Proteolysis of the Membrane Type-1 Matrix Metalloproteinase Prodomain

IMPLICATIONS FOR A TWO-STEP PROTEOLYTIC PROCESSING AND ACTIVATION*

Received for publication, July 31, 2007, and in revised form, September 26, 2007. Published, JBC Papers in Press, October 15, 2007. DOI 10.1074/jbc.M706290200

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Membrane type-1 matrix metalloproteinase (MT1-MMP) exerts its enhanced activity in multiple cancer types. Understanding the activation process of MT1-MMP is essential for designing novel and effective cancer therapies. Like all of the other MMPs, MT1-MMP is synthesized as a zymogen, the latency of which is maintained by its inhibitory prodomain. Proteolytic processing of the prodomain transforms the zymogen into a catalytically active enzyme. A sequential, two-step activation process is normally required for MMPs. Our in silico modeling suggests that the prodomain of MT1-MMP exhibits a conserved three helix-bundled structure and a “bait” loop region linking helices 1 and 2. We hypothesized and then confirmed that in addition to furin cleavage there is also a cleavage at the bait region in the activation process of MT1-MMP. A two-step sequential activation of MT1-MMP is likely to include the MMP-dependent cleavage at either P47GD or P80Q or at both sites of the bait region. This event results in the activation intermediate. The activation process is then completed by a protease initially cleaving the inhibitory prodomain at the R108RKR111 site, where Tyr112 is the N-terminal residue of the mature MT1-MMP enzyme. Our findings suggest that the most efficient activation results from a two-step mechanism that eventually is required for the degradation of the inhibitory prodomain and the release of the activated, mature MT1-MMP enzyme. These findings shed more light on the functional role of the inhibitory prodomain and on the proteolytic control of MT1-MMP activation, a crucial process that may be differentially regulated in normal and cancer cells.

Matrix metalloproteinases (MMPs), a family comprised of 25 individual zinc-dependent proteolytic enzymes, are classi-
catalytically active MT1-MMP enzyme involves proprotein convertases including furin, PACE4, PC6, and PC7 (23–25). The requirement for furin activity in the activation of the cellular MT1-MMP and MT3-MMPzymogens was convincingly demonstrated (26, 27). We, however, hypothesized that similar to the other MMPs, the activation pathway of MT1-MMP, in addition to the known proprotein convertase cleavage, involves a previously uncharacterized cleavage step that leads to the activation intermediate. The intermediate is then processed by proprotein convertases with the generation of the mature and fully active enzyme of MT1-MMP.

To determine whether this hypothesis is correct, we performed an extensive biochemical analysis of the cleavage events that target the prodomain of MT1-MMP. As a result of these studies, we suggest that the activation of MT1-MMP involves a two-step mechanism in which the processing of the prodomain sequence at the P17-GD↓L50 and P18-QS↓L61 cleavage sites generates the intermediate form. The activation intermediate is then processed by a proprotein convertase cleaving at the R108RKR↓Y112 site and generating the fully activated enzyme of MT1-MMP.

**MATERIALS AND METHODS**

**Antibodies, Reagents, and Cells**—Rabbit polyclonal antibody (AB815) against the hinge region of MT1-MMP and the murine monoclonal antibody (clone 3G4) against the catalytic domain of MT1-MMP and the hydroxamate inhibitor GM6001 were from Chemicon (Temecula, CA). The mouse monoclonal antibody against a V5 epitope was from Invitrogen. Sulfosuccinimidyl-6-(biotinamido)hexanoate (EZ-Link sulfo-NHS-Long Chain(LC)-biotin) was from Pierce. Human glioma U251 cells were originally from ATCC (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. As a control, we used U251 cells stably transfected with the two original pcDNA3.1-zeo and-neo plasmids (mock cells). For MT1-MMP overexpression, U251 cells transfected with the two original pcDNA3.1-zeo plasmids were constructed and randomly selected (numbers 5, 7, and 8) and then used for further analysis. MCF-7 cells stably expressing the wild-type MT1-MMP (MT1-WT cells) and the catalytically inert E240A mutant as well as the mock cells stably transfected with the original pcDNA3.1-zeo plasmids were constructed and described earlier (29).

