TIMP Independence of Matrix Metalloproteinase (MMP)-2 Activation by Membrane Type 2 (MT2)-MMP Is Determined by Contributions of Both the MT2-MMP Catalytic and Hemopexin C Domains*

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Received for publication, April 7, 2006, and in revised form, June 5, 2006 Published, JBC Papers in Press, July 6, 2006 Published, JBC Papers in Press, July 6, 2006 DOI 10.1074/jbc.M603331200

The important and distinct contribution that membrane type 2 (MT2)-matrix metalloproteinase (MMP) makes to physiological and pathological processes is now being recognized. This contribution may be mediated in part through MMP-2 activation by MT2-MMP. Using Timp2−/− cells, we previously demonstrated that MT2-MMP activates MMP-2 to the fully active form in a pathway that is TIMP-2-independent but MMP-2 hemopexin carboxyl (C) domain-dependent. In this study cells expressing MT2-MMP as well as chimera proteins in which the C-terminal half of MT2-MMP and MT1-MMP were exchanged showed that the MT2-MMP catalytic domain has a higher propensity than that of MT1-MMP to initiate cleavage of the MMP-2 prodomain in the absence of TIMP-2. Although we demonstrate that MT2-MMP is a weak collagenase, this first activation cleavage was enhanced by growing the cells in type I collagen gels. The second activation cleavage to generate fully active MMP-2 was specifically enhanced by a soluble factor expressed by Timp2−/− cells and was MT2-MMP hemopexin C domain-dependent; however, the RGD sequence within this domain was not involved. Interestingly, in the presence of TIMP-2, a MT2-MMP-MMP-2 trimolecular complex formed, but activation was not enhanced. Similarly, TIMP-3 did not promote MT2-MMP-mediated MMP-2 activation but inhibited activation at higher concentrations. This study demonstrates the influence that both the catalytic and hemopexin C domains of MT2-MMP exert in determining TIMP independence in MMP-2 activation. In tissues or pathologies characterized by low TIMP-2 expression, this pathway may represent an alternative means of rapidly generating low levels of active MMP-2.

Recruitment of secreted proteases to the cell surface not only increases the proteolytic repertoire of a cell but also results in high local concentrations of the proteases. A level of control is achieved through focal proteolysis (1), and this is of pivotal importance in many events mediated by matrix metalloproteinases (MMPs)2 such as tissue remodeling, angiogenesis, tumor metastasis, and leukocyte recruitment (2, 3). The MMP family is a large group of zinc-dependent endopeptidases comprising both secreted and membrane-anchored (referred to as membrane-type (MT)-MMPs) enzymes that are regulated by the tissue inhibitors of metalloproteinases (TIMPs). Control of secreted MMPs, such as MMP-2 (also known as gelatinase A), is also maintained through their expression as inactive zymogens, which require processing of the prodomain to attain full activity (4). Because of their location at the cell surface MT-MMPs, which include transmembrane-anchored MT-MMPs 1, 2, 3, and 5 (5–8) and glycosylphosphatidylinositol anchored MT-MMPs 4 and 6 (9–11), play a major role in focal proteolysis. Despite the high degree of structural similarity of the MT-MMPs, differences in substrate specificity (12–15), TIMP requirements for MMP-2 activation (16, 17), and tissue and cellular localization have been demonstrated (18, 19). The structural basis for these differences are not fully understood. The importance of MT1-MMP (MMP-14) and its role in MMP-2 activation and cell invasion have been extensively investigated (for reviews, see Refs. 20–22). In contrast, the role and function of MT2-MMP (MMP-15) is just beginning to be addressed. Studies using a cell line derived from a MT1-MMP knock-out mouse have shown the important contribution of MT2-MMP to cell invasion of fibrin matrices (23). Indeed, elevated expression of MT2-MMP has been reported in many cancers, such as glioblastomas (24–26), ovarian (27), urothelial (28), and breast (19, 29) carcinomas and correlated with increased invasiveness (25, 26). In addition, MT2-MMP is involved in endothelial tubulogenesis (30), malignant conversion of keratinocytes (31), and is an anti-apoptotic factor (32). As well, MT2-MMP plays an important and distinct role from MT1-MMP in normal physiological processes (33). MMP-2 is also a primary mediator of focal proteolysis due to its recruitment and activation at the cell surface. This occurs

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2 The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane type-MMP; sMT-MMP, soluble MT-MMP; TIMP, tissue inhibitor of matrix metalloproteinases; C domain, carboxyl domain; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified essential medium; LCD, linker and hemopexin C domain; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MES, 4-morpholineethanesulfonic acid.
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primarily through interaction of the MMP-2 hemopexin carboxyl (C) domain with the MT1-MMP/TIMP-2 receptor (34, 35) and the resultant formation of a trimolecular activation complex (5, 36, 37). Activation of MMP-2 by MT1-MMP occurs in a two step process; the first cleavage within the MMP-2 prodomain (between Asn^37^-Leu^38) is mediated by an active MT1-MMP molecule to generate the intermediate (68-kDa) form of MMP-2 (38, 39), and the second cleavage (between Asn^80^-Tyr^81) is mediated in an autocatalytic manner by active MMP-2 (38, 40) and generates fully active MMP-2 (66-kDa). Because the second activation step was blocked by exogenous MMP-2 hemopexin C domain, it was concluded that it had to be mediated by a membrane-localized rather than soluble, active MMP-2 molecule (41). Whereas the first activation step of MMP-2 by MT1-MMP can occur in the absence of TIMP-2, the second activation step is absolutely TIMP-2-dependent (16, 42). A number of alternative MT1-MMP-dependent activation pathways for MMP-2 have been reported involving proteases such as thrombin (43), neutrophil elastase (44), and plasmin (45), but the in vivo relevance of these pathways is unknown. Interestingly, a TIMP-independent MMP-2 activation pathway that is mediated by MT1-MMP and the tight-endothelial junction protein claudin-5 has been reported (46). In addition, with the exception of MT4-MMP (14), all the other MT-MMPs have been shown to activate MMP-2 (7, 16, 47–49).

