Analysis of tissue inhibitor of metalloproteinases-2 effect on pro-matrix metalloproteinase-2 activation by membrane-type 1 matrix metalloproteinase using baculovirus/insect-cell expression system

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Membrane-type 1 matrix metalloproteinase (MT1-MMP; MMP14) is known to activate pro-matrix metalloproteinase-2 (pro-MMP-2; progelatinase A) on the cell surface. To analyse the tissue inhibitor of metalloproteinases-2 (TIMP-2) effect on activation of pro-MMP-2 by MT1-MMP, we have expressed the full-size MT1-MMP (fMT1-MMP) and a transmembrane (TM)-domain-deleted soluble MT1-MMP (sMT1-MMP) in the baculovirus/Sf9 (*Spodoptera frugiperda* 9) insect-cell system, where neither endogenous gelatinolytic MMPs nor TIMP-2 are expressed. Both fMT1-MMP and sMT1-MMP expressed in the expression system were found not to contain the pro-domain and were able to activate the TIMP-2-free pro-MMP-2. Both in the insect cells and *in vitro*, activation of pro-MMP-2 by fMT1-MMP was enhanced at low concentrations of TIMP-2 and inhibited by its higher concentrations. The maximal enhancing effect was detected at 0.05 molar fraction of TIMP-2/fMT1-MMP. In contrast, activation of pro-MMP-2 by sMT1-MMP

was dose-dependently inhibited by TIMP-2. These results demonstrate that the TM domain of MT1-MMP is not required for the ability to activate pro-MMP-2, but is required for the enhancing effect of TIMP-2 on pro-MMP-2 activation by recruiting pro-MMP-2 to the MT1-MMP–TIMP-2 complex as a cell-surface pro-MMP-2 receptor. Moreover, our data strongly suggest that the pro-domain of MT1-MMP is not required for the TIMP-2-mediated enhancing effect on pro-MMP-2 activation. In addition, the pro-MMP-2 in the MT1-MMP–TIMP-2–pro-MMP-2 ternary complex was not activated without external activator, but readily by addition of sMT1-MMP. This result demonstrates that MT1-MMP free of TIMP-2 would be the enzyme responsible for activation of the pro-MMP-2 in the ternary complex under physiological conditions.

Key words: pro-domain, pro-MMP-2 receptor, ternary complex, transmembrane domain.

INTRODUCTION

Matrix metalloproteinases (MMPs) are an important group of zinc-containing enzymes responsible for degradation of extracellular-matrix components in normal processes such as embryonic development and wound healing and in pathological processes such as metastasis of cancer and arthritis [1–4]. MMPs commonly have pro-, zinc-binding catalytic, hinge and haemopexin-like domains. All MMPs, except membrane-type MMPs (MT-MMPs) which are anchored on the plasma membrane [5–7], are secreted as inactive precursors whose latency is derived from the N-terminal pro-domain [8]. Processing of the pro-domain by proteolytic cascades is thus an important step for regulation of MMP activity in the extracellular matrix [2,9].

Matrix metalloproteinase-2 (MMP-2; 72 kDa gelatinase; 72 kDa type IV collagenase; gelatinase A) can degrade various components of the extracellular matrix, including type IV collagen, which is a major component of basement membranes, as well as collagen types V, VII, and X, fibronectin, elastin and gelatin [3,10]. It is known that pro-MMP-2 can be activated by other soluble MMPs, such as MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1) [11,12], by the urokinase-type plasminogen activator and plasmin system [13] and by autoactivation at high concentrations [14,15]. Also, it has been reported that pro-MMP-2 can be activated by the invasive tumour-cell surface [16–18].