**Cloning and Expression of the Recombinant MT1-MMP Catalytic Domain, the Prodomain, and the Soluble, Catalytically Inert MT1-MMP E240A Constructs**—The MT1-MMP catalytic domain (MT1-CAT) was expressed and purified as described (31, 32). The catalytically inert E240A full-length MT1-MMP mutant cDNA (29) was used as a template to clone the MT1-MMP prodomain (MT1-PRO) and the soluble catalytically inert MT1-MMP E240A (MT1-PRO-CAT-PEX) constructs. The MT1-PRO-CAT-PEX construct included the propeptide sequence (PRO), the inert (E240) catalytic domain (CAT), and the hemopexin (PEX) domains. To facilitate the isolation and detection in the samples, MT1-PRO-CAT-PEX was tagged with a His6 tag both C- and N-terminal. In addition, a V5 epitope sequence was linked to the C-terminal His6 tag sequence.

Specifically, the forward (5′-CACCATGCATCATCATCATC-TCATCATACCGCGCTGGCTCTCCCTCGGCTC-3′) and the reverse (5′-TTAGCGCTTCCTTCTGGAACATTGG-3′) primers were used to obtain the MT1-PRO construct (the His6 tag sequence is underlined) in the PCR. The forward (5′-CACCATTGATCATATCATCTATCATCTGGTGGCCTGGCCCAAGAAGCAGAGCTTCCCCTCG-3′) and the reverse (5′-GGCTACGCGCCCGGCGGTC-3′) primers were used in the cloning of MT1-PRO-CAT-PEX. After confirming their authenticity by sequencing, the constructs were then re-cloned into the pET101 expression vector. Competent Escherichia coli BL21(DE3) Codon Plus cells (Strategene) were transformed with the recombinant vectors. Cells were grown at 37 °C in a Luria-Bertani broth containing ampicillin (0.1 mg/ml). Culture was induced with 1 mM isopropyl β-D-thiogalactoside for 6 h at 37 °C. E. coli cells (6 g/liter of E. coli culture) were then collected by centrifugation (5,000 × g; 15 min), re-suspended in 20 ml of 10 mM Tris-HCl, pH 8.0, containing 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and lysozyme (5 mg/ml), and disrupted on ice by sonication (30 s pulse, 30-s intervals; 8 pulses total).

The MT1-PRO was purified from the supernatant fraction using a 1.6 × 10-cm Co2+-chelating Sepharose Fast Flow column (Amersham Biosciences) equilibrated with PBS supplemented with 1 mM NaCl. MT1-PRO was eluted with an imidazole gradient (10–100 mM; 100 ml) in PBS, 1 mM NaCl. The MT1-PRO
fractions were concentrated using a 5-kDa cutoff concentrator (Millipore, Billerica, MA) and dialyzed against PBS containing 0.005% Brij35. A polyclonal antibody to the purified individual MT1-PRO was then raised in rabbits.

The MT1-PRO-CAT-PEX inert construct was purified from the inclusion bodies and then refolded to restore its native conformation. The inclusion bodies (10 mg of total protein) were washed in 10 mM Tris-HCl, pH 8.0, containing 1 M NaCl and 1% Triton X-100 and then dissolved in 10 mM Tris-HCl, pH 8.0, containing 6 M guanidine hydrochloride and 10 mM 2-mercaptoethanol. The refolded MT1-PRO-CAT-PEX was next concentrated using a 30-kDa cutoff concentrator (Millipore) and purified on a 1.6 × 10-cm Co2+/chelating Sepharose Fast Flow column (Amersham Biosciences) equilibrated with PBS, 1 M NaCl. The construct was eluted with an imidazole gradient (10–500 mM gradient; 100 ml) in PBS, 1 M NaCl, concentrated using a 30-kDa cutoff concentrator and dialyzed against PBS, containing 0.005% Brij35.

Cleavage of Synthetic Peptides, MT1-PRO, MT1-PRO-CAT-PEX, and Immunoprecipitated Cellular MT1-MMP—The peptides (Y44LPPGDL50RTHTQRSPO59 and H53TQRSPQSL61 SAAIAM68) that span the putative cleavage sites in the MT1-MMP prodomain and the corresponding mutant peptides (Y44LPPGDL50RTHTQRSPO59 and H53TQRSPQSL61 SAAIAM68; the mutant residues are underlined) were synthesized by GenScript (San Diego, CA). The peptides, MT1-PRO and MT1-PRO-CAT-PEX (1 µg each), were co-incubated with MT1-CAT or MMP-2 (20 nM each) for the indicated time at 37 °C in 50 mM HEPES, pH 6.8, supplemented with 10 mM CaCl2, 0.5 mM MgCl2, and 50 mM ZnCl2. The digest samples were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MS) using a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer to determine the mass of the cleavage products and, consequently, the location of the scissile bonds.