Using a TIMP-2-free cell line derived from the TIMP-2 knock-out mouse (50), we have shown that MT2-MMP activates MMP-2 in a TIMP-2-independent pathway that is clearly distinct from that of MT1-MMP (16). Full activation of MMP-2 by MT2-MMP proceeded via the 68-kDa intermediate but occurred more rapidly than MT1-MMP-mediated activation. TIMP-2 had no enhancing effect on the level of active MMP-2 generated by MT2-MMP. In addition, activation by MT2-MMP required localization of MMP-2 at the cell surface in a MMP-2 hemopexin C domain-dependent but TIMP-2-independent manner. In this study we use Timp2^-/-^ cells transfected for stable cell surface expression of MT2-MMP as well as chimera proteins in which the C-terminal half of MT2-MMP and MT1-MMP are exchanged to dissect the contribution that the different domains of MT2-MMP make to influence TIMP-independent activation of MMP-2.

EXPERIMENTAL PROCEDURES

Recombinant Protein Expression and Purification Hemopexin C Domains—The cDNA encoding the linker (L) and hemopexin C domain (CD) (Thr^305^-Cys^559) of MT2-MMP (MT2-MMP LCD) was amplified by PCR and cloned into the bacterial expression vector pGYMX (51). The pGYMX vectors contain SspI sites (AATATT) by silent mutation in the MT1-MMP and MT2-MMP (after 957 and 1110 bp, respectively) were exchanged. This was achieved using PCR to introduce unique SspI sites (AATATT) by silent mutation in the MT1-MMP and MT2-MMP cDNAs. The double Gly residues at each end of the tag were designed for flexibility. The FLAG-tagged MT1-MMP and MT2-MMP constructs were used to generate chimera constructs, in which the 3’ end of MT1-MMP and MT2-MMP (after 957 and 1110 bp, respectively) were exchanged. This was achieved using PCR to introduce unique SspI sites (AATATT) by silent mutation in the MT1-MMP and MT2-MMP cDNAs. These sites were then used in cloning. The constructs generated encode proteins in which the C-terminal half of MT1-MMP and MT2-MMP is exchanged after Cys^319^ and Cys^570^, respectively. The resulting chimera proteins are termed MT1catL/MT2CD-MMP and MT2catL/MT1CD-MMP. All MT1-MMP and MT2-MMP constructs described were cloned into the pGW1GH vector and were used to transf ect Timp2^-/-^ fibroblasts using conditions described in Morrison et al. (16). Positive clones were identified by flow cytometry using an anti-FLAG M2 antibody (Sigma) and by screening for MMP-2 activation in the presence and absence of 10 nM TIMP-2.

Soluble MT-MMP (sMT-MMP)—PCR was used to introduce a FLAG sequence at the 3’ end of the sMT1-MMP cDNA used previously in Bigg et al. (42). This construct was cloned into the pPIC9 vector (Invitrogen) for expression in Pichia GS115 cells (Invitrogen). Cells were grown in 500 ml baffled shaker flask cultures, and after 24 h, 0.5% methanol was added to induce sMT1-MMP expression. Culture medium was diluted in MES buffer (final concentration, 50 mM MES, 5 mM CaCl_2, 0.02% NaNO_3, 0.05% Brij, pH 6.2) and sMT1-MMP was purified upon binding to reactive red agarose (Sigma) (V_t = 30 ml). After
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washing, the column was eluted with 1 mM NaCl in MES buffer. Fractions containing sMT1-MMP were pooled and dialyzed into 50 mM Hepes, 150 mM NaCl, pH 7.2.

A cDNA construct was generated by PCR that encodes C-terminal FLAG-tagged sMT2-MMP truncated at Gin566. The construct was cloned into the pGW1GH vector and used to transfect Timp2−/− cells (16). Positive clones with stable expression of sMT2-MMP were identified by Western blot analysis using an anti-FLAG M2 antibody (Sigma). Anti-FLAG M2 affinity gel (Sigma) was used to purify sMT2-MMP to homogeneity.

TIMPs—Human TIMP-2 and TIMP-4 were expressed in Chinese hamster ovary cells and baby hamster kidney cells, respectively, and purified as described in Bigg et al. (42). Mouse TIMP-1 was expressed in Timp2−/− cells and purified as described in Shimokawa and Nagase (56). TIMP-3 was kindly provided by Dr. Roy Black (Angeles, Seattle). All TIMPs were active site-titrated against a standard preparation of active MMP-8 to determine their concentrations.

ProMMP-2—TIMP-2-free human proMMP-2 was expressed in Timp2−/− cells as previously described (16).

Cell Culture and MMP-2 Activation Assays—Timp2−/− cells were cultured, and cell assays were performed as described in Morrison et al. (16). In brief, Timp2−/− cells expressing the full-length MT1-MMP and MT2-MMP constructs were seeded in 96-well plates at 2 × 10⁴ cell/well. After 24 h of incubation at 37 °C, cells were washed extensively in PBS and incubated under serum-free conditions in the presence of 5 mM TIMP-2-free proMMP-2. In addition, the following were also added. (a) For hemopexin C domain competition assays, between 1 and 3750 nM MT2-MMP LCD, MT1-MMP LCD, and MMP-2 LCD pretreated with polymyxin B (10 μg/ml) (Sigma), (57) to remove bacterial endotoxin contamination, were added to cells expressing MT2-MMP and MT1-MMP. TIMP-2 (10 nM) was added to cells expressing MT1-MMP. (b) For RGD peptide competition assays, between 0.05 and 12.5 μg/ml cyclic RGD or RAD peptide (kindly provided by Dr. Shoukat Dedhar, BC Cancer Agency, Vancouver, Canada) were added to cells expressing MT2-MMP. (c) Conditioned medium from Timp2−/− cells transfected with pGW1GH vector alone was concentrated using a Centricon centrifugal filtration device (Millipore) with a molecular weight cut-off of 10,000. The concentrated conditioned medium was then filter sterilized and added to cells expressing MT2-MMP and MT1-MMP. Cells expressing MT1-MMP were incubated with and without 10 nM TIMP-2. (d) TIMPs 1–4 were added to cells expressing MT2-MMP at concentrations between 0.01 and 270 nM, and TIMP-2 was added to cells expressing MT2catL/MT1CD-MMP, MT1catL/MT2CD-MMP, MT2-MMP FLAG, and MT1-MMP FLAG, at concentrations between 0.3 and 80 nM. (e) Neutralized Vitrogen® soluble type I collagen (Cohesion Technologies, Inc.) (between 12.5 and 200 μg/ml) was added to cells expressing MT2-MMP.

