Internal Cleavages of the Autoinhibitory Prodomain Are Required for Membrane Type 1 Matrix Metalloproteinase Activation, although Furin Cleavage Alone Generates Inactive Proteinase

Vladislav S. Golubkov, Piotr Cieplak, Alexei V. Chekanov, Boris I. Ratnikov, Alexander E. Aleshin, Natalya V. Golubkova, Tatiana I. Postnova, Ilian A. Radichev, Dmitri V. Rozanov, Wenhong Zhu, Khatereh Motamedchaboki, and Alex Y. Strongin
From the Cancer Research Center, Sanford-Burnham Medical Research Institute, La Jolla, California 92037

The functional activity of invasion-promoting membrane type 1 matrix metalloproteinase (MT1-MMP) is elevated in cancer. This elevated activity promotes cancer cell migration, invasion, and metastasis. MT1-MMP is synthesized as a zymogen, the latency of which is maintained by its prodomain. Excision by furin was considered sufficient for the prodomain release and MT1-MMP activation. We determined, however, that the full-length intact prodomain released by furin alone is a potent autoinhibitor of MT1-MMP. Additional MMP cleavages within the prodomain sequence are required to release the MT1-MMP enzyme activity. Using mutagenesis of the prodomain sequence and mass spectrometry analysis of the prodomain fragments, we demonstrated that the intradomain cleavage of the PGD↓L50 site initiates the MT1-MMP activation, whereas the 108RRKR111↓Y112 cleavage by furin completes the removal and the degradation of the autoinhibitory prodomain and the liberation of the functional activity of the emerging enzyme of MT1-MMP.

Cells rely on pericellular proteolysis to penetrate through the tissue (1, 2). The initiation of the invasive phenotype most frequently represents a matrix metalloproteinase (MMP)-dependent process (3). Twenty four individual MMPs are encoded by the human genome (4, 5). MMPs are secreted (soluble MMP) or membrane-bound endoproteases (membrane-type MMPs) (6). They include a signal peptide, an 80-residue prodomain, an ~170-residue zinc- and calcium-dependent catalytic domain, a short hinge region, and a four-bladed β-propeller hemopexin-like C-terminal domain (7). MT1-MMP is distinguished from soluble MMPs by a transmembrane domain and a cytoplasmic tail.

The prodomain maintains the MMP proenzymes in their latent state (8). In the proenzyme, the catalytic zinc ion is chelated by the three His residues of the conserved HEXX-HXGXXH active site motif and by the conserved Cys residue of the cysteine-switch motif (PRC93GVPD in MT1-MMP) from the C-terminal portion of the prodomain (7, 9, 10). When the interaction of the Cys residue with the active site Zn2+ is disrupted by proteolytic removal of the prodomain (8, 10, 11), the active site becomes accessible to water. The coordination of the catalytic zinc with a water molecule is essential for the catalysis (12).

Since the discovery of MT1-MMP in 1994–1995 and the findings showing its role in the activation of MMP-2 (13, 14) the following question remains. What is the mechanism of the MT1-MMP activation? In contrast to soluble MMPs, which are secreted as inactive proenzymes and activated extracellularly, the latent MT1-MMP proenzyme is processed intracellularly by the furin-like proprotein convertases at the 108RRKR111↓Y112 sequence (the furin site) in the linker motif connecting the prodomain and the catalytic domain sequences (13, 15, 16). The cleavage by furin takes place in the course of the secretion pathway (17, 18) and results in the mature enzyme sequence commencing from the N-terminal Tyr112 (8). We recently demonstrated, however, that the activation of MT1-MMP in cancer cells represents a two-step process (19). This process includes the MMP-dependent cleavage at the “bait region” of the prodomain sequence as a first step and leads to the generation of the activation intermediate. The intermediate is then processed by furin at the 108RRKR111↓Y112 motif as a second step generating, as a result, the mature enzyme of MT1-MMP. However, the physiological significance of the two-step activation mechanism remained unidentified.

To shed additional light on the molecular mechanism of both the activation of MT1-MMP and the inactivation of the inhibitory prodomain in cancer cells, we performed extensive mutagenesis of the prodomain sequence and analyzed the mutants using in vitro and cell-based systems. As a result, we established the precise and well coordinated sequence of the proteolytic events that take place in the course of the activation
of MT1-MMP. These events result in the release of the fully functional enzyme of MT1-MMP in cancer cells.

MATERIALS AND METHODS

Antibodies, Reagents, and Cells—The murine monoclonal 3G4 antibody against the catalytic domain, the AB815 polyclonal antibody against the hinge region of MT1-MMP, and the GM6001 hydroxamate inhibitor were from Millipore. A rabbit polyclonal antibody to the MT1-MMP prodomain was described earlier (19). The murine monoclonal FLAG M2 antibody and anti-FLAG M2-agarose beads were from Sigma. EZ-Link sulfo-NHS-LC-biotin was from Pierce. The recombinant catalytic domain (CAT) and the recombinant prodomain (PRO) of MT1-MMP were expressed and isolated earlier (19, 20).

Fabricant and Reagents—The murine monoclonal 3G4 antibody against the catalytic domain, the AB815 polyclonal antibody against the hinge region of MT1-MMP, and the GM6001 hydroxamate inhibitor were from Millipore. A rabbit polyclonal antibody to the MT1-MMP prodomain was described earlier (19). The murine monoclonal FLAG M2 antibody and anti-FLAG M2-agarose beads were from Sigma. EZ-Link sulfo-NHS-LC-biotin was from Pierce. The recombinant catalytic domain (CAT) and the recombinant prodomain (PRO) of MT1-MMP were expressed and isolated earlier (19, 20). α1-Antitrypsin (AAT) was obtained from Calbiochem. Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone was from Bachem. Recombinant versions of the catalytic domains of MMP-2 and MMP-9 were expressed, purified, and activated as described previously (20). The catalytic domains of MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MT6-MMP were expressed in Escherichia coli, purified from the inclusion bodies, and refolded to restore its native conformation (21). Human HT1080 and MCF7 cells were from ATCC (Manassas, VA). Highly metastatic M4A4 clone of MDA-MB-435 cells was a gift from Dr. Virginia Urquidi (University of California, San Diego). Cells were grown in DMEM supplemented with 10% FBS.