To identify the N-terminal sequence of the cleavage fragments, the catalytically inert MT1-PRO-CAT-PEX E240A construct (5 µg) was co-incubated with MT1-CAT and furin (50 ng each) for 1 h at 37 °C in 50 mM HEPES buffer, pH 6.8. Recombinant human furin was prepared in the S2 Drosophila expression system (Invitrogen) and purified to homogeneity (33). The reactions were separated by SDS gel electrophoresis followed by the transfer of the protein bands on the polyvinylidene difluoride membrane and N-terminal microsequencing of the resulting bands. Microsequencing was performed at ProSeq (Boxford, MA).

For the subsequent cleavage experiments, cellular MT1-MMP was immunoprecipitated, using ABB15 antibody (1 µg) and Protein G-agarose beads (20 µl of a 50% slurry), for 12 h at 4 °C from the cell lysate aliquots (1 mg of total protein each) of the confluent MT1/PDX cells. The lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 1% IGEPAL, pH 7.4) was supplemented with a protease inhibitor mixture set III (Sigma) (1 mM phenylmethylsulfonyl fluoride and 10 mM EDTA). The beads were collected by centrifugation and then washed in 50 mM HEPES, pH 6.8. The samples were incubated for 30 min at 37 °C with MT1-CAT (20 ng) in 50 mM HEPES, pH 6.8, containing 10 mM CaCl2, 0.5 mM MgCl2, and 50 µM ZnCl2. The digest samples were analyzed by Western blotting with 3G4 antibody and a TMB/E substrate (Chemicon) to identify the cleavage products.

Cell Surface Biotinylation—Cell surface-associated MT1-MMP was biotinylated by incubating cells (80–90% confluency) for 30 min on ice in PBS containing 0.1 mg/ml EZ-Link NHS-LC-biotin. Excess biotin was removed by washing cells in ice-cold PBS and then quenched by incubating cells for 10 min in PBS containing 100 mM glycine. After washing with PBS, cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 1% IGEPAL, pH 7.4) supplemented with a protease inhibitor mixture set III. MT1-MMP was precipitated from cell lysates using streptavidin-agarose beads and analyzed by Western blotting with the MT1-MMP antibody (3G4) followed by the goat secondary horseradish peroxidase-conjugated IgG and a TMB/M substrate (Chemicon).

Gelatin Zymography—Gelatin zymography was used to determine the efficiency of MMP-2 activation by cellular MT1-MMP. Cells were plated in the wells of a 48-well plate (Costar/Corning) in serum-containing Dulbecco’s modified Eagle’s medium and grown to reach a 90% confluence. The medium was then replaced with serum-free Dulbecco’s modified Eagle’s medium supplemented with the purified MMP-2 proenzyme (100 ng/ml). After incubation for 12 h, the medium aliquots were analyzed by gelatin zymography on 10% acrylamide gels containing 0.1% gelatin (Novex) to detect the proenzyme and the activated species of MMP-2.

Modeling and Multiple Sequence Alignment—The propeptide size and related annotations were obtained from the UNIPROT data base (srs.ebi.ac.uk) (34). The structure parameters of the propeptide were obtained from the known atomic resolution structures of the proenzymes of MMP-1 (Protein Data Bank entry 1SU5) (5), MMP-2 (PDB 1CK7) (7), MMP-3 (PDB 1SLM) (8), and MMP-9 (PDB 1L6J) (6) and the catalytic. The sequence alignment was performed with CLASTALW (35) and edited with Jalview (36). The structure of the MT1-MMP proenzyme (residues 36–508) was modeled with the program MODEPIPE (37) using PDB entries 1SU5 (MMP-1), 1CK7 (MMP-2), 1SLM (MMP-3) and entries 1BQO and 1BVE (the catalytic domain of MT1-MMP) (38) as templates. The sequence identity of the MMP-1 template with MT1-MMP is 40% (E-value = 1e−17). The predicted structure of the MT1-MMP propeptide is in a good agreement with all structurally investigated MMP propeptides (root mean square deviation = 0.9 Å).