Timp2−/− cells expressing MT2-MMP or transfected with pGW1GH vector alone were also grown on or in a native type I collagen matrix Vitrogen® (Cohesion Technologies, Inc.) (2.3 mg/ml). Neutralized collagen alone or collagen premixed with cells at 5 × 10⁶ cells/ml (50 μl/well) were added to a 96-well plate, and the plate was incubated at 37 °C for 1 h to form collagen fibrils. Then 100 μl/well of cells at 5 × 10⁶ cells/ml in DMEM with 10% serum was added to wells containing collagen alone or to empty wells as a control. DMEM with 10% serum (100 μl/well) was added to the wells containing cells embedded in collagen. After 24 h of incubation at 37 °C, collagen gels with cells were washed extensively in PBS and incubated under serum-free conditions in the presence of 5 nm TIMP-2-free proMMP-2. In all cell assays, supernatants were collected after 24 h and analyzed by gelatin zymography using 8% polyacrylamide gels as described (16).

Enzyme Capture Assay—Enzyme-linked immunosorbent assay plates were coated with 2 μg/ml sMT2-MMP, TIMP-2, or ovalbumin in Voller’s buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) for 16 h at 4 °C. Wells were then blocked for 2 h in PBS with 3% ovalbumin. After washing in PBS with 0.05% Tween, MMP-2 was added at concentrations between 15 and 2000 ng/well and incubated for 24 h at 4 °C. After extensive washing, 25 μl of non-reducing SDS-PAGE sample buffer was added per well to elute bound proteins. Samples were analyzed by gelatin zymography.

Pulldown Assays—Gelatin-Sepharose was blocked in PBS with 0.5% bovine serum albumin for 2 h at 4 °C. Then 10-μl aliquots of blocked gelatin-Sepharose were incubated with 100 μl of proMMP-2 (100 μg/ml) or with PBS for 2 h at 4 °C. After washing in PBS, 10-μl aliquots of gelatin-Sepharose with bound proMMP-2 were incubated with 100 μl of TIMP-2 (200 μg/ml) or concentrated conditioned medium or PBS or DMEM for 2 h at 4 °C. Then, after washing, the various samples were incubated with 100 μl of sMT1-MMP or sMT2-MMP (50 μg/ml) for 16 h at 4 °C. After extensive washing, gelatin-Sepharose beads were eluted with 30 μl of 10% Me₃SO. Affi-Gel 10 (Bio-Rad) was coupled according to manufacturer’s instructions to MMP-2 LCD or TIMP-2 as a positive control (1 mg protein/ml Affi-Gel) and then, after blocking reactive groups with 1 M ethanolamine, incubated with 100 μl of sMT1-MMP or sMT2-MMP (50 μg/ml) for 16 h at 4 °C. Bound proteins were removed from the Affi-Gel with denaturing SDS-PAGE sample buffer. Samples were analyzed by Western blotting using an anti-FLAG M2 antibody (Sigma).

Reverse Transcription-PCR—Reverse transcription-PCR was carried out as described in Morrison et al. (16). Claudin-5 cDNA was kindly provided by Dr. Hiroshi Sato (Kanazawa University, Japan).

Collagenase Assays—Collagenase assays (30-μl reaction volume) were set up using between 0.25 and 250 nM activated sMT2-MMP, sMT1-MMP, or MMP-8 and 100 fmol of biotinylated native type I collagen in assay buffer (50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.05% Brij pH 7.4) at 28 °C for 18 h. Cleavage products were analyzed using 7.5% SDS-PAGE and an in-gel detection assay using streptavidin Alexa Fluor 680 conjugate (Molecular Probes) and a Odyssey infrared imaging system (LI-COR Biosciences) described in Pelman et al. (58). The kcat/KM ratios were calculated for sMT1-MMP and sMT2-MMP using the equation kcat/KM = (ln 2/t1/2)/[E], where t1/2 is the time taken for half the substrate to be cleaved, and [E] is the enzyme concentration.
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RESULTS

Activation of MMP-2 by MT2-MMP Is Blocked by Exogenous MT2-MMP Hemopexin C Domain—To characterize the molecular mechanism of MMP-2 activation by MT2-MMP, we produced a recombinant form of the MT2-MMP hemopexin C domain with linker (MT2-MMP LCD) in E. coli. The purified MT2-MMP LCD had a molecular mass of 31,400 Da as determined by MALDI-TOF mass spectrometry, within 2 Da of the predicted mass 31,402 (Fig. 1A). Increased electrophoretic mobility upon reduction (Fig. 1A) indicated correct disulfide bridge formation (51). The identity of MT2-MMP LCD was confirmed by N-terminal sequencing and Western blot analysis (data not shown). The MT1-MMP and MMP-2 LCDs used as controls had, as predicted, molecular masses of 27,894 and 26,924 Da, respectively (Fig. 1A).

Stable transfectants of Temp2+/− cells expressing MT2-MMP on the cell surface were incubated with increasing amounts of MT2-MMP LCD and exogenous TIMP-2-free human proMMP-2. As the concentration of MT2-MMP LCD was increased in culture, the level of activation of MMP-2 to the fully active form decreased (see Fig. 1B, comparing all lanes to 0 control), with MMP-2 activation blocked at intermediate concentrations greater than 0.38 μM (~30-fold molar excess MMP2-LCD:MMP-2). This result was similar to that obtained for the positive control MMP-2 LCD (Fig. 1B). When MT1-MMP LCD was added to cells expressing MT2-MMP, there was no effect on MMP-2 activation (Fig. 1B). In addition, adding the MT2-MMP LCD to cells expressing MT1-MMP in the presence of 10 nM TIMP-2 had no effect on MMP-2 activation (Fig. 1C), indicating that the competitive effect of the MT2-MMP LCD on MMP-2 activation is specific to activation by MT2-MMP. Hence, these data demonstrate that MT2-MMP-mediated activation of MMP-2 to the fully active form is both MT2-MMP- and MMP-2 hemopexin C domain-dependent. This differs from the MT1-MMP activation pathway of MMP-2, which is MMP-2 but not MT1-MMP hemopexin C domain-dependent (41).