As a specific activator of pro-MMP-2 on cell surface, Sato et al. [5] reported MT1-MMP (MMP-14), which is anchored on the cell surface through its transmembrane (TM) domain. Pro-MT1-MMP is likely processed at a RRKR sequence between the pro- and catalytic domains by a pro-protein convertase furin [6,7]. Although tissue inhibitor of metalloproteinases-2 (TIMP-2) is known to bind to the mature MMP-2, pro-MMP-2, and MT1-MMP at 1:1 stoichiometric ratio and inhibit their catalytic activities [19–22], TIMP-2 at low concentrations enhances activation of pro-MMP-2 by MT1-MMP [21,24]. The enhanced effect of TIMP-2 on pro-MMP-2 activation by MT1-MMP appears to involve formation of the MT1-MMP–TIMP-2 complex between the N-terminal domain of TIMP-2 and the catalytic domain of MT1-MMP [7,21,24]. The TM domain of MT1-MMP would thus be required for recruiting pro-MMP-2 to the cell surface via the MT1-MMP–TIMP-2 complex as a pro-MMP-2 receptor, but should not affect catalytic activity of MT1-MMP. Many studies performed *in vitro* showed that soluble forms of MT1-MMPs were able to activate pro-MMP-2 [6,7,25–27]. However, studies using transfection into COS-1 cells demonstrated that the TM-deleted MT1-MMP lost the ability to activate pro-MMP-2 [28]. In addition, it has been proposed that the pro-domain of MT1-MMP is also required for binding of TIMP-2 in its membrane-anchored form [23,29]. The C-terminal domain of pro-MMP-2 then binds to the C-terminal domain of TIMP-2, resulting in a ternary complex [21]. It is suggested that

Abbreviations used: MT1-, membrane-type 1; fMT1-, full-size MT1-; sMT1-, soluble MT1-; MMP, matrix metalloproteinase; m.o.i., multiplicity of infection; TBS, Tris-buffered saline; TIMP, tissue inhibitor of metalloproteinases; TM, transmembrane.

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the pro-MMP-2 within the ternary complex may be activated by the TIMP-2-free MT1-MMP near the complex and processed to the mature form by autolytic cleavage [24,25].

We previously demonstrated that a baculovirus/*Spodoptera frugiperda* 9 (Sf9) insect-cell system expresses neither gelatinolytic MMPs nor TIMP-2 [30]. In addition, the system was reported to be processed at the furin cleavage site of expressed proteins by endogenous furin [31]. We have expressed full-size MT1-MMP (fMT1-MMP), TM-deleted soluble MT1-MMP (sMT1-MMP) and TIMP-2 using the baculovirus/Sf9 insect-cell system. Through co-infection of these viruses into Sf9 cells as well as using the purified proteins, we have investigated whether the TM domain of MT1-MMP is required for the ability to activate pro-MMP-2 and whether the TM domain or the pro-domain is required for the TIMP-2 effect on pro-MMP-2 activation. In addition, we have examined how the pro-MMP-2 in the ternary complex is activated.

EXPERIMENTAL

Cell culture

Sf9 cells, derived from *Spodoptera frugiperda*, were cultured in the TNM-FH ('*Trichoplusia ni* Medium-Formulation Hink') insect medium (Sigma, St. Louis, MO, U.S.A.) containing 100 units/0.1 mg penicillin/streptomycin (Sigma) and 0.25 µg/ml fungizone (GIBCO BRL, Gaithersburg, MD, U.S.A.) in the presence of 10% (v/v) fetal-bovine serum (GIBCO BRL) at 27 °C [32]. Human fibrosarcoma HT-1080 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL) containing 100 units/0.1 mg penicillin/streptomycin and 2 mM glutamine in the presence of 10% fetal-bovine serum at 37 °C [33].