RESULTS

Structural Modeling of the Three Helix-bundled MT1-MMP Prodomain—The multiple sequence alignment of the prodomain peptide sequences of several MMPs is shown in Fig. 1. There is a significant sequence homology of the helical regions and loops 2 and 3 in the peptide sequence of MMPs. In contrast,
loop 1, which is the bait region in MMP-1, -2, and -9 (9), displays the least homology thus providing structural evidence of a unique means of the first proteolytic step of a two-step activation mechanism for each MMP. The second and the final activation step of MMPs including MT1-MMP involves cleavage at the C-terminal part of loop 3 (Fig. 1).

Because the crystal structure of the MT1-MMP proenzyme is not currently available, we used in silico modeling to model the spatial structure of the MT1-MMP prodomain. The available structures of the sequence homologous MMP-1, -2, -3, and 9 were used as a template. We also built the model of MT1-MMP using the structure of the MT1-MMP catalytic domain and the structures of the prodomain and the PEX domain of MMP-1, MMP-2, and MMP-3 (Fig. 1).

Computer modeling suggests that the triple helix bundle is highly conserved in MMPs including the MT1-MMP prodomain. In our model, in agreement with the solved structures for proMMP-1, proMMP-2, proMMP-3, and proMMP-9, the proMT1-MMP prodomain has a conserved 3-helix structure. Consistent with the exposure of the bait region in loop 1 in MMP-1, -2, -3, -9, and other MMPs, the loop 1 peptide sequence also appears highly accessible to the proteolytic attack in MT1-MMP. It has also been established that the MMP cleavage motifs predominantly exhibit the presence of the P3 Pro and a hydrophobic residue (especially Leu) at the P1’ position. There are two potential cleavage motifs, P47GD L50 and P58QS L61, in the loop 1 sequence of the prodomain of MT1-MMP. Because the furin cleavage of the R108RKR111 loop 3 sequence represents the final step of MT1-MMP proenzyme processing, we hypothesized that the loop 1 bait sequence is the target of either the autolytic cleavage or this processing is performed by an external protease with an MMP cleavage specificity.

**MT1-MMP Proteolysis of the Prodomain in Vitro**—To determine whether the loop 1 sequence is susceptible to MT1-MMP autoproteolysis, we synthesized the peptides (Y44LPPGD L50RTHTQRSPQ59 and H53TQRSPQS L61SAAIAM68) that overlap the putative cleavage sites. We also synthesized the mutant peptides in which the cleavage sites were inactivated by the L50D and L61D mutations, respectively. The peptides were subjected to proteolysis by MT1-CAT and MMP-2. The MS analysis of the digest reactions were followed to determine the
The overlapping peptides (Y44LPPGD ↓ \textit{L}^{50}\text{RTH} \text{QRSPQS}^7 \textit{L}^{51}\text{SAAIAAM}^8, underlined) that span the putative cleavage sites of the bait region of the MT1-MMP prodomain were cleaved by MT1-CAT and MMP-2. The mass of the reaction products was identified by MS, A, the 44–68 sequence of the prodomain. The sequence of the synthesized peptides (44–59 and 53–68) is underlined. The cleavage peptides (italicized) identified by MS are shown in the bottom part of the panel. B, the sequence and the molecular mass (shown in daltons) of the intact 44–59 and 53–68 peptides and the cleavage products (44–49, 50–59, 53–60, and 61–68).

To additionally confirm these results, we isolated the recombinant MT1-PRO and then subjected the construct to MT1-CAT proteolysis and MMP-2 proteolysis (Fig. 3). The digest reactions were analyzed by SDS electrophoresis in 10–20% polyacrylamide gels in the Tris-Tricine system (Invitrogen) and the 61–111 C-terminal fragment of the MT1-PRO construct was accessible to furin (Fig. 4), we concluded that following refolding the construct restored its native conformation.