MMP-2 and MT2-MMP Do Not Directly Interact via Their Hemopexin C Domains—We postulated that binding might occur via direct interaction between the MMP-2 and MT2-MMP hemopexin C domains to allow MMP-2 activation (Fig. 2A). However, an enzyme capture assay demonstrated that whereas both active and proMMP-2 bound TIMP-2, no binding to sMT2-MMP occurred (Fig. 3A). Because steric hindrance might have prevented binding, an assay using sMT2-MMP in solution was performed. Again, no binding of sMT2-MMP to proMMP-2 prebound to gelatin-Sepharose via the MMP-2 fibronectin-type II modules (51) was detected (Fig. 3B). Interestingly, sMT2-MMP could bind to MMP-2 in the presence of TIMP-2, demonstrating that sMT2-MMP-TIMP-2-MMP-2 can form a trimolecular complex (Fig. 3B). This complex could also be formed with the MMP-2 LCD immobilized on Affi-Gel (Fig. 3C), demonstrating that as in the MT1-MMP-TIMP-2-MMP-2 complex, MT2-MMP is captured by formation of an inhibitory complex with TIMP-2 bound via the TIMP-2 C domain to the MMP-2 LCD (53, 59). Although trimolecular complex formation can occur with MT2-MMP, it is not required for MMP-2 activation. Indeed, TIMP-2 does not enhance TIMP-2-mediated MMP-2 activation but only inhibits activation at higher concentrations (16) (see Fig. 6A).

The RGD Sequence of the MT2-MMP Hemopexin C Domain Does Not Play a Role in MMP-2 Activation—One major difference between MT2-MMP and MT1-MMP that could account for the difference in TIMP-2 dependence for MMP-2 activation

FIGURE 1. MT2-MMP LCD inhibits activation of MMP-2 by MT2-MMP. A, silver-stained 15% SDS-PAGE of MMP-2 LCD, MT2-MMP LCD, and MT1-MMP LCD with (+) and without (−) DTT. Molecular masses of LCDs were measured using MALDI-TOF or electrospray ionization-TOF mass spectrometry. The positions of molecular mass markers in kDa are shown. B, gelatin zymography of supernatants incubated under serum-free conditions in the presence of 5 nM TIMP-2-free proMMP-2, 0–1.5 μM MT2-MMP LCD, and 10 nM TIMP-2. Medium containing MMP-2 incubated alone (M) is shown, and the positions of pro, intermediate, and active MMP-2 are indicated.
is the presence of an RGD sequence in the MT2-MMP hemopexin C domain (amino acids 495–497). A model of the MT2-MMP hemopexin C domain generated with Pymol, using the hemopexin C domain structures available for other MMPs, predicts that the MT2-MMP RGD sequence is surface-exposed and potentially available for integrin binding (Fig. 4A). Binding to integrins might result in the formation of a co-receptor for MMP-2 at the cell surface (Fig. 2B). The addition of 12.5 μg/ml of a cyclic RGD peptide to Timp2/−/− cells expressing MT2-MMP had profound effects on the cell morphology compared with cells treated with the control RAD peptide (data not shown), indicating that the RGD peptide was effectively blocking integrins. However, even when up to 12.5 μg/ml RGD peptide was added to cells expressing MT2-MMP, no effect was seen on MMP-2 activation (Fig. 4B).

Concentrated Conditioned Medium Proteins from Timp2/−/− Cells Specifically Enhance MMP-2 Activation by MT2-MMP—MMP-2 activation by MT2-MMP occurs more rapidly than the TIMP-2-dependent activation mediated by MT1-MMP (16). However, in MT2-MMP-mediated activation, unlike that mediated by MT1-MMP in the presence of optimal levels of TIMP-2 (10 nM), the intermediate form of MMP-2 as well as the fully active form is always observed (Fig. 1, B and C). This indicates that the second cleavage at Asn80-Tyr81 in the prodomain of MMP-2 is the rate-limiting step in the MT2-MMP-mediated activation pathway. We hypothesized that Timp2/−/− cells might produce a soluble factor that is involved in the second activation step (Fig. 2C) but is present in limiting quantities. To test this hypothesis, concentrated serum-free conditioned medium from Timp2/−/− cells transfected with the vector alone was added to MT2-MMP transfectants. An enhancement in the activation of MMP-2 by MT2-MMP to the fully active form was observed in the presence of concentrated conditioned medium proteins (Fig. 5A). Moreover, the effect was rapid, with enhanced activation occurring within 2 h, and by 24 h the increase in activation of MMP-2 to the fully active form in the presence of 10-fold concentrated conditioned medium proteins was pronounced (Fig. 5B). When 4–10-fold concentrated culture medium alone (DMEM) was added to the MT2-MMP-expressing cells, a decrease in the second activation cleavage compared with controls was observed (Fig. 5, A and B). This enhancing effect of conditioned medium proteins was specific to MT2-MMP-mediated activation, as when added to cells expressing MT1-MMP in the presence of 10 nM TIMP-2, no alteration of MMP-2 activation was observed (Fig. 5, A and B). In addition, no activation of MMP-2 by MT1-MMP to the fully active form occurred in the presence of concentrated conditioned medium when TIMP-2 was not added (Fig. 5B). This again highlights the essential requirement of TIMP-2 for the second, but not the first step of MMP-2 activation mediated by MT1-MMP and demonstrates that the concentrated conditioned medium does not contain any resid-
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The enhancement of MMP-2 activation by MT2-MMP did not result from increased growth of these cultures, as the presence of concentrated conditioned medium had no effect on cell growth. Cell numbers of $2.9 \times 10^5$, $3.0 \times 10^5$, and $2.8 \times 10^5$ cells/well (n = 4 for all conditions) were determined for cells grown with and without 10-fold concentrated conditioned medium proteins or 10-fold concentrated DMEM, respectively. In case activation of MMP-2 by MT2-MMP was enhanced due to an increased concentration of MMP-2, we depleted MMP-2 from the concentrated conditioned medium by gelatin-Sepharose chromatography. The enhanced activation was unaltered using the MMP-2-depleted conditioned medium (data not shown), confirming the presence of a unique soluble activation enhancer.

