Direct Activation of Pro-Matrix Metalloproteinase-2 by Leukolysin/Membrane-type 6 Matrix Metalloproteinase/Matrix Metalloproteinase 25 at the Asn109-Tyr Bond

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ABSTRACT

Leukolysin/membrane-type 6 matrix metalloproteinase (leukolysin/MT6-MMP), a glycosylphosphatidylinositol-anchored neutrophil matrix metalloproteinase, is also abnormally expressed in brain cancer tissues. Yet, little is known about its role in cancer progression. Here we show that MT6-MMP is capable of activating pro-MMP-2, an enzyme implicated in tumor invasion and metastasis. Although MT6-MMP is only 10% as active as MT5-MMP in mediating pro-MMP-2 activation, it generates a higher ratio of mature/intermediate forms of MMP-2 than MT5-MMP. Consistently, purified CAT of MT6-MMP converts proMMP-2 into mostly the mature form. Using the catalytically inactive mutant MMP-2EA (the E404A mutant of pro-MMP-2), which cannot autocatalytically mature from the intermediate form into the mature one, we show that MT6-MMP cleaves not only the known MT-MMP-processing site at Asn66-Leu but also the previously unsuspected Asn109-Tyr to yield a fully mature molecule. Despite their difference in mediating pro-MMP-2 activation in transfected cells, the CAT of MT6-MMP appears to be as efficient as that of MT5-MMP in cleaving proMMP-2-2EA in buffer, suggesting that its CAT is a strong proMMP-2 activator. Indeed, the CAT of MT6-MMP can partially substitute the CAT of prototypical MT1-MMP in mediating proMMP-2 activation. Taken these facts together, we conclude that MT6-MMP may participate in tumor invasion and metastasis by directly converting proMMP-2 into active form.

INTRODUCTION

Malignant cancer cells can traverse densely packed ECM barriers such as the basement membrane by elaborating powerful proteolytic enzymes such as members of the MMP family (1, 2). Composed of more than 25 zinc-dependent proteinases, the MMPs can degrade a wide spectrum of protein substrates from the ECM (1, 3, 4). Given their purported proteolytic properties and observed expression profiles, members of the MMP family have been proposed to play key roles in tumor invasion and metastasis (1–3, 5–7). Therefore, detailed understanding of MMP function may lead to effective therapies against invasive and metastatic cancer.

Among the MMPs identified thus far, the MT-MMPs have received considerable attention in recent years. First, the archetypal MT1-MMP was dubbed a “master switch” for invasion and metastasis because it is a cellular activator of proMMP-2, an enzyme implicated in tumor invasion and metastasis (8, 9). Second, MT-MMPs can be targeted to the invasive front of tumor cells to create a privileged environment that favors the proteolysis of the ECM barrier (9–11). Third, MT1-MMP knockout mice exhibit severe developmental defects in contrast to the apparent normal ones displayed by mice harboring deletions in non-membrane-associated MMPs (12, 13). As a result, investigations of MT-MMPs, mostly MT1-MMP, have yielded considerable evidence suggesting that MT-MMPs facilitate invasiveness of tumor cells by juxtaposing cell surface proteolytic complexes to ECM barriers (8–10, 14, 15).

MT6-MMP was the last MT-MMP to be isolated, initially from the human peripheral blood cDNA library (16) but also found abnormally expressed by brain tumors (17). Along with its closest relative MT4-MMP, MT6-MMP is apparently anchored on plasma membrane through a GPI anchor (18), in contrast to those with type I transmembrane domains at their COOH termini, i.e., MT1, -2, -3, and -5-MMPs (8, 19–21). In resting human neutrophils, MT6-MMP is not only stored in the gelatinase granules and secretory vesicles but also displayed on cell surface (22). Activation of neutrophils by cytokines or chemokines such as IL-1 or IL-8 mobilizes MT6-MMP from the intracellular pools to the extracellular milieu in an yet to be determined mechanism (22). Upon secretion, MT6-MMP is believed to mediate proteolytic cleavage of ECM components from the basement membrane (16, 23), consistent with the hypothesis that MT6-MMP facilitates the transendothelial migration of neutrophils during inflammatory response (16, 22). In contrast, tumor cells are not known to contain such storage granules and thus may deploy MT6-MMP activity through a distinct mechanism.

