of the membrane-anchored enzyme by formation of enzyme-inhibitor complexes (17) may induce conformational changes, which may predispose the enzyme to a non-metalloprotease-mediated ectodomain shedding. However, the lack of a readily detectable counterpart to the 31–35-kDa species on the plasma membrane suggests the possibility that this fragment(s) is not a shedding product of the membrane-bound enzyme but a result of the turnover of the larger MT1-MMP soluble species, like the 50-kDa species, via a TIMP-2-insensitive process. Structural data and studies in cellular systems with defined proteolytic backgrounds will help to distinguish between these possibilities. Although the potential contribution of the 31–35-kDa species to the formation of the 18-kDa species cannot be disregarded, this is likely to be minimal when compared with the formation of the 18-kDa species generated by the autocatalytic processing of MT1-MMP on the cell surface. Taken together, these observations further underscore the complexity of the MT1-MMP shedding process and the unexpected consequences that TIMP-2 and synthetic MMP inhibitors may have on the regulation of MT1-MMP on the cell surface, as we have previously documented (20, 21).

Previous studies reported that the cytosolic domain of MT1-MMP plays a role in the stabilization of MT1-MMP on the cell surface by altering the rate of enzyme internalization (58) and is also involved in enzyme homodimerization (60), a process thought to favor autocatalytic turnover (61). Here we have shown that the pattern of MT1-MMP shedding was essentially unaltered in enzymes lacking the cytosolic domain. This finding suggests that homotypic physical interactions mediated by the cytosolic domain (60) are not essential for MT1-MMP autocatalytic and non-autocatalytic shedding.

The autocatalytic pathway of MT1-MMP shedding concludes with the release of an 18-kDa fragment that extends from Tyr212 to Ala255, 29 amino acid residues upstream of the Gly285 displayed at the N terminus of the membrane-tethered 44-kDa species (17). Therefore, shedding of the 18-kDa fragment would require cleavage at both the Ala255-Ile256 and the Gly284-Gly285 peptide bonds. The computational model of MT1-MMP shows that the Ala255-Ile256 peptide bond near the methionine turn is sheltered and thus is less accessible to proteolysis. This sug-
suggests that cleavage at the Gly\(^{284}\)-Gly\(^{285}\) peptide bond, which is on the surface, is likely to precede that at the Ala\(^{255}\)-Ile\(^{256}\) site. However, at present, it is unclear whether cleavage at the Gly\(^{284}\)-Gly\(^{285}\) site predisposes for hydrolysis at the second Ala\(^{255}\)-Ile\(^{256}\) site. Data from the recombinant 21-kDa fragment, which contains the Ala\(^{255}\)-Ile\(^{256}\) site but ends at Gly\(^{284}\), clearly indicates that disruption of the Gly\(^{284}\)-Gly\(^{285}\) site does not disturb the integrity and functionality of the Ala\(^{255}\)-Ile\(^{256}\) peptide bond. Indeed, we have shown that the 21-kDa fragment is stable and catalytically competent. Furthermore, the 21-kDa fragment was not hydrolyzed at the Ala\(^{255}\)-Ile\(^{256}\) site when was incubated alone or with HT1080 cells or their conditioned media (data not shown). We posit that the sequence of events leading to the cleavage of the Ala\(^{255}\)-Ile\(^{256}\) peptide bond after cleavage at the Gly\(^{284}\)-Gly\(^{285}\) site occur only within membrane-tethered active MT1-MMP molecules. Conceivably, cleavage at the Gly\(^{284}\)-Gly\(^{285}\) site within a membrane-anchored enzyme destabilizes the structure yielding the Ala\(^{255}\)-Ile\(^{256}\) peptide bond susceptible for subsequent hydrolysis. The inhibitor profile suggests that cleavage at the Gly\(^{284}\)-Gly\(^{285}\) site is an autocatalytic event because high affinity MT1-MMP protease inhibitors like TIMP-2 and TIMP-4 (19, 53, 62) prevented formation of the 44-kDa species starting at Gly\(^{285}\) (17). Additionally, a catalytic mutant of MT1-MMP was not processed to the 44-kDa species, as previously reported (22, 60). In regard to the Ala\(^{255}\)-Ile\(^{256}\) site, the data suggest that cleavage at that site most likely to be also autocatalytic because TIMP-1 does not prevent shedding of the 18-kDa species. In addition, if it were mediated by another metalloprotease or a serine protease, the presence of TIMP-1, marimastat, or serine protease inhibitors should have resulted in the appearance of the 21-kDa fragment extending from Tyr\(^{112}\) to Gly\(^{284}\), which was not detected. According to the N terminus of the membrane-bound 44-kDa species (Gly\(^{285}\)) and the C-terminal end of the soluble 18-kDa fragment (Ala\(^{255}\)), shedding of the catalytic domain should proceed via an intermediate species of ~21 kDa. However, such a species could not be detected on the cell surface or in the media of cells expressing recombinant or natural MT1-MMP. A plausible explanation for the absence of a soluble 21-kDa fragment during the shedding of the 18-kDa species is that the cleavages at the Ala\(^{255}\)-Ile\(^{256}\) and Gly\(^{284}\)-Gly\(^{285}\) sites occur rapidly and sequentially and thus would preclude accumulation of a 21-kDa intermediate form and thus ends with the 18-kDa fragment as the final product. Attempts to induce accumulation of the intermediate 21-kDa species by generating A255V or A255I substitutions at the Ala\(^{255}\)-Ile\(^{256}\) cleavage site were unsuccessful as these mutants failed to undergo activation and processing, demonstrating the importance of this site for catalytic competence.