Role of Candidate Molecules Identified in Conditioned Medium of Timp2

Because both the hemopexin C domains of MMP-2 and MT2-MMP and a soluble factor are involved in the MT2-MMP-mediated activation pathway of MMP-2, we attempted to isolate the soluble factor from conditioned medium using hemopexin C domain affinity columns. An 11.7-kDa protein was eluted from a MMP-2 hemopexin C domain affinity column in 1M NaCl and was identified by N-terminal Edman sequencing as secretory leukocyte protease inhibitor (SLPI). Although we demonstrated that MMP-2 like MT1-MMP (60) cleaved SLPI, activation of MMP-2 by MT2-MMP was not enhanced by SLPI (data not shown). We did not detect any proteins bound to a MT2-MMP hemopexin C domain column. Using a gelatin-Sepharose pull-down assay, 20-fold concentrated conditioned medium did not promote interaction of MMP-2 and sMT2-MMP via the formation of a "novel" trimolecular complex as proposed in Fig. 2C (data not shown). These data indicate that although a soluble factor expressed by

FIGURE 3. Interaction of MT2-MMP and MMP-2. A, enzyme capture assay in which 0–2000 ng/well MMP-2 was added to wells coated with active sMT2-MMP, TIMP-2 (positive control), or ovalbumin (OVA) (negative control). After incubation for 16 h at 4 °C and extensive washing, bound proteins were eluted in SDS-PAGE sample buffer and analyzed by gelatin zymography for the presence of MMP-2. Position of pro and active MMP-2 are shown (Std). B, pulldown assays for sMT2-MMP or sMT1-MMP control using MMP-2 bound to gelatin-Sepharose in the presence and absence of TIMP-2. Affi-Gel coupled to TIMP-2 was also incubated with sMT2-MMP and sMT1-MMP as a positive control. Gelatin-Sepharose and Affi-Gel controls without MMP-2 and TIMP-2, respectively, are shown. C, pulldown assay for sMT2-MMP using MMP-2 LCD coupled to Affi-Gel in the presence and absence of TIMP-2. TIMP-2 coupled to Affi-Gel was included as a positive control. FLAG-tagged sMT-MMPs were detected in bound (B and C) and unbound (C only) fractions by Western blot analysis using anti-FLAG M2 antibody. The positions of molecular mass markers in kDa are shown.
At concentrations greater than 10 nM, inhibition of activation was observed, similar to that found for TIMP-2 and TIMP-4. As expected, TIMP-1 had no effect on activation of MMP-2 (Fig. 6A), as it is a poor inhibitor of MT2-MMP (16). Not surprisingly, since the Timp2−/− cells are skin fibroblasts (16, 50), we could not detect expression of the endothelial tight junction protein claudin-5 by reverse-transcribed-PCR (Fig. 6B), ruling out its involvement. We then investigated the possibility that the soluble factor may be an extracellular matrix protein, like fibronectin or collagen, that could bind to a cell surface receptor, such as an integrin, to form a co-receptor (Fig. 2C). The addition of fibronectin to the MT2-MMP-expressing cells did not enhance MMP-2 activation (data not shown); therefore, we examined the effect of collagen.

Type I Collagen Promotes Activation of MMP-2 by MT2-MMP to the Intermediate Form—We and others have shown that collagen plays a pivotal role in MT1-MMP activation of MMP-2 through clustering MT1-MMP at the cell surface (54, 61, 62) and, as shown more recently, through stabilization of MT1-MMP at the cell surface (63). Type I collagen was isolated and identified from conditioned medium of Timp2−/− cells (data not shown). Incubating MT2-MMP-expressing cells in the presence of ascorbic acid to promote fibril formation of the endogenous collagen had no effect on MMP-2 activation by MT2-MMP (data not shown). However, when MT2-MMP-expressing cells were grown on or in a type I native collagen matrix, we observed increased activation of MMP-2 compared with cells grown on plastic (Fig. 7A) or cells grown in the presence of soluble type I collagen (Fig. 7B). This enhanced activation, in contrast to that seen with concentrated conditioned medium proteins (Fig. 5, A and B), primarily resulted in more intermediate rather than fully active MMP-2 (Fig. 7A). This result indicates that collagen may function to increase the local concentration of MT2-MMP and, thus, potentiate the first activation cleavage within the MMP-2 prodomain. We then stud-
ied the collagenolytic activity of sMT2-MMP and found that the $k_{cat}/K_M$ of MT2-MMP for type I collagen is ~44 $\text{M}^{-1} \text{s}^{-1}$, 100-fold less efficient than MT1-MMP ($k_{cat}/K_M = 4400 \text{M}^{-1} \text{s}^{-1}$) and MMP-8 (Fig. 7C). Whereas we have shown that growth of MT2-MMP-expressing cells on or within collagen gels enhances MMP-2 activation, the lack of effect on MMP-2 activation by soluble collagen that is not present as microfibrils suggests that it is unlikely to be the enhancing factor found in conditioned medium.

**TIMP-2 Independence in MMP-2 Activation by MT2-MMP**

**Is Determined by Contributions of Both the MT2-MMP Catalytic and Hemopexin C Domains** — We have shown that MT2-MMP can form a trimolecular complex with TIMP-2 and MMP-2 (Fig. 3, B and C), and yet, unlike MT1-MMP-mediated activation of MMP-2, TIMP-2 does not enhance activation by MT2-MMP. We postulated that this fundamental difference in the activation pathways might be due to differences in the hemopexin C domains of MT1-MMP and MT2-MMP. To test this hypothesis, constructs of MT1-MMP and MT2-MMP were made in which the C-terminal half of these proteins (after Cys$^{319}$ and Cys$^{370}$ in MT1-MMP and MT2-MMP, respectively) were exchanged (Fig. 8A). Of the 22 positive clones identified that expressed MT2catL/MT1CD-MMP, all activated MMP-2 to the fully active form but only in the presence of TIMP-2 (shown for 4 clones in Fig. 8B). In the absence of TIMP-2, activation proceeded to the intermediate form only (Fig. 8B).