One established mechanism by which tumor cells deploy some members of the MT-MMP family is through the activation of proMMP-2, a protease long implicated in tumor invasion and metastasis (4, 8, 9, 15, 24). Specifically, the type I MT-MMPs, including MT1-,-2,-3, and -5-MMPs, cleave proMMP-2 at the Asn66-Leu bond in its prodomain to generate an intermediate form, which then converts to the mature form autocatalytically (8, 9, 19–21, 25). However, the ability of the GPI-anchored MT4- and MT6-MMPs to activate proMMP-2 has not been documented mechanistically (17, 23). In fact, neither enzymes were considered as proMMP-2 activators (17, 23), a conclusion consistent with the sequence divergence between type I and GPI-anchored MT-MMPs (16, 17). Here we present evidence that MT6-MMP behaves like a proMMP-2 activator capable of cleaving not only the classic MT-MMP-processing site at Asn66-Leu but also the activation site Asn109-Tyr.

MATERIALS AND METHODS

Chemicals, Cell Lines, Cell Culture, and Immunological Reagents.

Chemicals were from Sigma-Aldrich (St. Louis, MO). Cell lines and cell cultures were used as described (26). Recombinant human TIMP-2 and human ProMMP-2 were gifts from R&D Systems (Minneapolis, MN). BB-94 was a gift from British Biotech (Oxford, United Kingdom). Antibody against MT1-MMP-CAT was a gift from Dr. Jorma Keski-Oja (University of Helsinki, Finland). Antibodies against MT5-MMP and MT6-MMP were generated against bacterially expressed CATs with His-tagged (see below) in rabbits.

Bacterial Expression of Leukolysin Catalytic Domain (Tyr109-Gly289).

We modified the pET15b vector (Novagen, Madison, WI) by inserting a palindrome sequence, GCCCGGCC, into the Xhol site repaired by Klenow polymerase. The resulting vector, pET15Shs, contains a Small site in which a blunt-end fragment can be inserted in frame with an NH2-terminal His tag (see Fig. 3A). The CAT of leukolysin from residue 107 to 280 was isolated with 2 primers, TAC GCT CTG AGC GGC AGC GTG, CCC ATA GAG TTG CTG CAG CCC, by PCR with pfu (Stratagene, La Jolla, CA), and inserted into...
RESULTS

Activation of ProMMP-2 by Transiently Transfected Leukolysin/MMP25/MT6-MMP. Although the hallmark function of MT1, -2, -3, and -5-MMPs is their ability to activate proMMP-2 (9), it is generally accepted that the GPI-anchored MT4 and 6-MMPs do not possess similar activity (16, 17, 23). However, our sequence analysis (Fig. 1) indicates that the CAT of MT6-MMP is more closely related to MT1-MMP than MT4-MMP, raising the possibility that MT6-MMP may share some of the functions of MT1, -2, -3, and -5-MMPs. On the basis of this analysis, we reexamined the ability of MT6-MMP to activate proMMP-2 in transient transfection assays (19). Indeed, we were able to demonstrate the activation of proMMP-2 by MT6-MMP (Fig. 2, Lane 2). Relatively weak compared with MT1-MMP and MT5-MMP, MT6-MMP reproducibly activates proMMP-2 in different cell lines tested, including MDCK, COS-7, HEK293, H11080, and C6 (data not shown). We estimated that MT6-MMP is ∼10% as effective as MT5-MMP in activating proMMP-2 under identical experimental settings (Fig. 2, Lane 2 versus 3), whereas MT5-MMP is about 25% as active as MT1-MMP (Fig. 2, Lane 3 versus 4). Two major species of MT6-MMP, at 58 and 54 kDa, respectively, were detected in cell lysates (Fig. 2, Lane 6), presumably representing the pro and active species of this enzyme. The pro and active species of MT5-MMP and MT1-MMP were also observed as reported previously (Fig. 2, Lanes 7 and 8) (8, 19). These observations are consistent with the hypothesis that MT6-MMP is activated inside the cells and able to process proMMP-2 into intermediate and active forms.