The 18-kDa fragment ends just one residue upstream of the conserved Met\(^{257}\) known to be part of the methionine turn, a structural feature characteristic of all members of the MMP family and of the super family of metzincins (55). Topologically, the methionine turn is positioned near the three histidines that coordinate with the catalytic zinc ion and is on the opposite side of these residues with respect to the active site cavity. Thus, the methionine turn is thought to be critical for catalysis, based on its close proximity to the coordination site for the catalytic zinc ion. Furthermore, the side chain of Ile\(^{256}\), upstream of Met\(^{257}\), forms a portion of the S1’ pocket of this enzyme. The loss of the 29-amino acid fragment during the formation of the 18-kDa MT1-MMP species would by necessity truncate the S1’ pocket; hence, it has the ability to potentially impair or alter substrate binding properties. Furthermore, the proximity of the surface loop that bears the methionine residue to the TIMP-2 binding region (43) indicates that a disorder in this location would likely impair TIMP-2 binding. Our results with the 18-kDa fragment support this notion and provide experimental documentation of the importance of the methionine turn for MMP-mediated catalysis and TIMP binding. It is worth noting that, as far as we know, the proteolytic inactivation at the methionine turn as it occurs during MT1-MMP processing has not been reported for other members of the MMP family including soluble MMPs, despite the conserved nature of this motif. This suggests the possibility that MT1-MMP specifically developed a self-controlling mechanism to allow the enzyme to function principally as a membrane-anchored protease, and any perturbation in its cellular localization would result in specific enzyme inactivation. This may explain why a transmembrane-deleted soluble MT1-MMP expressed in HT1080 cells was processed at the Ala\(^{255}\)-Ile\(^{256}\) site,

\(^{3}\) P. Osenkowski and R. Fridman, manuscript in preparation.
possibly by the endogenous MT1-MMP (15). Together, these data reveal the importance of the Ala255–Ile256 site for the maintenance of catalytic competence in MT1-MMP. Sequence alignment of the transmembrane MT-MMPs (MT1-, MT2-, MT3-, and MT5-MMP) (26) reveals a complete homology around the A-I peptide bond and the residues near the methionine turn (depicted in Fig. 5C). Presently, the shedding mechanisms of the MT-MMP family members have not been completely elucidated. It would be interesting to determine whether cleavage at the conserved Ala-A-I peptide bond represents a common and specific mechanism designed to terminate MT-MMP-dependent catalysis and TIMP interactions at the cell surface.

In summary, we have identified the major soluble forms of MT1-MMP and demonstrated the complexity of MT1-MMP shedding and its regulation by natural and synthetic MMP inhibitors. Fig. 9 depicts the autocatalytic processing of MT1-MMP on the cell surface leading to the release of the inactive 18-kDa species and the non-autocatalytic shedding leading to the release of the entire ectodomain by a yet unknown protease. Inhibitors of MT1-MMP block autocatalytic shedding and thus stabilize the active enzyme on the cell surface (17). The autocatalytic shedding terminates MT1-MMP-dependent pericellular proteolysis, independently of endogenous inhibitors, by specific hydrolysis at vital conserved sites (methionine turn). On the other hand, the non-autocatalytic shedding, as represented by the 50-kDa species (24) and possibly the 31–35-kDa species (not shown in Fig. 9), may still contribute to pericellular proteolysis and partly compensate for the removal of enzyme from the cell surface by shifting the proteolytic machinery to a new front, possibly with different substrates and functional consequences. Finally, the shed ectodomains of MT1-MMP may bind TIMPs (24) and hence alter the enzyme-inhibitor balance at the cell surface and/or may play new unexpected roles (3).
Complex Pattern of Membrane Type 1 Matrix Metalloproteinase Shedding: REGULATION BY AUTOCATALYTIC CELL SURFACE INACTIVATION OF ACTIVE ENZYME
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J. Biol. Chem. 2002, 277:26340-26350.
doi: 10.1074/jbc.M200655200 originally published online May 9, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200655200

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