This activation pattern no longer resembled that of MT2-MMP (Figs. 6A and 8C) (16) but now mimicked that of MT1-MMP in its requirement for TIMP-2 to complete the second step of MMP-2 activation (42). These data again revealed the critical role that the MT2-MMP hemopexin C domain plays in TIMP-2-independent MMP-2 activation, where the second activation cleavage of MMP-2 proceeds in the absence of TIMP-2.

Surprisingly, when we examined the activation of MMP-2 by MT1catL/MT2CD-MMP, we found that MMP-2 activation mediated by this chimera was also TIMP-2-dependent. In the absence of TIMP-2, no activation occurred, optimal activation was seen between 1.3 and 5 nM TIMP-2, and at concentrations
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above 10 nM activation was inhibited (Fig. 8C). The pattern of MMP-2 activation by MT1catL/MT2CD-MMP and wild type MT1-MMP were essentially the same (Fig. 8C). Despite the importance of the MT2-MMP hemopexin C domain in TIMP-2-independent activation found in the domain competition experiments (Fig. 1), in the chimera protein MT1catL/MT2CD-MMP, the MT2-MMP hemopexin C domain alone was not sufficient to confer TIMP-2 independence. This indicates that the domain-domain interface in MT2-MMP is important or that both the hemopexin C domain and the catalytic domain contribute to TIMP-2-free activation. Indeed, these chimera results show that for MT1-MMP-mediated MMP-2 activation, both the catalytic and hemopexin C domains of MT1-MMP dictate the requirement for TIMP-2 to complete the second activation step.

When low concentrations of TIMP-2 were present, activation of MMP-2 by MT2catL/MT1CD-MMP to the intermediate form only was primarily observed, similar to activation in the absence of TIMP-2. The presence of such a strong band corresponding to the intermediate form of MMP-2 in the absence of TIMP-2 indicates the higher propensity that the MT2-MMP catalytic domain has to initiate cleavage of the MMP-2 prodomain compared with that of MT1-MMP, where relatively little MMP-2 intermediate was seen in the absence of TIMP-2 (Fig. 8C) (16). With the addition of 1.3–20 nM TIMP-2, MT2catL/MT1CD-MMP generated both intermediate and fully active MMP-2, the proportions of each form varying with TIMP-2 concentration. Optimal activation was seen at 2.5–10 nM TIMP-2, as shown for wild type MT1-MMP but not wild type MT2-MMP (Fig. 8C) (16). Hence, this second step of MMP-2 activation appears to be driven by the MT1-MMP C-terminal portion of the chimera and TIMP-2. At TIMP-2 concentrations >20 nM, inhibition of MMP-2 activation was seen. Shown in Fig. 8C are the results for two independently derived clones. These results contrast to those seen for wild type MT2-MMP, where equal proportions of intermediate and active MMP-2 were seen in the absence of and at concentrations below 20 nM TIMP-2 (Figs. 6A and 8C). Using FLAG-tagged versions of MT2-MMP and MT1-MMP as controls ruled out the possibility that changes in activation patterns were due to the presence of the tag (Fig. 8C).

These data together with the competition experiments shown in Fig. 1 indicate that the TIMP-2-independent activation of MMP-2 by MT2-MMP is in part determined by the MT2-MMP hemopexin C domain. However, it is clear that the catalytic domain and/or linker must also influence TIMP dependence in MMP-2 activation, since activation by MT1catL/MT2CD-MMP remained TIMP-2-dependent. In addition, in the presence of TIMP-2, MMP-2 activation by MT2catL/MT1CD-MMP was less efficient at generating fully active MMP-2 compared with MT1catL/MT2CD-MMP or wild type MT1-MMP (Fig. 8C), suggesting that more complex domain interactions are involved.

DISCUSSION

The activation mechanisms of MMP-2 mediated by MT-MMPs are extremely complex. In this study we have shown that activation of proMMP-2 by MT2-MMP occurs in a TIMP-2-independent manner due in part to the greater propensity of the MT2-MMP versus MT1-MMP catalytic domain to make the initial first cleavage of the MMP-2 prodomain in the absence of TIMP-2 (Fig. 8C). Growing cells on or within collagen gels increased the amount of the intermediate form of MMP-2 generated by this first cleavage (Fig. 7A). In addition, the second cleavage of the MMP-2 prodomain to generate fully active MMP-2 was enhanced by a secreted soluble protein (Fig. 5, A and B) and was MT2-MMP and MMP-2 hemopexin C domain-dependent (Fig. 1B). We demonstrated that for MT2-MMP the catalytic domain and/or linker as well as the hemopexin C domain contribute in a non-catalytic manner to influence TIMP independence in MMP-2 activation (Fig. 8, B and C).

The data we generated using chimera proteins of MT2-MMP and MT1-MMP are consistent with studies from other groups (64–66). When MT1-MMP was expressed without a hemopexin C domain, MT1-MMP could still activate MMP-2 in a TIMP-2-dependent manner, indicating the influence of the catalytic domain in TIMP-2 dependence (66). This study however, contrasts to that of Itoh et al. (64) in which the same hemopexin C domain deletion construct of MT1-MMP did not activate MMP-2. Interestingly, both groups demonstrated the negative influence a hemopexin C domain exerts on MMP-2 activation, when they expressed a chimera of MT1-MMP containing the MT4-MMP hemopexin C domain (MT4-MMP cannot dimerize or activate MMP-2) and showed that the chimera did not activate MMP-2 (64, 66). A construct of MT2-MMP without a hemopexin C domain was expressed and shown to activate MMP-2, but the requirement for TIMP-2 was not investigated (66). In our chimeras we also exchanged the stalk, transmembrane domain, and cytoplasmic tail of MT1-MMP and MT2-MMP. The MT1-MMP cytoplasmic tail plays a critical role in MT1-MMP endocytosis and turnover, with its deletion resulting in increased MMP-2 activation (67). The cytoplasmic domains of MT1-MMP and MT3-MMP are functionally interchangeable (65). Given the high level of identity of the MT2-MMP and MT3-MMP cytoplasmic tails, we would predict that to be the case for MT2-MMP as well.