Cloning and Mutagenesis—The genetic constructs we used are shown in Fig. 1. The recombinant constructs were obtained in PCRs using the primers shown in supplemental Table S1. MDA-MB-435 cells were stably transfected with the wild-type (WT) full-length MT1-MMP in the pcDNA3.1 plasmid (MDA-MB-435-MT1 cells). The WT full-length MT1-MMP with a FLAG tag inserted in the hinge region (MT1-FLAG) was characterized earlier (22). The MT1-FLAG construct was then subcloned into the pcDNA3.1D/V5-His-TOPO directional expression vector (Invitrogen). Where indicated, the 108RRKR furin cleavage site was inactivated by the 108AAAA sequence to generate the AAAA mutant. The furin-resistant AAAA mutant was used as a template to generate the W38E, L39E, L45D, L50D, L61D, I65A, M68A, L74D, M85D, M88D, I114D, L117D, the inactivating mutants of the predicted MMP cleavage sites in the prodomain; L50D/I114D, the double mutant; G48-FLAG, the mutant with the FLAG tag inserted immediately after Gly48; T52-FLAG, the mutant with the FLAG tag inserted immediately after Thr52; PRO, the individual recombinant prodomain; CAT, the individual recombinant catalytic domain.

Activation of MT1-MMP in Cancer Cells
Activation of MT1-MMP in Cancer Cells

the FLAG tag was inserted immediately after the Thr52 in the sequence that is downstream from the PGD ↓ L50 cleavage site. The MT1-FLAG, E240A, AAAA, AAAA/E240A, G48-FLAG, and T52-FLAG constructs were expressed in MCF7 cells. The MT1-FLAG, G48-FLAG and T52-FLAG constructs were precipitated using anti-FLAG M2-agarose beads from the total cell lysates and analyzed by Western blotting with the MT1-MMP 3G4 antibody. The TIMP-2 free-proenzyme of MMP-2 (68 kDa) was isolated as described earlier (14).

Cell Surface Biotinylation—Cell surface proteins were biotinylated by incubating cells for 1 h on ice in PBS containing 0.1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin. Cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 1% deoxycholate, 1% IGEPA, pH 7.4, supplemented with a protease inhibitor mixture set III (Sigma), 1 mM phenylmethylsulfon fluoride, and 10 mM EDTA. Biotin-labeled proteins were precipitated from cell lysates using streptavidin-agarose beads (Sigma).

Immunoprecipitation of the MT1-MMP-FLAG Constructs and LC/MS/MS—Cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 1% deoxycholate, 1% IGEPA, pH 7.4, supplemented with a protease inhibitor mixture set III, 1 mM phenylmethylsulfon fluoride, and 10 mM EDTA. The lysates were centrifuged (10 min; 20,000 g). The supernatant fraction was incubated for 16 h using anti-FLAG M2-agarose beads. The proteins were eluted from the beads using 0.2 mg/ml FLAG peptide. The samples were separated in the NuPAGE 4–12% gel with a probability score >0.95 and a cross-correlation (Xcorr) value of >2.0 were further analyzed and annotated.

Proteolysis of the Recombinant Prodomain (PRO) in Vitro—The PRO (1 µg) was co-incubated for 30 min at 37 °C with the recombinant catalytic domains of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24), and MT6-MMP (MMP-25) at a 1:100 MMP/PRO molar ratio in 50 mM HEPES, pH 6.8, containing 10 mM CaCl2 and 50 µM ZnCl2. The PRO was also co-incubated with the recombinant catalytic domain of MT1-MMP (CAT) at a 2:1 CAT/PRO molar ratio. The digests were analyzed by SDS-PAGE and also by matrix-assisted laser desorption ionization time-of-flight MS using a Bruker Daltonics AutoFlex II TOF/TOF mass spectrometer.

Enzymatic Assays—The CAT activity was measured in triplicate in wells of a 96-well plate in 0.1 ml of 50 mM HEPES, pH 7.5, containing 10 mM CaCl2 and 50 µM ZnCl2. Mca-PLGL-Dpa-AR-NH2 (20 µM, R & D Systems) was used as a fluorescent substrate. The concentrations of the catalytically active CAT were quantified by active site titration using GM6001 (k = 0.5 nm) (21). Briefly, the CAT (20 nm) was incubated with increasing concentrations of GM6001. Residual activity of the CAT was then measured by determining the rate of cleavage of Mca-PLGL-Dpa-AR-NH2. The data were plotted versus the amounts of GM6001, and a line was fitted through the data points. The intercept on the x axis equals to the concentration of the active enzyme. Based on these measurements, we used 20 nm active CAT in the reactions. The steady-state rate of substrate hydrolysis was monitored continuously (λex = 320 nm and λem = 400 nm) at 37 °C for 3–75 min using a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices).

To determine the k values of the PRO constructs, the CAT (300 pm) was preincubated for 30 min at 4 °C with increasing concentrations of the PRO (2–340 nm). The residual activity of the CAT was then measured using Mca-PLGL-Dpa-AR-NH2.

To analyze the molar ratio at which the PRO inhibits the cleavage of ATT by the CAT, the CAT-PRO complex at indicated molar ratio was formed for 30 min at 4 °C followed by the incubation with ATT at 37 °C for 1 h. The reaction was stopped by adding the 5× SDS sample buffer. The ATT cleavage products were analyzed by SDS-PAGE with subsequent Coomassie staining.