Construction of plasmids

The full-length cDNA encoding pro-MT1-MMP (Met¹-Val⁵⁸²) was obtained by PCR amplification from a human fibroblast cDNA library [34] with *Pfu* polymerase; a 5' primer with an *EcoRI* site, 5'-GAATTCAGACCCGGTGGTCTCGG-3' (non-MT1-MMP cDNA sequences, italic; restriction-enzyme site, underlined) and a 3' primer with a *PstI* site, 5'-CTGCAGAGGCAAAGTCCTTGTTG-3'. The PCR product containing the full-length cDNA was subcloned into a *HincII* site of pGEM-3Zf(+) vector (Promega, Madison, WI, U.S.A.), and the sequence of the resultant clone (pGEM-3Zf-MT1-MMP) was confirmed to avoid PCR errors. The cDNA encoding the TM-deleted sMT1-MMP (Met¹-Ser⁵³⁸) with hexahistidine tag was amplified by PCR of pGEM-3Zf-MT1-MMP using a T7 primer upstream of the cDNA and a 3' primer with hexahistidine codons and a termination codon (5'-ATCAATGATGATGATGATGATGGCTCACCGCCCCGCCGCC-3') with *Pfu* polymerase. The PCR product containing the sMT1-MMP cDNA was cleaved at an *EcoRI* site introduced at the 5'-untranslated region during PCR of the full-length MT1-MMP cDNA. The large fragment with a half *EcoRI* site at the 5' end and a blunt end at the 3' end was cloned into *EcoRI*-*HincII* sites of pGEM-3Zf(+) vector and sequenced. The 1.8 kb *XbaI*-*PstI* fragment of MT1-MMP cDNA and the 1.7 kb *XbaI*-*PstI* fragment of sMT1-MMP cDNA were subcloned into the unique *NheI* and *PstI* sites of a baculoviral transfer vector, pBlueBac4 (Invitrogen, Carlsbad, CA, U.S.A.).

The 5'-partial human TIMP-2 cDNA corresponding to nucleotides 262-352 [9] was made as a synthetic adapter which contains a half *HindIII* site at the 5' end and a half *PstI* site at the 3' end. The 3'-partial TIMP-2 cDNA corresponding to nucleotides 343-962 [9] was obtained by PCR from the human fibroblast cDNA library using a 5' primer containing a *PstI* site

(5'-GACGCCTGCAGCTGCTCCCCGGGTGC-3') and a 3' primer (5'-AGTTGGCCACAGGGGCGTTG-3') with *Pfu* polymerase. The PCR product was cleaved at the unique *PstI* site and the large fragment with a half *PstI* site at the 5' end and a blunt end at the 3' end was eluted. The *HindIII*-*PstI* 5'-cDNA adapter and the *PstI*-blunt end 3'-cDNA fragment were co-ligated between the *HindIII* and *SmaI* sites of pUC19. The resultant plasmid (pUC19-TIMP-2) was sequenced to avoid PCR errors. The 0.7 kb *HindIII*-*EcoRI* fragment of pUC19-TIMP-2 was subcloned into the *HindIII* and *EcoRI* sites of pcDNA3 vector (Invitrogen). The 0.7 kb *NcoI*-*XhoI* fragment of pcDNA3-TIMP-2 was subcloned into the *NcoI* and *Sall* sites of pBlueBac4 (Invitrogen).

Production of recombinant baculoviruses

Procedures for transfection, plaque isolation and multiplication of the recombinant baculoviruses to express fMT1-MMP, sMT1-MMP and TIMP-2 were followed as described in the Bac-N-Blue Transfection kit manual (Invitrogen). Recovery of the culture medium from the Sf9 cells infected with pro-MMP-2 baculovirus and purification of the recombinant pro-MMP-2 was as described previously [30].