MT2-MMP and MT1-MMP have a high degree of similarity (73.9%), but the linker of MT2-MMP is almost double the length of the MT1-MMP linker (63 versus 32 amino acids). Potentially the increased length could influence MMP-2 activation through steric means, thus explaining the inability of the MT1catL/MT2CD-MMP chimera to fully activate MMP-2 without TIMP-2. Indeed, changing the glycosylation pattern (55) or composition (68) of the MT1-MMP linker has been shown to affect trimeric complex formation and autocatalytic processing, respectively. The most likely region of the catalytic domain to influence TIMP-2 dependence of MMP-2 activation is the MT-loop (69), created by the insertion of an additional 7–8 amino acids in the catalytic domain of MT-MMPs 1, 2, 3, and 5. The MT-loop of MT1-MMP forms a pocket that can interact with the AB-loop of TIMP-2 (69) or form contacts with substrates such as MMP-2. Mutation of the eight N-terminal amino acids of the MT1-MMP MT-loop affects TIMP-2 binding and MMP-2 activation (70). The
sequence of the MT-loop is not completely conserved between MT-MMPs 1–3. Interestingly, Miyamori et al. (71) reported that human MT2-MMP was defective in MMP-2 activation and that mutations of residues Pro\textsuperscript{183} and Glu\textsuperscript{185} within the human MT2-MMP MT-loop to Ser\textsuperscript{183} and Asp\textsuperscript{185} found in mouse MT2-MMP conferred the ability to activate MMP-2. Unfortunately, the results of this study are unclear since these substitutions were not made in wild type human MT2-MMP but in a mouse/human chimera. Also, confusion arises as they and others had previously reported that human MT2-MMP activates MMP-2 (47, 72–74). Although further verification is required, it seems likely that the MT-loop and linker of MT2-MMP could engender an enhanced ability to initiate cleavage in the MMP-2 prodomain in the absence of TIMP-2.

As first shown using *Timp2<sup>−/−</sup>* cells, in MT1-MMP-mediated MMP-2 activation the second cleavage to generate fully active MMP-2 is driven by TIMP-2 through localization of MMP-2 at the cell surface (16, 42). This does not occur for MT2-MMP-mediated MMP-2 activation. Here, the addition of a soluble factor present in concentrated conditioned medium from *Timp2<sup>−/−</sup>* cells specifically and rapidly enhanced the generation of fully active MMP-2 by MT2-MMP. We showed that *Timp2<sup>−/−</sup>* cells express TIMP-3 (16), but the addition of exogenous TIMP-3 to MT2-MMP-expressing cells did not enhance MMP-2 activation (Fig. 6A). The MT2-MMP activation pathway of MMP-2 is, therefore, distinct not only to that of MT1-MMP but also to that of MT3-MMP. MT3-MMP activates MMP-2 in a TIMP-2-dependent manner, but TIMP-3 was also reported to support activation (17).

Type I collagen is expressed by *Timp2<sup>−/−</sup>* cells and has been shown to promote MMP-2 activation by MT1-MMP in the presence of TIMP-2 (54, 61, 62) after cell surface localization of proMMP-2 via the fibronectin type II modules (75). We found that growing MT2-MMP-expressing cells on or within type I collagen gels primarily enhanced the first cleavage step to generate more intermediate rather than promote the generation of fully active MMP-2 (Fig. 7A). We demonstrated that MT2-MMP has collagenolytic activity toward native type I collagen (Fig. 7C). In this respect MT2-MMP also differs from MT3-MMP, which does not cleave type I collagen (13). However, cleavage of collagen by MT2-MMP was ~100-fold less efficient than MT1-MMP. The interaction of the MT2-MMP hemopexin C domain with type I collagen may be much weaker than that of MT1-MMP (54, 76), which could weaken the clustering or stabilizing effect that collagen might exert on MT2-MMP. With the increased propensity of the MT2-MMP catalytic domain to initiate activation of MMP-2, a weaker interaction with collagen might be sufficient to promote the first cleavage but not the final MMP-2 intermolecular cleavage. We have identified a role for collagen in MT2-MMP mediated MMP-2 activation; however, the identity of the soluble enhancer remains under investigation.

Although the second activation step, which is enhanced by the soluble factor in conditioned medium, is MT2-MMP and MMP-2 hemopexin C domain-dependent, we showed that the soluble factor does not bind either hemopexin C domain alone. Moreover, it does not function as a bridging molecule to promote trimolecular complex formation (data not shown). Perhaps instead the soluble factor serves to increase the local concentration of active MT2-MMP through other means, such as reducing turnover via clathrin-dependent and independent mechanisms, blocking auto-processing, or by promoting dimerization of MT2-MMP. These processes have been well documented for MT1-MMP (for review, see Ref. 77) but not addressed for MT2-MMP. Alternatively, the soluble factor may function to indirectly promote binding of MMP-2 to a cell-surface receptor.

Interestingly, we found that MT2-MMP, at least in a soluble form, can form a trimolecular complex with TIMP-2 and MMP-2, and yet TIMP-2 does not enhance MMP-2 activation. In the absence of TIMP-2, MMP-2 was detected at the cell surface of *Timp2<sup>−/−</sup>* cells expressing MT2-MMP but not MT1-MMP or cells transfected with vector alone (16). We, therefore, proposed that MT2-MMP might (i) form a receptor for MMP-2 (Fig. 2A) or (ii) together with a second cell surface molecule form a receptor for MMP-2 (Fig. 2, B and C). We have now shown that MT2-MMP alone does not form a receptor for MMP-2 (Fig. 3). We also showed that integrin binding via the MT2-MMP hemopexin C domain RGD does not play a role in MMP-2 activation (Fig. 4). The receptor for MMP-2 in the TIMP-independent MT2-MMP pathway, with the MT1-MMP activation pathway of MMP-13 (78), remains elusive.