**MT1-MMP Proteolysis of the Inert, Soluble MT1-MMP Construct—**To corroborate our cleavage data we isolated and then refolded the inert (E240A) soluble MT1-MMP construct (MT1-PRO-CAT-PEX) to restore its native conformation. The construct was efficiently cleaved by furin at the R108RKR \textit{111} ↓ Y112 cleavage site and generated the mature MT1-MMP enzyme N-terminal sequence following from Tyr112. In the MT1-MMP proenzyme the N-terminal putative furin cleavage site (R\textit{108}RPR \textit{92}) is a part of the cysteine switch-motif (R\textit{90}RPRCGVPD \textit{97}; the switch motif sequence is underlined). It is well established that the cysteine-switch sequence region interacts with the active site histidines, maintains the latent proenzyme state of MT1-MMP, and in the native proenzyme is inaccessible to external furin (23). Because only the conventional R\textit{108}RKR \textit{111} ↓ Y112 cleavage site of the MT1-PRO-CAT-PEX construct was accessible to furin (Fig. 4), we concluded that following refolding the construct restored its native conformation.

We next subjected MT1-PRO-CAT-PEX to MT1-MMP proteolysis. The cleavage reactions were analyzed by SDS-gel electrophoresis and then the N-terminal sequence of the resulting cleavage fragments was determined by N-terminal amino acid microsequencing. As expected, the sequence of the intact

MT1-PRO-CAT-PEX construct (Fig. 4A, upper band) commenced from the N-terminal MHHHHHHHG sequence (the His6 tag sequence is underlined; amino acid numbering starts from the signal peptide sequence). The identified N-terminal sequence of the cleavage product (Fig. 4B, lower band) was LSAAIAAMXXF, thus suggesting that proteolysis at the P58QS↓L61 took place and that this proteolysis generated the fragment commencing from the N-terminal Leu61.

To analyze the cleavage products in more detail, the cleavage reaction aliquots were subjected to MS. The peptides, the molecular mass of which corresponded well to peptides 26-50 (LRTHTQRSPQS) were identified in the reactions. Taken together, these results suggest that autolytic cleavage of the prodomain sequence of MT1-MMP may involve proteolysis at either the P7GD↓L50 site or the P58QS↓L61 site or at both sites generating, as a result, an intermediate activation species of MT1-MMP.

**Activation Intermediate of Cellular MT1-MMP**—To additionally confirm the presence of the activation intermediate of MT1-MMP, we immunoprecipitated cellular MT1-MMP from MT1/PDX cells using the rabbit Ab815 antibody. These cells co-express MT1-MMP with the inhibitor of furin, α1-antitrypsin variant Portland (PDX). We specifically selected MT1/PDX cells for our analysis because of the high levels of the MT1-MMP zymogen in these cells. Earlier, we demonstrated that the expression of PDX significantly repressed the activity of the cellular furin-like proprotein convertases and, in a result, enhanced the levels of the MT1-MMP in MT1/PDX cells when compared with the PDX-negative glioma cells (30). The precipitated material (IP) was subjected to MT1-CAT hydrolysis. The reaction products were analyzed by Western blotting using the 3G4 monoclonal antibody that was raised against MT1-CAT (Fig. 5). The intact samples of cellular MT1-MMP demonstrated the presence of the major bands that represent the conventional MT1-MMP proenzyme and the enzyme and a minor band of the intermediate molecular weight. Following MT1-CAT proteolysis, the concentrations of the proenzyme were reduced with a concomitant increase in the levels of the intermediate.

To additionally confirm these results, we determined the status of MT1-MMP in the cells that stably express the wild-type proteasome and the proteasine mutants in which the furin cleavage motifs were inactivated by mutations (ARAA and R99A) (29). As controls, we used the cells stably transfected with the original plasmid (mock) as well as the MT1/PDX cells. To evaluate the status of the cell surface-associated MT1-MMP, cells were surface-biotinylated with membrane-impermeable biotin and then the biotin-labeled proteins were immunoprecipitated using streptavidin-agarose beads. MT1-MMP was identified in
the precipitates using the 3G4 antibody. To determine the status of the total cell MT1-MMP pool, the cells were lysed and the lystate aliquots were analyzed by Western blotting with the 3G4 antibody (Fig. 5). The enzyme of MT1-MMP was predominantly observed in mock cells. In the cells expressing the wild-type MT1-MMP, there were three bands that represented the proenzyme and the intermediate and the enzyme (the major band, especially on the cell surface). The presence of PDX and the resulting inhibition of furin stimulated the accumulation of the MT1-MMP proenzyme in the MT1/PDX cells. ARAA cells were similar to MT1/PDX cells; the proenzyme and the intermediate dominated the activated enzyme in the total cell lystate. In contrast to other cell types and similar to MT1/PDX cells, significant levels of the proenzyme and the intermediate were present on the surface of ARAA cells. The presence of naturally synthesized MT1-MMP contributed to the levels of the enzyme in ARAA cells. The R89A mutant accumulated, especially in the cell lystate, the increased levels of the intermediate. The double ARAA/R89A mutant was similar to ARAA except that the levels of the intermediate were insignificantly higher in the first when compared with the second.

