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Direct Activation of Pro-Matrix Metalloproteinase-2 by Leukolysin/Membrane-type 6 Matrix Metalloproteinase/Matrix Metalloproteinase 25 at the Asn^{109}-Tyr Bond[1]

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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 proMMP-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 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 Asn^{66}-Leu but also the previously unsuspected Asn^{109}-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-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[3] 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 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 Asn^{66}-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 Asn^{66}-Leu but also the activation site Asn^{109}-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 (Tyr^{107}-Gly^{280}). We modified the pET15b vector (Novagen, Madison, WI) by inserting a palindrome sequence, GCCCGGGC, into the *Xho*I site repaired by Klenow polymerase. The resulting vector, pET15bS, contains a *Sma*I site in which a blunt-end fragment can be inserted in frame with an NH_2-terminal His tag (see Fig. 3*A*). 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 GCC, by PCR with *pfu* (Stratagene, La Jolla, CA), and inserted into

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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.

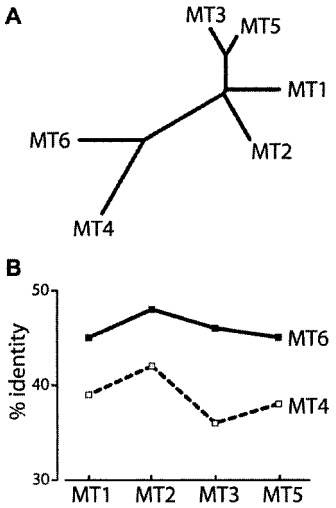

Fig. 1. Leukolysin/MT6-MMP is more closely related to the type I transmembrane MT-MMPs than MT4-MMP. *A*, dendrogram of MT-MMPs. The amino acid sequences of MT-MMP CATs were aligned by ClustalW (http://clustalw.genome.ad.jp/), and the resulting data set was used to construct a dendrogram to illustrate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

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^{280}), 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 Ni^{+} column (2×0.5 cm). Protein concentrations were determined with bicinchoninic acid reagents (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-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys (Dnp)-NH_2, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (Dnp)-NH_2 according to the supplier (R&D Systems). ProMMP-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 as described for MT5-MMP (19). A FLAG (FLAG peptide DYKDDDDK)-tagged proMMP-2EA mutant was stably expressed in MDCK cells and purified to homogeneity as described (26).

Protein NH_2-terminal Sequencing. Protein samples were transblotted to polyvinylidene difluoride membranes and submitted to the Michigan State University Macrochemical Facility for sequencing as described (25).

Expression Constructs and Transfection. pCR3.1uni-MT6-MMP, pCR3.1uni-MT1-MMP, pCR3.1-MMP2, and pCR3.1uni-MT5-MMP were described previously (16, 19). Domain swaps between MT6-MMP and MT1-MMP were performed by high-fidelity PCR with *pfu* polymerase and subcloned into pCR3.1uni vector as described previously (28). The resulting MT6/MT1 chimera is derived from sequences corresponding to Met^{1}-Gly^{280} of MT6-MMP and Gly^{285}-Val^{582} of MT1-MMP. Liposome-mediated DNA transfections into MDCK cells or HEK293 cells were performed by using LipofectAMINE (Invitrogen, San Diego, CA) as described (28).

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, HT1080, 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 Asn^{66}-Leu bond at its prodomain 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-galactopyranoside (0.4 mM), purified as inclusion bodies and refolded into soluble proteins as described in "Materials and Meth-

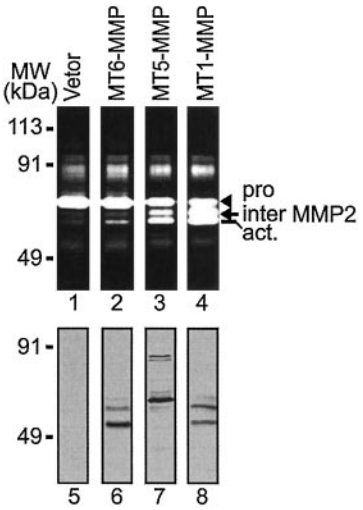

Fig. 2. Activation of proMMP2 by transient transfected leukolysin/MMP-25/MT6-MMP. proMMP-2 expression plasmid was cotransfected with the control vector *(Lanes 1* and *5*), MT6-MMP expression vector *(Lanes 2* and *6*), MT5-MMP expression vector *(Lanes 3* and *7*) or MT1-MMP expression vector *(Lanes 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.