Studies of MT1-MMP knock-out mice provided in vivo evidence for alternative MT1-MMP-independent MMP-2 activation pathways (79). We have shown that in situations characterized by low TIMP-2 concentrations (<10 nM), MT2-MMP can rapidly activate MMP-2 to its fully active form (16). This activation pathway appears to be under the control of a soluble factor found in conditioned medium, as limiting the expression of the factor controls the amount of active MMP-2 generated. Dissection of this complex MMP-2 activation pathway has revealed that multiple domains of MT2-MMP likely contribute to the process. In this study we have identified the critical role of the MT2-MMP hemopexin C domain and the high propensity for the catalytic domain to initiate MMP-2 activation in the absence of TIMP-2. With an increasing number of studies now highlighting the importance of MT2-MMP in many physiological and pathological situations (26, 30–33), understanding how MT2-MMP functions and elucidating control mechanisms is of key importance.

**Acknowledgments**—We thank Michael McCall, Yili Wang, and Andrea Connor for technical assistance and Dr. Oded Kleifeld for help with the MT2-MMP hemopexin domain model.

**REFERENCES**

1. Itoh, Y., and Seiki, M. (2006) *J. Cell. Physiol.* **206**, 1–8
2. Nagase, H., and Woessner, J. F., Jr. (1999) *J. Biol. Chem.* **274**, 21491–21494
3. Egeblad, M., and Werb, Z. (2002) *Nat. Rev. Cancer* **2**, 161–174
4. Gomis-Ruth, F. X. (2003) *Mol. Biotechnol.* **24**, 157–202
5. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370**, 61–65
6. Will, H., and Hinzmann, B. (1995) *Eur. J. Biochem.* **231**, 602–608
7. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995) *J. Biol. Chem.* **270**, 23013–23020
TIMP-independent Activation of MMP-2 by MT2-MMP

8. Puente, X., Pendas, A., Llanos, E., Velasco, G., and Lopez-Otín, C. (1996) 
Cancer Res. 56, 944–949

9. Itoh, Y., Kajita, M., Kinoshita, T., Imai, K., Okada, Y., Stevenson, W. G. S., and Seiki, M. (1999) 
J. Biol. Chem. 274, 34260–34266

10. Kojima, S., Itoh, Y., Matsumoto, S., Masuho, Y., and Seiki, M. (2000) 
FEBS Lett. 480, 142–146

11. Pei, D. (1999) 
Cell Res. 9, 291–303

12. d’Ortho, M. P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, I., Smith, B., Timpl, R., Zardi, L., 
and Murphy, G. (1997) 
Eur. J. Biochem. 250, 751–757

13. Ogiwara, K., Takano, N., Shinohara, M., Murakami, M., and Takahashi, T. (1998) 
J. Cell. Physiol. 175, 50–56

14. Shimada, T., Nakamura, H., Ohuchi, E., Fujii, Y., Murakami, Y., Sato, H., Seiki, M., and Okada, Y. (1999) 
J. Biol. Chem. 262, 907–914

15. English, W. R., Puente, X. S., Freije, J. M., Knauper, V., Amour, A., Merryweather, A., Lopez-Otín, C., 
and Murphy, G. (2000) 
J. Biol. Chem. 275, 10406–10455

16. Wang, Y., Johnson, A. R., Ye, Q.-Z., and Dyer, R. D. (1999) 
Biochimie (Paris) 81, 329–342

17. Sato, H., Takino, T., and Miyahori, M. (2005) 
Cancer Sci. 96, 212–217

18. Hotary, K. B., Yana, I., Sabeh, F., Li, X. Y., Takino, T., Seiki, M., and Okada, Y. (1999) 
Proc. Natl. Acad. Sci. U. S. A. 94, 5155–5160

19. Ueno, H., Nakamura, H., Iida, E., Fujimoto, N., Yamashita, J., Sato, H., Seiki, M., and Okada, Y. (1999) 
Am. J. Pathol. 154, 417–428

20. Zhang, J., Sarkar, S., and Yong, V. W. (2005) 
Cell Res. 15, 142–146

21. Sounni, N. E., and Noel, A. (2005) 
J. Biol. Chem. 280, 2069–2077

22. Sato, H., Takino, T., Iwata, K., Inoue, M., and Seiki, M. (1997) 
Cell Res. 7, 751–757

23. Lafleur, M. A., Mercuri, F. A., Ruangpanit, N., Seiki, M., Sato, H., and Namiki, M. (1998) 
J. Cell. Physiol. 174, 212–217

24. Lampert, K., Machein, U., Machein, R. M., Conca, W., Peter, H. H., and Volk, B. (1998) 
Am. J. Pathol. 153, 429–437

25. Nordkvist, B., Sato, H., Takino, T., Nakamura, H., Iida, E., Fujimoto, N., Yamashita, J., Sato, H., Seiki, M., 
and Okada, Y. (1999) 
Am. J. Pathol. 154, 417–428

26. Zhang, J., Sarkar, S., and Yong, V. W. (2005) 
Carcinogenesis 26, 2069–2077
TIMP-independent Activation of MMP-2 by MT2-MMP

73. Butler, G. S., Will, H., Atkinson, S. J., and Murphy, G. (1997) *Eur. J. Biochem.* **244**, 653–657
74. Kolkenbrock, H., Hecker-Kia, A., Orgel, D., Ulbrich, N., and Will, H. (1997) *Biol. Chem.* **378**, 71–76
75. Steffensen, B., Bigg, H. F., and Overall, C. M. (1998) *J. Biol. Chem.* **273**, 20622–20628
76. Tam, E. M., Moore, T. R., Butler, G. S., and Overall, C. M. (2004) *J. Biol. Chem.* **279**, 43336–43344
77. Osenkowski, P., Toth, M., and Fridman, R. (2004) *J. Cell. Physiol.* **200**, 2–10
78. Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G. (1996) *J. Biol. Chem.* **271**, 17124–17131
79. Zhou, Z., Apte, S. S., Soininen, R., Cao, R., Baaklini, G. Y., Rauser, R. W., Wang, J., Cao, Y., and Tryggvason, K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4052–4057