Modeling of the MT1-MMP Structure—The propeptide size and related annotations were obtained from the UNIPROT database (23). The structural parameters of the propeptide were obtained from the known atomic resolution structures of the proenzymes of MMP-1 (PDB 1SU3) (10), MMP-2 (PDB 1CK7) (24), MMP-3 (PDB 1SLM) (25), and MMP-9 (PDB 1L6J) (26). The structure of the MT1-MMP proenzyme (residues 36–508) was then modeled by MODEPIE (27) using 1SU3, 1CK7, and 1SLM, and also the PDB entries 1BQQ and 1BUV (the catalytic domain of MT1-MMP) as templates (19, 28). The modeled structure was visualized using PyMOL (DeLano Scientific).

Gelatin Zymography—Cells were plated in the wells of a 48-well plate (Costar/Corning) in serum-containing DMEM and grown to reach a 90% confluence. The medium was then replaced with serum-free DMEM supplemented with the purified MMP-2 proenzyme (100 ng/ml). In 12 h, the medium aliquots were analyzed by gelatin zymography using 10% acrylamide gels containing 0.1% gelatin (Invitrogen). The TIMP-2 free-proenzyme of MMP-2 (68 kDa) was isolated as described earlier (14).

RESULTS

Prodomain Is an Autoinhibitor of the Emerging MT1-MMP Enzyme—To determine whether the prodomain released by the furin cleavage alone is an inhibitor of the emerging MT1-MMP enzyme, we co-incubated the increasing amounts of the purified 26–111 full-length PRO with the purified CAT and then measured the residual activity of the CAT using the Mca-PLGL-Dpa-AR-NH2 substrate. The PRO construct sequence was identical to that of the prodomain resulting from the cleavage of ATT by the CAT, the CAT-PRO complex at indicated molar ratio was formed for 30 min at 4 °C followed by the incubation with ATT at 37 °C for 1 h. The reaction was stopped by adding the 5× SDS sample buffer. The ATT cleavage products were analyzed by SDS-PAGE with subsequent Coomassie staining.

Activation of MT1-MMP in Cancer Cells

27728 JOURNAL OF BIOLOGICAL CHEMISTRY
CAT ratio, the ability of the CAT to cleave AAT was quantitatively repressed.

Conversely, if the CAT was in excess over the PRO, the PRO itself became a target of CAT proteolysis. Thus, at a 3:1 or 2:1 CAT/PRO ratio, both AAT and the PRO became extensively proteolyzed (Fig. 2B). Co-incubation of the CAT with the PRO alone confirmed that at a 2:1 CAT/PRO ratio the PRO was cleaved in a matter of minutes (Fig. 2C). Overall, our data imply that in the stoichiometric PRO/H18528 CAT complex the intact PRO functions as an inhibitor. In turn, if the CAT is in excess, the PRO is readily proteolyzed, and its inhibitory activity is inactivated. According to the MT1-MMP structure model (19), the furin cleavage site is located in the unstructured loop between the prodomain and the catalytic domain (Fig. 2D). Thus, it is unlikely that furin site cleavage alone affects both the prodomain integrity and the prodomain association with the enzyme. Accordingly, furin cleavage alone is insufficient for liberating the full proteolytic activity of MT1-MMP. These data suggest that an additional activation cleavage step is required to inactivate the inhibitory capacity and the association of the prodomain with the emerging enzyme of MT1-MMP (Fig. 2E).

The MMP Cleavage Sites in the Prodomain Sequence—To predict the potential cleavage sites in the prodomain sequence, we used a specialized software we developed (21). The software determines the contribution of each amino acid residue at each of the P3-P2/H positions to the efficiency of the proteolysis and assigns a numerical score to every peptide bond in the protein sequence. The score is based on the positional weight matrix approach that we developed for individual MMPs using the high volume data from the substrate phage library cleavage.3 The elements of the positional weight matrix define the probability of the presence of each amino acid type at each of the P3-P2 positions relative to the cleavage-resistant substrates. The offset and threshold values are specific for

3 B. I. Ratnikov, P. Cieplak, A. M. Eroshkin, M. D. Kazanov, J. Pierce, Q. Sun, B. Stec, A. L. Osterman, A. Y. Strongin, and J. W. Smith, manuscript in preparation.

FIGURE 2. Prodomain is both an inhibitor and a substrate of MT1-MMP. A, determination of the kᵢ value of the PRO. The CAT was preincubated for 30 min at 4 °C with increasing concentrations of the PRO. The residual activity of the CAT was then measured using Mca-PLGL-Dpa-AR-NH₂. A representative experiment is shown. B, enzymatic assay of the CAT-PRO complex at the indicated molar ratio. The CAT was preincubated for 30 min at 4 °C with increasing concentrations of the PRO. The residual activity of the CAT was then measured at 37 °C for 1 h AAT as a substrate. C, CAT excess cleaves the PRO. The CAT was incubated for the indicated time at 37 °C with the PRO at a CAT/PRO molar ratio of 2:1. The reactions were then analyzed by SDS-PAGE. D, structure of MT1-MMP. PRO, prodomain (green); CAT, catalytic domain (yellow); PEX, hemopexin domain (gray); Zn, catalytic zinc atom coordinated with His²³⁹, His²⁴³, and His²⁴⁹ of the autoactive site and with Cys⁹³⁹ of the cysteine-switch sequence. The furin cleavage site (blue) is located in the unstructured loop connecting the PRO and the CAT. E, schematic representation of the one- and two-step activation mechanism of MT1-MMP. We hypothesize that the one-step processing at the furin cleavage site (a blue dot) alone is insufficient to liberate the CAT proteolytic activity because of the presence of the intact autoinhibitory PRO. An additional MMP-dependent cleavage (a red dot) is required as a second activation step leading to the degradation of the autoinhibitory PRO.
the individual MMPs. These values have been determined using a 10-fold cross-validation test. The predicted cleavage sites in the MT1-MMP prodomain sequence are shown in Fig. 3A and Table 1. According to our in silico analysis, the PGD L50 and PQS L61 sequence regions with the high positional weight matrix score were the most probable MMP cleavage sites.