The Analysis of the Double L50D/L61D MT1-MMP Mutant—To address the question if the cleavage at the bait region of MT1-MMP is necessary before furin cleavage to accomplish the efficient activation of MT1-MMP, we inactivated, by mutagenesis, the P57GD ▪ L50 and P58QS ▪ L61 cleavage sites of the bait region and, as a result, generated the double MT1-L50D/L61D mutant. We then expressed the mutant and the wild-type MT1-MMP in MCF-7 cells. We specifically selected MCF-7 for our studies because they do not naturally express wild-type MT1-MMP in MCF-7 cells. We specifically selected MCF-7 cells for our studies because they do not naturally express MT1-MMP and MMP-2. In addition, we generated the rabbit polyclonal antibody to the recombinant prodomain of the MT1-MMP sequence. Because the prodomain is absent in the mature MT1-MMP enzyme, the availability of this antibody greatly facilitates the identification of the MT1-MMP proenzyme alone in cell samples.

Fig. 5C shows that according to Western blotting with the antibody against the individual MT1-MMP prodomain the L50D/L61D mutant construct efficiently accumulates in the proenzyme form in MCF-7 cells suggesting that the mutant resists furin processing. In contrast, the insignificant levels of the MT1-MMP proenzyme were detected in cells expressing either the inert mutant (Glu240.V5) or the wild-type MT1-MMP (MT1-V5).

These results correlate well with the reduced efficiency of the L50D/L61D mutant in activating MMP-2. Thus, according to the gelatin zymography of medium aliquots the independent clones of MT1-L50D/L61D cells were significantly less efficient in generating the activated species of MMP-2 when compared with the wild-type construct. Consistent with our observations and many others, the inert E240A construct was incapable of MMP-2 activation (Fig. 5C). These results confirm that the processing of MT1-MMP at the P57GD ▪ L50 and P58QS ▪ L61 cleavage sites of the bait region facilitates either the subsequent cleavage of the R108KRR111 ▪ Y112 site of the prodomain by furin or the release of the prodomain by MT1-MMP or both to generate the mature MT1-MMP enzyme with full proteolytic activity. Overall, our results suggest that a "furin alone, one-step mechanism" is less efficient in activating MT1-MMP when compared with the two-step activations that involves furin and an additional proteinase.

To address a question if other proteinases in addition to MT1-MMP are the activating enzymes for the Ser-Leu61 or the Asp-Leu50 cleavage, we compared, by using Western blotting with the 3G4 antibody to the catalytic domain, the activation pattern of the wild-type MT1-MMP with that of the inert Glu240 mutant. Consistent with our other results, the proenzyme, intermediate, and mature enzyme species were each detected in the MT1-WT cell lysates. In turn, the activated mature enzyme was the predominant MT1-MMP form in E240A cells. Because MCF-7 cells do not naturally synthesize MT1-MMP and MMP-2 and because the activity of the Glu240 mutant is nearly completely repressed by the mutation of the catalytically essential Glu240, these results suggest that the bait region of the prodomain of MT1-MMP may be accessible by and then sensitive to multiple cellular proteinases rather than only to MT1-MMP itself or MMP-2.