ods." Shown in Fig. 3*B* 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. 3*B*, *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. 4*A*, 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-Arg-Lys(Dnp)-NH_2 (30, 31), a finding contradicting the recent report that the CAT of 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 α-convertase (TACE) substrate Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(Dnp)-NH_2 (Fig. 4*A*). Using the gelatinase substrate, we determined the IC_{50} of BB-94 against leukolysin (~10 nM) at ~10 nM (Fig. 4*B*). These results suggest that leukolysin encodes a very active proteinase.

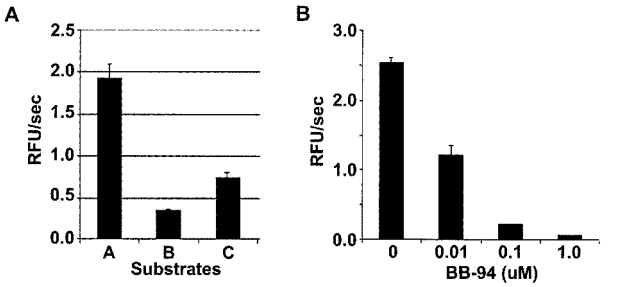

Fig. 4. Purified recombinant leukolysin is catalytically active against synthetic substrates. In *A*, active leukolysin (10 nM) was analyzed against fluorescent substrates for gelatinase (*Column A*), stromelysin (*Column B*) or TACE (*Column C*) in assay buffer (20 mM Tris, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$) 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 μM as described in *A* ($n = 3$). We estimate that the IC_{50} for BB-94 against leukolysin is ~10 nM.

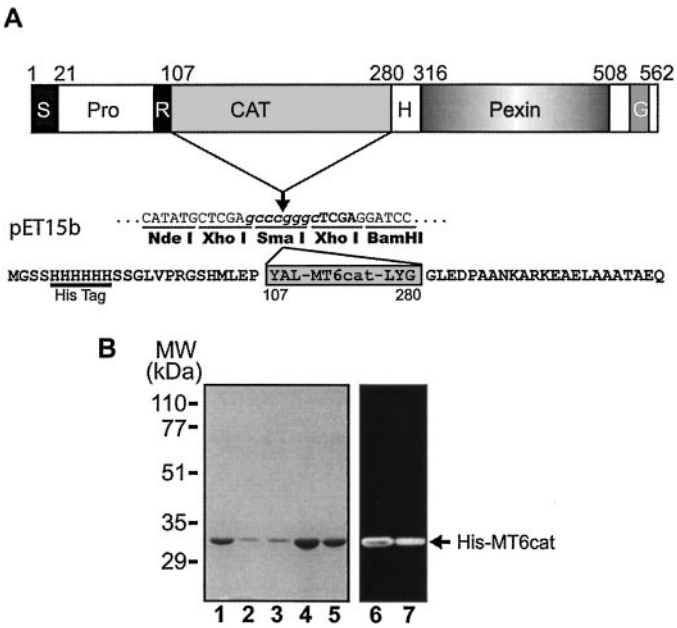

Fig. 3. Expression and purification of recombinant leukolysin. *A*, Schematic presentation of MT6-MMP and expression vector for its CAT. *Top*, open reading frame of MT6-MMP in domains from signal peptide (*S*), prodomain (*pro*), the furin motif (*R*), CAT, hinge region (*H*), hemopexin-like domain (*pexin*), and GPI anchor (*G*). *Middle*, modified polylinker region of the expression vector pET15bS with a new *Sma*I site and two flanking *Xho*I sites. MT6-MMP-CAT (residues 107–280) was inserted into the *Sma*I site to create an in-frame fusion with the upstream His tag (*bottom*). *B*, purification of recombinant leukolysin. Refolded recombinant leukolysin *(Lane 1)* was loaded onto a charged Ni^+ 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.