To test these predictions, we subjected the PRO to MMP proteolysis, including MMP-2, MMP-9, MT1-MMP, MT2-MMP, MT3-MMP, MT5-MMP, and MT6-MMP. The digests were then analyzed by MALDI-TOF MS to identify the mass of the resulting peptides and, consequently, the scissile bonds. The MS analysis detected the presence of the expected fragments in the cleavage reactions. The MS analysis confirmed that both the PGD L50 and the PQS L61 sites of the prodomain were cleaved by the multiple individual MMPs. The GAE I103 sequence was cleaved by MMP-2, MMP-17, and MMP-25. The FYG L74 and PEA W38 sites were cleaved by MT1-MMP. The MKA M88 site was cleaved by MMP-2, MT1-MMP, and MT6-MMP (Table 2).

**Processing of Furin-resistant MT1-MMP in HT1080 Cells**

Because the full-length prodomain excised by the furin cleavage alone performed as a potent MT1-MMP inhibitor, additional cleavages were required to inactivate its inhibitory capacity and to release the enzyme activity of MT1-MMP. To investigate the MMP-dependent step of the prodomain processing in more detail, we constructed mutant MT1-MMP. In this AAAA mutant, the 108RRKR111 Y112 furin cleavage site was inactivated by Ala substitution of the positively charged 108 Arg and Lys residues (8). The use of the AAAA mutant allowed us to focus our experiments on the MMP-dependent processing of
the prodomain. A FLAG tag was inserted in the hinge region of the AAAA construct to distinguish the mutant from the endogenous wild-type (WT)-MT1-MMP. The mutant was expressed in fibrosarcoma HT1080 cells. To evaluate the status of the cell surface-biotinylated with membrane-impermeable biotin, and then biotin-labeled proteins were immunoprecipitated using streptavidin-agarose beads. The mutant was identified in the precipitates using the antibodies to FLAG, to the prodomain of MT1-MMP, and to TIMP-2. The 55-kDa mutant enzyme and the residual amounts of the 63-kDa intact proenzyme and the bound TIMP-2 were observed in the cell lysates. To identify the nature of the activation intermediate, its band was excised and subjected to in-gel tryptic digestion. The resulting peptides were analyzed by LC/MS/MS. The 57SPQSLSAAIAAMQK70 peptide with the intact PQSL sequence (underlined) was readily identified in the activation intermediate samples. In turn, the peptides that included the intact PGDL50 sequence were absent in the activation intermediate of both MT1-FLAG and AAAA samples (supplemental Table S2).

Based on these data, we suggested that the cleavage of the PGDL50 site, but not the PQSL61 site, of the prodomain generated the activation intermediate of MT1-MMP. According to our modeling of the membrane-tethered MT1-MMP, the PQSL61 site of the prodomain is proximal to the plasma membrane, whereas the PGD L50 site is clearly exposed. The proximity to the membrane may limit both the access to and the cleavage of the PQSL61 site by the proteinases. These limitations played no role in our in vitro, in-solution, tests in which the accessible PQSL61 site was cleaved by the multiple MMPs. 

**Processing of the Furin-resistant MT1-MMP in MCF7 Cells**—MCF7 cells were stably transfected with the AAAA mutant. The cells transfected with the MT1-FLAG construct in which the furin cleavage remained unmodified and the FLAG tag was inserted in the hinge region served as a control. The cells were lysed; the lysates were immunoprecipitated using anti-FLAG M2-agarose beads, and the precipitates were separated by gel electrophoresis (Fig. 3C). The proenzyme, the enzyme, and also the activation intermediates of both MT1-FLAG and AAAA were observed in the cell lysates. To identify the nature of the activation intermediate, its band was excised and subjected to in-gel tryptic digestion. The resulting peptides were analyzed by LC/MS/MS. The 57SPQSLSAAIAAMQK70 peptide with the intact PQSL sequence (underlined) was readily identified in the activation intermediate samples. In turn, the peptides that included the intact PGD L50 sequence were absent in the samples (supplemental Table S2).

**TABLE 1**

| N-end of the resulting peptide (the original P1' position) | C-end of the resulting peptide (the original P1 position) | Calculated mass (Da) | Determined mass (Da) |
|----------------------------------------------------------|----------------------------------------------------------|----------------------|----------------------|
| **Leu**<sup>64</sup> Gly<sup>73</sup> | 1370.62 | 1369 |
| **Met**<sup>65</sup> Gly<sup>102</sup> | 1719.01 | 1719 |
| **Phe**<sup>71</sup> Ala<sup>87</sup> | 1816.04 | 1815 |
| **Met**<sup>65</sup> Gly<sup>102</sup> | 2049.46 | 2050 |
| **Leu**<sup>60</sup> Gly<sup>73</sup> | 2663.04 | 2661 |
| **Trp**<sup>68</sup> Ser<sup>60</sup> | 2729.27 | 2728 |
| **His of a His tag** Asp<sup>49</sup> | 3526.76 | 3527 |
| **Leu**<sup>60</sup> Ala<sup>87</sup> | 3526.76 | 3527 |
| **His of a His tag** Ser<sup>60</sup> | 4093.68 | 4094 |
| **Leu**<sup>60</sup> Arg<sup>111</sup> | 5794.69 | 5796 |
| **His of a His tag** Gly<sup>73</sup> | 6917.07 | 6915 |

**TABLE 2**

**The cleavage fragments of the PRO**

The PRO was digested for 30 min at 37 °C by the individual MMPs at a 1:100 MMP/PRO molar ratio. The molecular mass of the fragments was then determined by MALDI-TOF MS analysis of the individual cleavage reactions. N-end (P1) and C-end (P1), the N-terminal and the C-terminal residues of the cleavage fragment, respectively.

**Activation of MT1-MMP in Cancer Cells**
cleavage of which by MMPs may be involved in the prodomain processing and MT1-MMP activation, we used extensive mutagenesis of the prodomain sequence. Mutagenesis was used to inactivate the predicted MMP cleavage sites in the prodomain using the AAAA mutant sequence as a template. As a result, we prepared the W38E, L39E, L45D, L50D, L61D, I65A, M68A, L74D, M85D, M88D, I103D, I114D, and L117D mutants that were then expressed in HT1080 cells (Fig. 4A).