Expression and Purification of Leukolysin Catalytic Domain. Mechanistically, MT1-MMP is known to activate proMMP-2 by cleaving the Asn66-Leu bond at its protomain to yield an intermediate form at 66 kDa which autoactivates itself into the fully active 62-kDa form (9). However, MT6-MMP generated very little of the intermediate form of proMMP-2 (Fig. 2, Lane 2), suggesting that MT6-MMP may activate proMMP-2 through a different mechanism. To test this idea, we wished to determine the cleavage site on proMMP-2 by MT6-MMP. To this end, we attempted to produce a leukolysin CAT in *Escherichia coli* as described for MT1-MMP (29). The recombinant MT6-MMP was expressed in BL21DE3 cells by induction with isopropyl-1-thio-β-D-galactopyranosidase (0.4 mM), purified as inclusion bodies and refolded into soluble proteins as described in “Materials and Meth-

**Fig. 1.** Leukolysin/MT6-MMP is more closely related to the type I transmembrane MT-MMPs than MT4-MMP. A, dendrogram of MT-MMPs. The amino acid sequences of MT-MMP CATs were aligned by ClustalW (http://clustalw.genome.ad.jp/), and the resulting data set was used to construct a dendrogram to illustrate the relatedness among MT-MMPs. B, percentage of sequence identity between MT4-MMP (16) or MT6-MMP (17) and MT1, -2, -3, and -5-MMPs, calculated within the CATs and plotted to show that MT6-MMP is more closely related to the type I transmembrane MT-MMPs than MT4-MMP.

**Fig. 2.** Activation of proMMP-2 by transient transfected leukolysin/MMP25/MT6-MMP. proMMP-2 expression plasmid was cotransfected with the control vector (Lanes 1 and 5), MT6-MMP expression vector (Lanes 2 and 6), MT5-MMP expression vector (Lanes 3 and 7) or MT1-MMP expression vector (Lanes 4 and 8) into MDCK cells. 24 h later, cells were washed three times with PBS and then incubated with serum-free medium. Conditioned media (24 h) were collected, and cells lysed in radioimmunoprecipitation assay buffer in the presence of proteinase inhibitors. Conditioned media were analyzed by gelatin zymogram. Cell lysates were analyzed by Western blotting with rabbit antibodies against MT6-MMP (Lane 5 and 6), MT5-MMP (Lane 7) or MT1-MMP (Lane 8) respectively. The pro, intermediate (inter) and active (act.) species of MMP-2 are indicated on the right side of the zymogram. MW, molecular mass.
charged Ni \( / H \) 1001

recombinant leukolysin. Refolded recombinant leukolysin (Lane 1) was loaded onto a charged Ni \( / H \) column. After an extensive washing in Tris-buffered saline, the column was eluted with 1 M imidazole. The unbound materials (Lane 2) and eluted fractions 1–3 (Lanes 3–5) were analyzed by SDS-PAGE and stained with R250 (Lanes 1–5). Aliquots from fractions 2 and 3 (Lanes 6 and 7) were analyzed by zymography. Note the single species active on zymography (arrow). MW, molecular mass.

**Characterization of MT6-MMP Catalytic Activity.** We evaluated the proteolytic activity of bacterially derived leukolysin CAT against fluorescent synthetic substrates as described (30). As shown in Fig. 4A, leukolysin appears to prefer the gelatinase substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH\(_2\) (31), over the stromelysin substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Ary-Lys(Dnp)-NH\(_2\) (30, 31), a finding contradicting the recent report that the CAT of leukolysin is a good gelatinase (16).

Fig. 4. Purified recombinant leukolysin is catalytically active against synthetic substrates. In A, active leukolysin (10 nM) was analyzed against fluorescent substrates for gelatinase (Column A), stromelysin (Column B) or TACE (Column C) in assay buffer (20 mM Tris, 5 mM CaCl\(_2\), and 1 mM ZnCl\(_2\)) on an automated plate reader (Beckman, CA).

The relative fluorescent unit per second (RFU/sec) for each substrate is presented as indicated (n = 3). B. IC\(_{50}\) for BB-94 is ~10 nM. Active leukolysin (10 nM) were assayed in the presence of BB-94 at 0, 0.01, 0.1, 1.0 \mu M as described in A (n = 3). We estimate that the IC\(_{50}\) for BB-94 against leukolysin is ~10 nM.