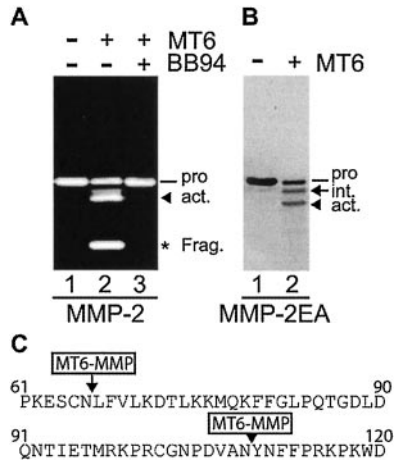

Fig. 5. Processing of proMMP-2 by leukolysin. *A*, processing of proMMP-2. proMMP-2 (100 ng) was incubated alone (*Lane 1*) or with leukolysin (10 ng, *Lanes 2* and *3*) in the absence *(Lane 2)* or presence (5 μM, *Lane 3*) of BB-94 for 2 h at 37°C. Aliquots (~5 ng) of the reaction mixtures were analyzed by zymography. *Arrowhead*, active (*act.*) form of MMP-2; *, fragmented (*Frag.*) form of MMP-2. *B*, processing of proMMP-2EA. The catalytically inactive mutant of MMP-2 (MMP-2EA, 1 μg) was incubated alone *(Lane 1)* or with leukolysin (20 ng, *Lane 2*) for 1 h at 37°C. Portions (100 ng) of the reaction mixes were analyzed by Western blotting. Both the intermediate (*int.*, *arrow*) and mature (*mat.*, *arrowhead*) forms of proMMP-2 were observed. *C*, NH_2-terminal sequencing of the intermediate and mature forms of MMP-2EA. ProMMP-2EA (2 μg) were cleaved by leukolysin as described in *B*, and resulting products were transferred to polyvinylidene difluoride membrane and submitted to sequencing as described (25). The cleavage sites were marked by *arrow* and *arrowhead* over the amino acid sequence of proMMP-2 from residues 61 to 120. Leukolysin cleaves both the Asn^{66}-Leu and Asn^{109}-Tyr bonds to generate the intermediate and mature forms, respectively.

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. 5*A*, leukolysin activated proMMP-2 efficiently (Fig. 5*A*), 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. 5*A*, *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 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 Asn^{66}-Leu bond.[4] Under identical conditions, leukolysin converted this mutant into two distinct products at 68 and 62 kDa (Fig. 5*B*), 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 these two products. As summarized in Fig. 5*C*, 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.

[4] X. Wang and D. Pei, unpublished observation.

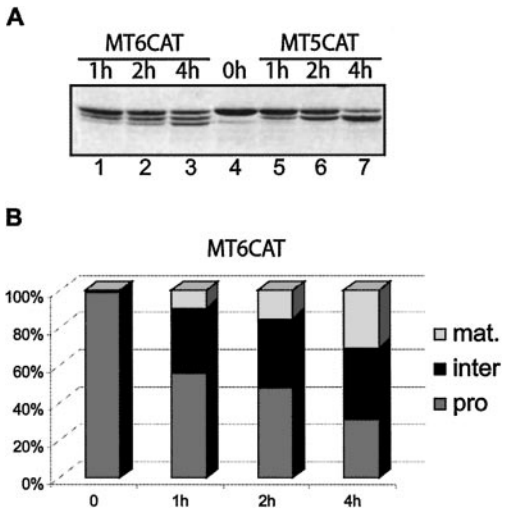

Fig. 6. Comparative cleavage of proMMP-2EA by MT5-MMP and MT6-MMP. *A*, time course study of MMP-2EA cleavage. Comparable amounts of MT5CAT (100 ng) and MT6CAT (150 ng) were incubated with proMMP-2EA (4 μg) for 1, 2, and 4 h, respectively. Reaction mixtures were analyzed with SDS-PAGE under reducing condition and stained with R250. *B*, quantification of MT6-MMP-mediated cleavage of MMP-2EA. The intact and the two cleaved forms of MMP-2 were quantified by densitometry and graphed to show the time-dependent increase of mature (*mat.*) MMP-2. *Inter*, intermediate.