The mutants were also co-expressed with WT-MT1-MMP in MDA-MB-435-MT1 cells. The AAAA/E240A catalytically inert double mutant, which was incapable of self-proteolysis, and the unmodified AAAA mutant were used as controls. The cells were surface-biotinylated and then, following cell lysis and immunoprecipitation using streptavidin beads, the status of the precipitated MT1-MMP was determined with the FLAG antibody. Our results showed that the 63-kDa AAAA proenzyme (but not the L50D proenzyme) was largely converted into the 55-kDa enzyme in HT1080 cells (Fig. 4A).

The mutants, however, demonstrated a more intricate activation pattern (Fig. 4B). Thus, the W38E and L39E mutations (both in the helix H1 of the prodomain) led to the accumulation of the 60-kDa activation intermediate and the proenzyme in HT1080 cells. The 60-kDa intermediate, but not the proenzyme, of W38E and L39E was also observed in MDA-MB-435-MT1. The L50D mutation, but not the L45D (both in the L1 loop region of the prodomain), led to the accumulation of the proenzyme in HT1080 cells, whereas in MDA-MB-435-MT1 cells the L50D proenzyme was extensively converted into the enzyme without the generation of the activation intermediate. The L61D and, especially I65A and M68A, mutations in the H2 helix region of the prodomain led to the accumulation of the activation intermediate in HT1080 and MDA-MB-435-MT1 cells. The presence of the intermediate alone in HT1080 and MDA-MB-435-MT1 cells resulted in the L74D mutation in the L2 loop. The M85D mutation in the H3 helix of the prodomain had no effect when compared with the original AAAA construct. An increase in the levels of the intermediate was the result of the M88D mutation (also in the H3 helix). From the three mutations in the loop region, which links the prodomain with the catalytic domain and is downstream from the cysteine-switch motif, only I103D resulted in the generation of the intermediate in HT1080 and MDA-MB-435-MT1 cells. Although the L117D mutation had no dramatic effect, the I114D mutation increased the level of the proenzyme in the cells.

It became clear, however, that only the L50D mutation blocked the generation of the activation intermediate but not the MT1-MMP enzyme and that only the I103D mutation blocked the generation of the mature enzyme and led to the
accumulation of the activation intermediate in the cells. Based on these data, we concluded that the activation intermediate was the result of the cleavage of the PGD ↓ L50 sequence of the prodomain. The cleavage of the GAE ↓ I103 sequence should result in the enzyme of MT1-MMP, which would commence from Ile103 and which would be 9 residues longer from the N terminus compared with the conventional MT1-MMP Tyr112 enzyme (Fig. 4A). These results correlate well with the autocatalytic activation of MT1-MMP we observed earlier in furin-deficient colon carcinoma LoVo cells (33).

Importance of the PGD ↓ L50 Cleavage—We next assessed if the PGD ↓ L50 cleavage took place in cis or in trans. For this purpose, we expressed the AAAA and L50D constructs in MDA-MB-435 and MDA-MB-435-MT1 cells. Cell surface mutant MT1-MMP was isolated from the biotin-labeled cells using streptavidin beads. To discriminate the mutants from the WT-MT1-MMP, the samples were then analyzed using a FLAG antibody. The AAAA and L50D mutants were largely represented by their proenzyme species in MDA-MB-435 cells. In contrast, the AAAA mutant proenzyme was processed into both the 55-kDa enzyme and the 45-kDa degradation products. The L50D mutation in the AAAA background significantly stabilized the 55-kDa enzyme and reduced the levels of the 45-kDa degraded form in MDA-MB-435-MT1 cells (Fig. 4C). Based on these data, it may be suggested that the in trans cleavage we clearly observed in MDA-MB-435-MT1 cells relative to MDA-MB-435 cells played a primary role in the prodomain processing and MT-MMP activation.

To corroborate further the importance of the PGD ↓ L50 cleavage in the MT1-MMP activation process, we inserted the FLAG tag in the WT-MT1-MMP construct immediately after the Gly48 residue position of the prodomain. Importantly, the original WT-MT1-MMP and mutants constructs we used here exhibited the intact furin cleavage site. Because of this insertion, the PGD ↓ L50 cleavage site was inactivated in the resulting G48-FLAG construct. As a control, we used the T52-FLAG construct in which the FLAG tag was inserted after Thr52 that is downstream from the PGD ↓ L50 cleavage site in the prodomain sequence. As an additional control, we used the MT1-FLAG construct in which the FLAG tag was inserted in the hinge region. The constructs were expressed in MCF7 cells. The constructs were then precipitated from the total cell lysates using anti-FLAG M2-agarose beads. In addition, mutant MT1-MMP was isolated from the biotin-labeled cells using streptavidin beads. In both cases, the precipitates were analyzed by Western blotting with the MT1-MMP 3G4 antibody (Fig. 5).

As it became evident from the analysis of the FLAG- and streptavidin-pulldown samples, the MT1-FLAG construct was readily processed into the MT1-MMP enzyme. The G48-FLAG insertion blocked the intradomain cleavage of the prodomain. As a result, the proenzyme and the enzyme alone were observed in the FLAG-pulldown and the streptavidin-pulldown samples in the G48-FLAG cells, respectively. In turn, in the T52-FLAG cells the activation intermediate was observed in the FLAG-pulldown samples prepared from the total cell lysates, thus suggesting the intracellular processing of the PGD ↓ L50 site. The mature enzyme alone was observed in the streptavidin-pulldown samples that represented the cell surface proteins in T52-FLAG cells. GM6001 reduced the conversion of the proenzyme in the MT1-MMP activation intermediate in the MT1-FLAG and T52-FLAG cells, respectively. These data confirmed again that the PGD ↓ L50 (but not the PQS ↓ L61) cleavage led to the generation of the activation intermediate of MT1-MMP.