MT6-MMP (i.e., leukolysin) behaves like stromelysin (23). In agreement with English and colleagues, we found that leukolysin also cleaves the tumor necrosis factor \( \alpha \)-converting enzyme (TACE) substrate Mca-Ser-Pro-Leu-Ala-Val-Arg-Ser-Ser-Lys(Dnp)-NH\(_2\) (Fig. 4A). Using the gelatinase substrate, we determined the IC\(_{50}\) of BB-94 against leukolysin (~10 nM) at ~10 nM (Fig. 4B). These results suggest that leukolysin encodes a very active proteinase.

**Leukolysin Cleaves ProMMP-2 at Asn\(^{66}\)-Leu and Asn\(^{109}\)-Tyr.** To test the hypothesis that leukolysin can activate proMMP-2 directly, we incubated the purified CAT (10 ng) with proMMP-2 (100 ng; R&D Systems) at 37°C for 2 h. As shown in Fig. 5A, leukolysin activated proMMP-2 efficiently (Fig. 5A), generating the fully activated gelatinase A and a further processed fragment (Lane 2). This activation process was inhibited by BB-94 (5 \mu M) as expected (Fig. 5A, Lane 3) and TIMP-1 and TIMP-2 (10 nM; data not shown). Interestingly, the pattern generated with purified leukolysin is consistent with that of cellular MT6-MMP as shown in Fig. 2 with very little accumulation of the intermediate form of proMMP-2, raising the possibility that leukolysin may be able to directly convert proMMP-2 into fully mature form, in contrast to the known two-step process mediated by the type I MT-MMPs (8, 15, 25). To test this possibility, we generated a catalytically inactive proMMP-2 mutant by mutating its active site (see “Materials and Methods”), rendering it incapable of autolysis as reported (29). Like MT1-MMP, MT5-MMP cleaved this proMMP-2 mutant at the expected Asn\(^{66}\)-Leu bond. Under identical conditions, leukolysin converted this mutant into two distinct products at 68 and 62 kDa (Fig. 5B), arguing that it cleaves proMMP-2 at two distinct sites to yield both the intermediate and the fully mature forms. To confirm this prediction, we performed NH\(_2\)-terminal sequencing of one of these products. As summarized in Fig. 5C, the cleaved proMMP-2 species have Leu\(^{67}\) and Tyr\(^{110}\) at their NH\(_2\) termini, respectively, thus representing the intermediate and the fully mature forms of MMP-2.

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4 X. Wang and D. Pei, unpublished observation.
This result demonstrates that leukolysin not only recapitulates the hallmark activity of MT1-MMP, i.e., the processing of proMMP-2 at the Asn109-Tyr bond (8, 25), but also exhibits a previously unsuspected hallmark activity of MT1-MMP, i.e., MT6-MMP can activate proMMP-2, a function previously ascribed only to the type I MT-MMPs (9, 23). Contrary to the expected two-step activation process mediated by type I MT-MMPs, MT6-MMP can activate proMMP-2 by directly cleaving the Asn109-Tyr bond, in addition to the known Asn 66-Leu bond in the prodomain of proMMP-2 (Fig. 5). To the best of our knowledge, this novel activity provides a mechanistic explanation of the observed pattern of proMMP-2 activation by MT6-MMP at the cellular level (Fig. 2).

MT6-MMP and MT5-MMP Catalytic Domains Activate ProMMP-2 with Comparable Efficiency but Distinct Patterns of Cleavage. At the cellular level, we demonstrated that MT6-MMP is only 2.5 or 10% as active as MT1-MMP or MT5-MMP, respectively, in activating proMMP-2 (see Fig. 2, Lanes 2 versus 3 and 2 versus 4), which may reflect the difference in catalytic activities of these enzymes. To test this idea, we directly compared the proteolytic activity of the two MT6-MMP and MT5-MMP. Comparable amounts of active site titrated MT5-CAT (100 ng) and MT6-CAT (150 ng) were used to cleave the same amount of proMMP2EA (2 μg) in a time course experiment. Although MT6-MMP is only 10% as efficient as MT5-MMP in cellular activation of proMMP-2, surprisingly, their CATs appear to have similar catalytic activity against proMMP-2EA in buffered assays. As shown in Fig. 6, MT5-CAT converted ~70% of proMMP-2EA into the 68-kDa intermediate form whereas MT6-CAT cleaved proMMP-2EA into both the intermediate (~30%) and fully mature (~30%) forms in 4 h (Fig. 6, Lane 3 versus Lane 7). Consistent with the data shown in Fig. 5, MT6-CAT converted proMMP-2 into the fully mature form, whereas MT5-CAT did not (Fig. 6, Lanes 1–3 versus 4–7). These data confirm that MT6-MMP encodes a highly efficient CAT with unique substrate specificity.