The Activity of MT1-MMP at Acidic pH—Overall, our results suggest that non-furin proteolysis generates the activation intermediate of MT1-MMP and that the relative level of the activation intermediate is highest inside the cellular compartment when compared with that present on the cell surface. Based on these data, we hypothesized that the intermediate is generated in the secretory vesicle in the course of secretion of the MT1-MMP proenzyme from the Golgi compartment to the plasma membrane. Because of the presence of the acidic interior in the secretory vesicles, we next determined if MT1-MMP is capable of exhibiting its proteolytic activity at acidic pH. To determine whether MT1-MMP is active at acidic pH, we performed the refolding procedure of MT1-CAT at pH 5.0, 5.4, 6.0, and 7.0 and we then tested the proteolytic activity of the refolded MT1-CAT samples against α1-antitrypsin (AAT), a convenient and sensitive protein substrate of MT1-MMP (39). Fig. 6 shows that either the refolding of MT1-CAT or the cleavage reactions or both procedures were performed at pH 5.0, AAT was remaining in its intact form. The refolding at either pH 6.0 or 7.0 followed by the cleavage reactions performed at pH 5.4, 6.0, or 7.0 resulted in significant proteolytic activity of MT1-CAT. The refolding of MT1-CAT at pH 5.4 followed by the pH 5.4 cleavage reaction was sufficient to generate a high MT1-CAT activity that led to a significant level of AAT cleavage. We infer from these results that the acidic interior of the secretory vesicle is compatible with autoproteolysis of MT1-MMP and that self-proteolysis of MT1-MMP may occur in the secretory vesicle budding from the trans-Golgi compartment.
**Activation of MT1-MMP**

**DISCUSSION**

Because the discovery of MT1-MMP in 1994–1995 and the findings showing its role in the activation of MMP-2 there has been a question: what is the mechanism of the activation of the activator (19, 20)? Volumes of evidence that have been generated through the years suggest that MT1-MMP is a key player in tumor cell migration and that MT1-MMP is a likely drug target in multiple pathologies. A precise and complete understanding of the activation and regulation mechanisms of MT1-MMP is required for the design of effective therapies.

It is known that the R108RKR111 ↓ Y112 motif of the prodomain sequence of the latent MT1-MMP proenzyme is processed by furin and several additional furin-like proprotein convertases in the course of the secretion pathway and that this single-step processing results in the mature enzyme sequence commencing from N-terminal Tyr112 (25). It is also well established that the cysteine residue of the prodomain cysteine-switch motif maintains the latent status of the proenzyme by chelating the active site zinc (4). The cysteine-switch peptide sequence itself and the prodomain are inhibitory for MMPs including MT1-MMP (9, 40, 41). A single-step mechanism suggests that the excised prodomain likely remains associated with the mature proteinase. Thus, following cleavage of the ADAM12 prodomain in the trans-Golgi by a furin proteinase, the prodomain remains non-covalently associated with the mature molecule (42).

To inactivate the excised inhibitory prodomain and to liberate the processed active enzyme species from the inhibition of the prodomain, several members of the MMP family have adopted a two-step mechanism. This mechanism involves the cleavage of the prodomain peptide sequence both by an external proteinase and by autocatalysis. Based on these considerations and on the conserved three-helix bundle structure of the MMP prodomains (9), we have hypothesized that, in addition to a furin-dependent step, there is an additional and previously uncharacterized step in the MT1-MMP activation process. Accordingly, we suggest that detection of the activation intermediate of MT1-MMP that is generated as a result of the cleavage in the bait region of the prodomain will bring us a step closer to a full understanding of the activation mechanism of this proteinase.

The results of our biochemical studies supported our hypothesis. To prove our hypothesis, we used synthetic peptides, the recombinant prodomain, and the soluble MT1-MMP constructs, the furin-cleavage resistant MT1-MMP mutants, and the MT1-MMP mutants with the inactivated bait region cleavage sites. The proteolytic processing of these constructs was analyzed by N-terminal sequencing and MS of the resulting cleavage fragments. Our results provide substantial evidence that supports a two-step mechanism of MT1-MMP activation and prodomain sequence processing.

Our results suggest that there is a proteolytic processing of the bait region of the prodomain sequence of MT1-MMP (either at the P47G/GD ↓ L50 or the P58QS ↓ L61 or at both sites). This event results in the activation intermediate of MT1-MMP, the presence of which we have demonstrated using *in vitro* tests and cell-based assays. In agreement, the processing of the prodomain by furin was impaired in the L50D/L61D mutant in which both cleavage sites of the prodomain bait region were inactivated by mutations. The stepwise activation of MT1-MMP also involves the action of a furin proteinase cleaving the inhibitory prodomain at the R108RKR111 ↓ Y112 site, where Tyr112 is the N-terminal residue of the mature MT1-MMP enzyme. This two-step mechanism eventually facilitates the degradation of the inhibitory prodomain and the release of the activated, mature MT1-MMP enzyme. We believe that our findings shed more light on the potentially important functional role of the inhibitory prodomain and on the proteolytic control of MT1-MMP activation, a crucial process that may be differentially regulated in normal and cancer cells.

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