To assess the activation status of the mutants, we also used gelatin zymography of MMP-2 that is a direct cleavage target of MT1-MMP (14). For this purpose, the purified MMP-2 proenzyme was added to MCF7 cells expressing the mutant constructs (Fig. 5). After incubation, the medium aliquots were analyzed to determine the conversion of the 68-kDa pro-MMP-2 into the 62-kDa MMP-2 enzyme. Although the E240A, AAAA/E240A, and G48-FLAG cells were incapable of MMP-2

FIGURE 5. Expression of the MT1-FLAG, G48-FLAG, and T52-FLAG mutants in MCF7 cells. Top, the constructs were immunoprecipitated (IP) from the total cell lysate with anti-FLAG M2-beads. The precipitates were analyzed by Western blotting (WB) with the MT1-MMP 3G4 antibody. Where indicated, the cells were co-incubated with GM6001 (25 μM) and decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-cmk) (30 μM). Middle, biotin-labeled cell surface proteins were immunoprecipitated (IP) with streptavidin-agarose beads. The precipitates were analyzed by Western blotting with the MT1-MMP 3G4 antibody. Bottom, gelatin zymography of MMP-2. The MMP-2 proenzyme was co-incubated with MCF7 cells expressing the MT1-FLAG, E240A, AAAA, AAAA/E240A, G48-FLAG, and T52-FLAG constructs. Medium aliquots were next analyzed by gelatin zymography. Mock, MCF7 cells transfected with the original plasmid without the MT1-MMP insert.
activation, both the MT1-FLAG and the T52-FLAG cells readily activated MMP-2.

Our results also suggested that the PGD ↓ L50 cleavage of the T52-FLAG mutant occurred prior to the cleavage of the 108RRKR111 ↓ Y112 motif by the intracellular furin. Otherwise, the T52-FLAG intermediate would not be observed in the MCF7 total cell lysate. Other potential implications are that the PGD ↓ L50 cleavage occurs in the course of the secretory pathway of MT1-MMP to the cell surface and that the PGD ↓ L50 cleavage initiates the prodomain degradation and the activity release of the emerging enzyme. In the absence of the PGD ↓ L50 cleavage (as in the G48-FLAG mutant), the resulting mature enzyme remains inhibited by the intact prodomain; therefore, this complex is incapable of MMP-2 activation. These results also support the intracellular generation of the MT1-MMP activation intermediate that we described earlier (19). We conclude that the PGD ↓ L50 cleavage of the MT1-MMP prodomain is required to trigger the follow-on MT1-MMP activation.

**Furin-independent Excision of the Prodomain**—Based on our results, we hypothesized that the L50D and the I103D mutations, if combined, should block the MMP-dependent in trans processing of the prodomain and stabilize the MT1-MMP proenzyme. To test our hypothesis, the L50D mutation was combined in the AAAA background with the I103D (L50D/I103D) and the E240A (L50D/E240A) mutations. The resulting L50D/I103D and L50D/E240A constructs were expressed in HT1080 cells and analyzed in parallel with the MT1-FLAG, AAAA/E240A, L50D, and I103D mutants. To determine the status of cell surface MT1-MMP, the cells were surface-biotinylated and lysed. The lysates were precipitated using streptavidin-agarose beads, and the FLAG-tagged constructs were detected using Western blotting with a FLAG antibody. In HT1080 cells, the MT1-FLAG construct was fully converted into the enzyme, although both the proenzyme and the enzyme of MT1-MMP were present in the L50D and AAAA cells. The activation intermediate alone was observed in the I103D cells. Similar with the inert AAAA/E240A and L50D/E240A mutants and consistent with our hypothesis, the L50D/I103D mutant proenzyme remained intact in HT1080 cells (Fig. 6A).

To test if prodomain mutants used in our study also inactive active MT1-MMP, we directly compared the inhibitory potency of the wild-type and mutant L50D PRO. For these purpose, the increasing amounts of the PRO constructs were co-incubated with the purified CAT. The residual activity of the CAT was then measured using the Mca-PLGL-Dpa-AR-NH2 substrate. In contrast to the wild-type PRO, the L50D mutant was resistant to MT1-MMP proteolysis, and the mutant construct performed as a superior inhibitor (the apparent $k_i = 53$ nM) relative to the wild-type PRO (the apparent $k_i = 160–200$ nM). As a result, we concluded that the prodomain mutation did not negatively affect both the prodomain folding and the binding affinity of the prodomain-MT1-MMP active site interactions.

In sum, we concluded that the MMP-dependent cleavage at the PGD ↓ L50 site triggers the MT1-MMP activation, the prodomain proteolytic degradation, and the liberation of the enzyme activity, whereas the cleavages at the GAE ↓ I103 and the 108RRKR111 ↓ Y112 sites complete the prodomain removal in a furin-independent and -dependent manner, respectively.

**DISCUSSION**

Ubiquitously expressed MT1-MMP, an archetype member of the MMP family, plays a key role in cell locomotion (6, 34, 35). As other MMPs, MT1-MMP is synthesized de novo as a latent proenzyme with the N-terminal prodomain. The C-terminal portion of the prodomain exhibits the cysteine-switch motif (PRC93GVPD in MT1-MMP) that directly interacts with the active site zinc. These interactions maintain the latency of the proenzyme (9). In general, proteolytic removal of the N-terminal prodomain by an external proteinase is required for the MMP enzyme to emerge. The 108RRKR111 ↓ Y112 furin cleavage motif is localized in the prodomain sequence of MT1-MMP downstream from the PRC93GVPD cysteine-switch. The cleavage of this characteristic motif by furin and related proprotein convertases plays a vital role in both the proteolytic removal of the prodomain and the activation of the MT1-MMP zymogen (16, 36). The C-terminal cysteine-switch peptide sequence remains intact in the furin-excised prodomain. The cysteine-switch peptide sequence itself is inhibitory for MMPs (37, 38).
The direct interaction of the prodomain with the catalytic and hemopexin domains was revealed in the crystal structure of the MMP-1 proenzyme (10). Because of the conservation of the domain structure and the amino acid sequence, it is highly likely that similar interactions take place in the MT1-MMP proenzyme. These interactions may significantly increase the overall affinity of the binding of the cysteine-switch motif to the active site of the proenzyme. In the MT1-MMP proenzyme, the \( ^{108}\text{RRKR}^{111} \downarrow \text{Y}^{112} \) furin cleavage motif is localized in the unstructured loop that links the prodomain sequence with the catalytic domain. Therefore, the furin cleavage alone cannot significantly affect the affinity of the interactions of the prodomain with the catalytic and hemopexin domains in the MT1-MMP proenzyme.