The Catalytic Domain of MT6-MMP Can Partially Substitute the Function of MT1-MMP Catalytic Domain. To reconcile the apparent discrepancy between the weak activity of MT6-MMP at the cellular level and the relatively strong activity observed in vitro against proMMP-2 (see Figs. 2 and 6), we generated a MT6/MT1 chimera construct, as shown in Fig. 7A, by swapping the pro- and CATs of MT6-MMP in front of the hinge region of MT1-MMP to see whether the CAT of MT6-MMP can functionally substitute the CAT of MT1-MMP. When cotransfected with a MMP-2 expression vector, the MT6/MT1 chimera was able to activate proMMP-2 efficiently as shown in Fig. 7. Because similar amounts of proteinases were expressed as observed by Western blotting (Fig. 7B, lower panels), we estimate that the MT6/MT1 hybrid is ~80% as efficient as MT5-MMP in activating proMMP-2 (Fig. 7B, Lanes 2 versus 3). Therefore, these data further verified that the CAT of MT6-MMP encodes an efficient proMMP-2 activator, suggesting that tumor cells may deploy MT6-MMP in generating active MMP-2 during invasion and metastasis.

DISCUSSION

In this report, we presented evidence that MT6-MMP, a GPI-anchored MT-MMP, can activate proMMP-2, a function previously ascribed only to the type I MT-MMPs (9, 23). Contrary to the established two-step activation process mediated by type I MT-MMPs, MT6-MMP can activate proMMP-2 by directly cleaving the Asn109-Tyr bond, in addition to the known Asn66-Leu bond in the prodomain of proMMP-2 (Fig. 5). To the best of our knowledge, this is the first definitive report on proMMP-2 activation by a member of the GPI-anchored MT-MMP. This finding provides a mechanistic understanding on how MT6-MMP can promote tumor invasion and metastasis when abnormally expressed by malignant tumor tissues.

Activation of ProMMP-2 by MT6-MMP. The proteolytic activity of MT6-MMP has been characterized by our group and others (16, 22,
23). It has been shown that MT6-MMP is capable of degrading ECM components such as laminin, fibronectin, fibrinogen, and type IV collagen (16, 22, 23). Previous attempts to demonstrate that MT6-MMP can activate proMMP-2 have yielded conflicting results. In the first paper describing the expression of MT6-MMP in tumor tissues, Velasco et al. (17) observed the conversion of proMMP-2 to lower molecular species similar to the activated MMP-2 species. Yet, no detailed studies have been reported on MMP-2 activation by MT6-MMP. A biochemical study of MT6-MMP proteolytic activity concluded that MT6-MMP encodes matrix-degrading activity but lack proMMP-2 activation activity (22). Coupled with a similar conclusion reached for MT4-MMP (32), another GPI-anchored MT-MMP, it was generally believed that GPI-anchored MT-MMPs do not possess proMMP-2 activation activity. In light of the data presented in this report, we suggest that MT6-MMP is a reasonable proMMP-2 activator.

**MT6-MMP Cleave proMMP-2 at the Asn**<sup>109</sup>-Leu Bond**

The type I-MT-MMPs activate proMMP-2 in a two-step process by cleaving the Asn<sup>66</sup>-Leu bond of proMMP-2 to generate the 62-kDa intermediate (9). However, recent studies have shown that type I MT-MMPs activate proMMP-2 in a two-step process by cleaving the Leu and Asn<sup>109</sup>-Tyr bonds directly (23). Coupled with a similar conclusion reached for MT4-MMP (22), another GPI-anchored MT-MMP, it was generally believed that GPI-anchored MT-MMPs do not possess proMMP-2 activation activity. In light of the data presented in this report, we suggest that MT6-MMP is a reasonable proMMP-2 activator.

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Direct Activation of Pro-Matrix Metalloproteinase-2 by Leukolysin/Membrane-type 6 Matrix Metalloproteinase/Matrix Metalloproteinase 25 at the Asn\textsuperscript{109}-Tyr Bond

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