This result demonstrates that leukolysin not only recapitulates the hallmark activity of MT1-MMP, *i.e.*, the processing of proMMP-2 at the Asn^{66}-Leu bond (8, 25), but also exhibits a previously unsuspected activity to cleave the Asn^{109}-Tyr to generate the fully mature form of this enzyme directly. To see whether the cleavage at Asn^{66}-Leu is a prerequisite for the second cleavage by MT6-MMP, we further mutated the Asn^{66}-Leu sequence into Ala^{66}-Ala in MMP-2EA. This mutant was cleaved into the fully mature form by MT6-MMP, but much less efficiently than the wild type MMP-2EA (data not shown), suggesting that the cleavage at Asn^{66}-Leu may relax the prodomain to allow MT6-MMP to contact and cleave the Asn^{109}-Tyr bond rapidly (Fig. 5, *B* and *C*). Nevertheless, 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.[4] 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. 7*A*, 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. 7*B*, *lower panels*), we estimate that the MT6/MT1 hybrid is ~80% as efficient as MT5-MMP in activating pro-MMP-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 Asn^{109}-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 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,

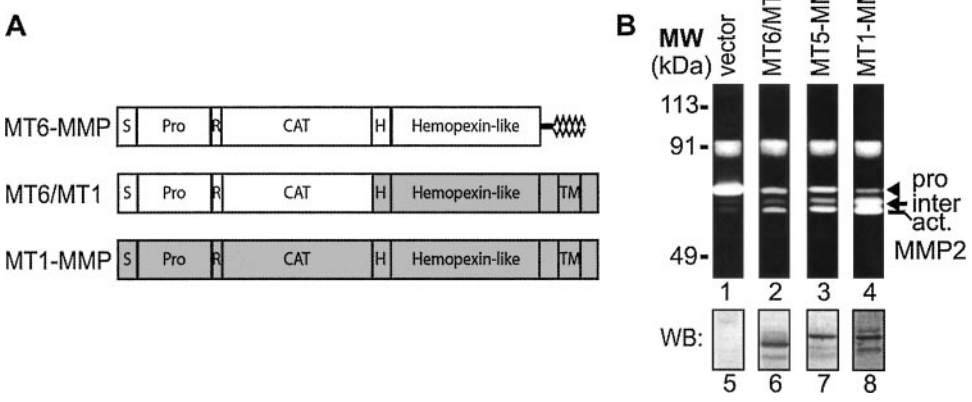

Fig. 7. MT6-MMP-CAT can partially substitute the function of MT1-MMP-CAT. *A*, schematic presentation for MT1-MMP, MT6-MMP, and MT6/MT1 chimera. *S*, signal peptide; *Pro*, prodomain; *H*, hinge region; *TM*, transmembrane domain. *B*, proMMP-2 expression plasmid was cotransfected with the control expression vector *(Lanes 1* and *5*), MT6-MMP expression vector *(Lanes 2* and *6*), MT1-MMP expression vector *(Lanes 3* and *7*) or MT6/MT1 chimera expression vector *(Lanes 4* and *8*) into MDCK cells as described in "Materials and Methods." The proMMP-2 activation profiles and the expression of the transfected MT-MMPs were analyzed by zymography (*top*) and Western blotting (*WB*; *bottom*) as described in Fig. 2. *Inter*, intermediate; *act.*, active.

23). 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 into 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 Asn^{109}-Tyr Bond. The type I MT-MMPs activate proMMP-2 in a two-step process by cleaving the Asn^{66}-Leu bond of proMMP-2 to generate the 62-kDa intermediate form, which in turn autocatalytically cleaves itself at the Asn^{109}-Tyr 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 Asn^{66}-Leu bond, not the Asn^{109}-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 Asn^{66}-Leu and Asn^{109}-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 Asn^{66}-Leu into Ala^{66}-Ala appears to be cleaved less efficiently than the wild type MMP-2 (data now shown), suggesting that cleavage at the Asn^{66}-Leu may facilitate the cleavage of the Asn^{109}-Tyr site for full activation. Nonetheless, MT6-MMP activates proMMP-2 by cleaving both Asn^{66}-Leu and Asn^{109}-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.[4] Further studies will help to establish MT6-MMP as a potential tumor maker and perhaps a therapeutic target for drug development.

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

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