As a result, we suggest that the furin cleavage alone would not be sufficient for liberating the functional activity of the emerging enzyme of MT1-MMP. Our current studies demonstrated that the prodomain released by the furin cleavage alone functions as a potent autoinhibitor of the enzyme in the resulting equimolar prodomain-enzyme complex. The insertion of the L50D mutation into the individual recombinant PRO construct did not affect its inhibitory conformation. The L50D mutation made the PRO construct resistant to MT1-MMP proteolysis. As a result, the ability of the mutant PRO construct to inhibit the proteolytic activity of the individual CAT construct became superior relative to the intact PRO.

Additional cleavage steps are required to inactivate the inhibitory capacity and the high affinity association of the prodomain with the emerging enzyme of MT1-MMP. Using extensive mutagenesis of the predicted cleavage sites in the prodomain sequence and mass spectrometry analysis of the prodomain fragments, which have been generated in the \textit{in vitro} cleavage reactions and by the respective cancer cells, we determined that the activation of MT1-MMP requires, as the first step, the intracellular processing of the PGD \( \downarrow \text{L}^{50} \) cleavage site of the prodomain (Fig. 4). This cleavage destroys the prodomain structure, generates the activation intermediate, and initiates the further intradomain cleavages (e.g. FYG \( \downarrow \text{L}^{74} \)). Finally, the remaining C-terminal portion of the prodomain is removed by the MMP-dependent processing at the GAE \( \downarrow \text{I}^{103} \) cleavage site that is 9 residues upstream of the \( ^{108}\text{RRKR}^{111} \downarrow \text{Y}^{112} \) furin site or at the \( ^{108}\text{RRKR}^{111} \downarrow \text{Y}^{112} \) site by furin. These events, only if combined, result in the conventional active MT1-MMP enzyme.

Interestingly, in the absence of furin, the MT1-MMP autolytic form commencing at Ile\(^{114} \) was earlier observed by several groups (28, 41). Based on the analysis of the I103D and I114D mutants and these earlier data, we believe that the GAE \( \downarrow \text{I}^{103} \) cleavage in the prodomain is followed by the additional cleavage at the RYA \( \downarrow \text{I}^{114} \) site and that these consecutive cleavages lead to the unconventional Ile114 MT1-MMP active enzyme that can be generated in the absence of the furin cleavage.

Our data suggest that the initiating cleavage at the PGD \( \downarrow \text{L}^{50} \) site and the additional cleavages of the prodomain are performed by the MMP activity, including MT1-MMP itself as we observed in the MT1-MMP overexpressing MDA-MB-435-MT1 cells. The individual MMPs, including MMP-2, MMP-9, MT2-MMP, MT3-MMP, MT6-MMP, and others, appear highly efficient in cleaving the PGD \( \downarrow \text{L}^{50} \) motif (Table 2), which is similar, in fact, to the consensus recognition motif for many members of the MMP family.

If MT1-MMP itself is involved in the prodomain cleavages, the cleavage takes place in trans rather than intramolecularly. Our data also suggest that the initializing cleavage PGD \( \downarrow \text{L}^{50} \) takes place inside the cells and that this cleavage occurs prior to the furin cleavage of the prodomain, and, as a result, the PGD \( \downarrow \text{L}^{50} \) cleavage leads to the activation intermediate of MT1-MMP that is readily detected in the total cell lysates but not on the cell surface. From these perspectives, the PGD \( \downarrow \text{L}^{50} \) cleavage triggers the activation process leading to the follow-on successful furin proteolysis of the autoinhibitory prodomain and to the release of the mature MT1-MMP enzyme. The processing of the prodomain leading to MT1-MMP activation is predominantly accomplished, whereas the \textit{de novo} synthesized MT1-MMP proenzyme is on its way to the plasma membrane. Because of the known association of furin with the Golgi compartment and the secretory vesicles, it is likely that the MMP-dependent step of the prodomain processing and MT1-MMP activation also take place either in the endoplasmic reticulum and the Golgi compartment or in the secretory vesicles or both. As a result, it is possible now to hypothesize that MT1-MMP becomes active inside the cancer cells and before its presentation at the cell surface. The implications of this suggestion are that, in addition to playing a role in the pericellular proteolysis, MT1-MMP cleaves its co-compartmentalized substrate proteins directly in the course of the section process in cancer cells. This suggestion explains the near quantitative processing of the multiple cleavage targets of MT1-MMP, including CD44, tissue transglutaminase, and integrins observed by us and others in many cancer cell types (33, 39, 40). We believe that our findings shed light on the potentially important and novel functional role of the intracellular activity of MT1-MMP. The presence of the intracellular catalytically active MT1-MMP may be a critical parameter that may lead to the unequal level of degradation of the targets of MT1-MMP proteolysis in cancer versus normal cells.

