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 normally expressed in brain cancer tissues. Yet, little is known about its role in tumor 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 proMMP-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 proMMP-2), which cannot autacatalytically 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 proMMP-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 proteases, 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 files, 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 archetype 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 proteinase 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-Gly280).
We modified the pET15b vector (Novagen, Madison, WI) by inserting a palindromic sequence, GGCCTGGCC, into the Xhol site repaired by Klenow polymerase. The resulting vector, pET15bS, contains a Smal 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 and Mca, (7-methoxycoumarin-4-yl) acetyl; Dpa, 3-N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Dnp, 2,4-dinitrophenyl.

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3 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; MDCK, Madin Darby canine kidney; GPI, glycosylphosphatidylinositol; IL, interleukin; TIMP, tissue inhibitor of metalloproteinase, CAT, catalytic domain; Mca, (7-methoxycoumarin-4-yl) acetyl; Dpa, N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Dnp, 2,4-dinitrophenyl.
pET15bS. The resulting clones were screened by restriction analysis, and four clones were identified in a sense orientation. An error-free clone, pET15bS-leukolysin (Tyr^{107}Gly^{286}), was introduced into BL21DE3 for induction as suggested (Novagen). The His-tagged CAT was expressed as inclusion bodies which were purified to almost homogeneity by repeated sonication and washing in PBS containing 1% Triton. The purified inclusion bodies were dissolved in 8 M urea and refolded by gradual removal of urea. The refolded proteins were further purified by affinity chromatography through a Ni²⁺-NTA column (Pierce, IL). Active leukolysin was determined by active site titration with TIMP-2 as described (27).

Enzyme Assays, SDS-PAGE, Western Blotting, and Zymography. These procedures were conducted as described previously (19, 25). Enzyme activities were determined using fluorescent peptide substrates (10 μM for each substrate), Mca-Ser-Pro-Leu-Ala-Glu-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys (Dnp)-NH₂, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (Dnp)-NH₂ according to the supplier (R&D Systems). MMP-2 activation by leukolysin was effected at 37°C and analyzed on zymography as described (19, 25). MMP-2EA, the catalytically inactive mutant of proMMP-2, was constructed by converting the active site HEFGH into HAFGH 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 Asn^{66}-Leu bond at its prodomain to yield an intermediate form (9). However, MT6-MMP generated very little of the intermediate form of proMMP-2 (2). Lane 2 versus 3), 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-β-D-thiogalactopyranoside (0.4 mM), purified as inclusion bodies and refolded into soluble proteins as described "Materials and Methods."
ods." Shown in Fig. 3B is a representative purification process on the Ni+ affinity column with the input (Lane 1), output (Lane 2), and elution fractions (Lanes 3–5) analyzed by SDS-PAGE. On zymogram, the purified materials were active against gelatin (Fig. 3B, Lanes 6, 7), consistent with our previous reports that leukolysin is a good gelatinase (16).

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-NH2 (31), over the stromelysin substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH2 (30, 31), a finding contradicting the recent report that the CAT of leukolysin appears to prefer the gelatinase substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (31), over the stromelysin substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH2 (30, 31), a finding contradicting the recent report that the CAT of leukolysin is a representative purification process on the Ni+ affinity column with the input (Lane 1), output (Lane 2), and elution fractions (Lanes 3–5) analyzed by SDS-PAGE. On zymogram, the purified materials were active against gelatin (Fig. 3B, Lanes 6, 7), consistent with our previous reports that leukolysin is a good gelatinase (16).

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Leukolysin Cleaves ProMMP-2 at Asn66-Leu and Asn109-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 μM) 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 autoactivation as reported (29). Like MT1-MMP, MT5-MMP cleaved this proMMP-2 mutant at the expected Asn66-Leu bond.4 Under identical activation as reported (29). Like MT1-MMP, MT5-MMP cleaved this proMMP-2 mutant at the expected Asn66-Leu bond.4 Under identical

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4 X. Wang and D. Pei, unpublished observation.
This result demonstrates that leukosyn is not only recapitulates the hallmark activity of MT1-MMP, i.e., the processing of proMMP-2 at the Asn109-Leu bond (8, 25), but also exhibits a previously unsuspected activity to cleave the Asn66-Leu bond (8, 25), but also exhibits a previously unsuspected hallmark activity of MT1-MMP, suggesting that the cleavage at Asn66-Leu may relax the prodomain to the processing of proMMP-2 at the Asn109-Leu 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 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.

**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,
It has been shown that MT6-MMP is capable of degrading ECM components such as laminins, 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 (23). 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 Cleaves ProMMP-2 at the Asn109-Leu Bond.** The type I MT-MMPs activate proMMP-2 in a two-step process by cleaving the Asn109-Leu bond of proMMP-2 to generate the 62-kDa intermediate form, which in turn autocatalytically cleaves itself at the Asn109-Leu bond into a fully active species at 59 kDa (9, 15, 24, 25). Using a bacterially derived CAT of MT1-MMP, Will et al. (29) demonstrated that MT1-MMP only cleaves at the Asn109-Leu bond, not the Asn109-Tyr one of a catalytic inactive mutant proMMP-2EA. In this report, the CAT of MT6-MMP generated in a similar manner, on the other hand, is able to cleave at both the Asn109-Leu and Asn109-Tyr bonds of proMMP-2EA, suggesting that MT6-MMP can mediate the activation of proMMP-2 both through the two-step process or directly. On the basis of the cleavage pattern presented in Figs. 5 and 6, we estimate that MT6-MMP cleaves both sites with almost equal efficiencies. A mutant converting the Asn109-Leu into Ala appears to be cleaved less efficiently than the wild type MMP-2 (data now shown), suggesting that cleavage at the Asn109-Leu may facilitate the cleavage of the Asn109-Tyr site for full activation. Nonetheless, MT6-MMP activates proMMP-2 by cleaving both the Asn109-Leu and Asn109-Tyr bonds directly.

**Implications of MT6-MMP-mediated ProMMP-2 Activation in Inflammation and Cancer.** The expression of MT6-MMP in granulocytes and brain tumor tissues supports the notion that it may play an important role in inflammation and cancer (16, 17, 22). In acute inflammation, MT6-MMP is mobilized by chemokines to the extracellular milieu where it can cleave components of the ECM barrier to allow neutrophils to migrate to the sites of infection (22). Our observation that MT6-MMP can activate proMMP-2, a MMP readily detected in the plasma and extracellular fluid, suggests that MT6-MMP can orchestrate a powerful cascade of proteolytic activities against the ECM barriers as neutrophils migrate to and reach the inflammatory sites. More significantly, the ability of MT6-MMP to activate proMMP-2, a MMP previously known to promote tumor invasion and metastasis (4), provides a conceptual framework on how MT6-MMP participates in the complex process of tumor invasion and metastasis. Although the only reported tumor tissues that express MT6-MMP were brain tumors, we have recently extended our studies into tumors of the prostate and breast by immunohistochemistry. Further studies will help to establish MT6-MMP as a potential tumor maker and perhaps a therapeutic target for drug development.

**REFERENCES**

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