REFERENCES

1. Friedl, P., and Wolf, K. (2009) Cancer Metastasis Rev. 28, 129–135
2. Friedl, P., and Wolf, K. (2010) J. Cell. Biol. 188, 11–19
3. Rowe, R. G., and Weiss, S. I. (2009) Annu. Rev. Cell. Dev. Biol. 25, 567–595
4. Murphy, G., and Nagase, H. (2008) Mol. Aspects Med. 29, 290–308
5. Nagase, H., Visse, R., and Murphy, G. (2006) Cardiovasc. Res. 69, 562–573
6. Kessenbrock, K., Plaks, V., and Werb, Z. (2010) Cell 141, 52–67
7. Maskos, K. (2005) Biochimie 87, 249–263
8. Yana, I., and Weiss, S. I. (2008) Mol. Biol. Cell 11, 2387–2401
9. Van Wart, H. E., and Birkedal-Hansen, H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5578–5582
10. Ojic, D., Bourenkov, G., Lim, N. H., Visse, R., Nagase, H., Bode, W., and Maskos, K. (2005) J. Biol. Chem. 280, 9578–9585
11. Rosenblum, G., Meroueh, S., Toth, M., Fisher, J. F., Fridman, R., Mobashery, S., and Sagi, I. (2007) J. Am. Chem. Soc. 129, 13566–13574
12. Vallee, B. L., and Auld, D. S. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 220–224
13. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) Nature 370, 61–65
14. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) J. Biol. Chem. 270, 5331–5338
15. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996)
Activation of MT1-MMP in Cancer Cells

27736

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 285 • NUMBER 36 • SEPTEMBER 3, 2010

FEBS Lett. 393, 101–104
16. Pei, D., and Weiss, S. J. (1995) Nature 375, 244–247
17. Thomas, G. (2002) Nat. Rev. Mol. Cell Biol. 3, 753–766
18. Seidah, N. G., Mayer, G., Zaid, A., Rousselet, E., Nassoury, N., Poirier, S., Essalmani, R., and Prat, A. (2008) Int. J. Biochem. Cell Biol. 40, 1111–1125
19. Golubkov, V. S., Chekanov, A. V., Shiryaev, S. A., Aleshin, A. E., Ratnikov, B. I., Gavlik, K., Radychev, I., Motamedchaboki, K., Smith, J. W., and Strongin, A. Y. (2007) J. Biol. Chem. 282, 36283–36291
20. Kridel, S. J., Sawai, H., Ratnikov, B. I., Chen, E. I., Li, W., Godzik, A., Strongin, A. Y., and Smith, J. W. (2002) J. Biol. Chem. 277, 23788–23793
21. Shiryaev, S. A., Savinov, A. Y., Cieplak, P., Ratnikov, B. I., Motamedchaboki, K., Smith, J. W., and Strongin, A. Y. (2009) PLoS One 4, e4952
22. Golubkov, V. S., Boyd, S., Savinov, A. Y., Chekanov, A. V., Osterman, A. L., Remacle, A., Rozanov, D. V., Doxsey, S. J., and Strongin, A. Y. (2005) J. Biol. Chem. 280, 25079–25086
23. Zdobnov, E. M., Lopez, R., Apweiler, R., and Etzold, T. (2002) Bioinformatics 18, 368–373
24. Morganova, E., Tuuttila, A., Bergmann, U., Isupov, M., Lindqvist, Y., Schneider, G., and Tryggvason, K. (1999) Science 284, 1667–1670
25. Becker, J. W., Marcy, A. L., Rokosz, L. L., Axel, M. G., Burbain, J. I., Fitzgerald, P. M., Cameron, P. M., Esser, C. K., Hagmann, W. K., Hermes, J. D., et al. (1995) Protein Sci. 4, 1966–1976
26. Elkins, P. A., Ho, Y. S., Smith, W. W., Janson, C. A., D’Alessio, K. J., McQueney, M. S., Cummings, M. D., and Romanic, A. M. (2002) Acta Crystallogr. D Biol. Crystallogr. 58, 1182–1192
27. Eswar, N., John, B., Mirkovic, N., Fiser, A., Ilyin, V. A., Pieper, U., Stuart, A. C., Marti-Renom, M. A., Madhusudhan, M. S., Yerkovich, B., and Sali, A. (2003) Nucleic Acids Res. 31, 3375–3380
28. Fernandez-Catalan, C., Bode, W., Huber, R., Turk, D., Calvete, J. J., Lichte, A., Tschesche, H., and Maskos, K. (1998) EMBO J. 17, 5238–5248
29. Mast, A. E., Enghild, J. I., Nagase, H., Suzuki, K., Pizzo, S. V., and Salvesen, G. (1991) J. Biol. Chem. 266, 15810–15816
30. Li, W., Savinov, A. Y., Rozanov, D. V., Golubkov, V. S., Hedayat, H., Postnova, T. I., Golubkova, N. V., Linli, Y., Krajewski, S., and Strongin, A. Y. (2004) Cancer Res. 64, 8657–8665
31. Will, H., Atkinson, S. I., Butler, G. S., Smith, B., and Murphy, G. (1996) J. Biol. Chem. 271, 17119–17123
32. Chernov, A. V., Sounni, N. E., Remacle, A. G., and Strongin, A. Y. (2009) J. Biol. Chem. 284, 12727–12734
33. Deryugina, E. I., Ratnikov, B. I., Yu, Q., Baciu, P. C., Rozanov, D. V., and Strongin, A. Y. (2004) Traffic 5, 627–641
34. Hotary, K. B., Allen, E. D., Brooks, P. C., Datta, N. S., Long, M. W., and Weiss, S. J. (2003) Cell 114, 33–45
35. Nagase, H., and Woessner, J. F., Jr. (1999) J. Biol. Chem. 274, 21491–21494
36. Remacle, A. G., Rozanov, D. V., Fugere, M., Day, R., and Strongin, A. Y. (2006) Oncogene 25, 5648–5655
37. Fotouhi, N., Lugo, A., Visnick, M., Lusch, L., Walsky, R., Coffey, J. W., and Hanglow, A. C. (1994) J. Biol. Chem. 269, 30227–30231
38. Hanglow, A. C., Lugo, A., Walsky, R., Finch-Arietta, M., Lusch, L., Visnick, M., and Fotouhi, N. (1993) Agents Actions 39, C148–C150
39. Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I., and Strongin, A. Y. (2001) J. Biol. Chem. 276, 18415–18422
40. Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., and Seiki, M. (2001) J. Cell Biol. 153, 893–904
41. Lichte, A., Kolkenbrock, H., and Tschesche, H. (1996) FEBS Lett. 397, 277–282