Western-blot analysis

Sf9 cells were infected with 1 multiplicity of infection (m.o.i.) of the wild-type, fMT1-MMP, sMT1-MMP or TIMP-2 baculovirus in the serum-free Sf9 medium. At 4 days post-infection, the culture medium was collected at 4 °C, and cell debris was removed by centrifugation for 5 min at 1000 g. Cells were scraped into PBS, centrifuged for 5 min at 1000 g, solubilized by boiling for 5 min in SDS/gel sample buffer [2% (w/v) SDS/60 mM Tris/HCl (pH 6.8)/0.01% Bromophenol Blue/10% (v/v) glycerol/100 mM β-mercaptoethanol]. The medium samples and cell lysate samples were separated on SDS/10 or 12.5%-polyacrylamide gel under reducing conditions. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) at 150 mA for 3 h. The membrane was blocked using Tris-buffered saline [TBS; 20 mM Tris/HCl (pH 7.6)/137 mM NaCl] containing 5% BSA and 0.1% Tween 20 for 1 h. Then the membrane was incubated with a mouse monoclonal antibody recognizing the catalytic domain (amino acid residues 160-173) of the human MT1-MMP (catalogue no. 1M39L; Calbiochem, La Jolla, CA, U.S.A.), a rabbit polyclonal antibody recognizing the pro-domain of the human MT1-MMP (catalogue no. AB8101; Chemicon International, Temecula, CA, U.S.A.), or a mouse monoclonal antibody recognizing the human TIMP-2 (catalogue no. 1M11L; Calbiochem) for 2 h at room temperature. The membrane was washed with TBS containing 0.1% Tween 20 and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse IgG antibody (catalogue no. A-6782; Sigma). The positive signals were detected using ECL® (enhanced chemiluminescence) Western-blotting detection reagents (Amersham).

Processing of pro-MMP-2 by MT1-MMPs in Sf9 insect cells

Either the culture medium of the HT-1080 cells or the culture medium of the Sf9 cells infected with the pro-MMP-2 baculovirus was used as pro-MMP-2 source. To collect the HT-1080 culture medium containing pro-MMP-2, HT-1080 cells were incubated for 24 h in serum-free medium. The culture medium was recovered by centrifugation for 5 min at 1000 g and replaced with the serum-free Sf9 medium by ultrafiltration using Centriprep-30 (Amicon, Bedford, MA, U.S.A.). Alternatively, to

collect the Sf9 culture medium containing the recombinant pro-MMP-2, Sf9 cells were infected with 1 m.o.i. of pro-MMP-2 baculovirus [30] in the serum-free medium, and the medium at 4 days post-infection was collected by centrifugation for 5 min at 1000 g. The culture medium was overlaid on the Sf9 cells infected with 1 m.o.i. of fMT1-MMP or sMT1-MMP baculovirus for 1 h and samples were withdrawn at various time intervals.

To analyse the effects of TIMP-2 on pro-MMP-2 activation by MT1-MMPs, the medium containing pro-MMP-2 was overlaid on the Sf9 cells co-infected with 1 m.o.i. of fMT1-MMP or sMT1-MMP baculovirus and various m.o.i. of TIMP-2 baculovirus for 1 h. The culture medium was recovered at 4 days after infection. Activation of pro-MMP-2 was examined by gelatin zymography by the method described previously [30].

Purification of the recombinant sMT1-MMP

All procedures were performed at 0–4 °C unless specified otherwise. The protein fraction containing sMT1-MMP was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ up to 65% saturation to the culture medium of Sf9 cells at 4 days after infection with 1 m.o.i. of sMT1-MMP baculovirus. The pellet was collected by centrifugation for 20 min at 12000 g and dialysed against MT1-MMP assay buffer [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM CaCl_2 /100 μM ZnCl_2 /0.02% Na_3V /0.025% Brij 35]. The protein solution was loaded on an Ni^{2+} -nitrilotriacetate affinity column (Qiagen, Valencia, CA, U.S.A.) equilibrated with the assay buffer, washed with the same buffer containing 40 mM imidazole, and eluted with assay buffer containing 300 mM imidazole. The eluate was tested by Western-blot analysis using a mouse monoclonal antibody recognizing the catalytic domain of the human MT1-MMP for the presence of sMT1-MMP. The positive fractions were pooled and dialysed twice against the MT1-MMP assay buffer. The concentration of protein during the purification process was quantified with a Micro BCA (bicinchoninic acid) Protein Assay Reagent kit (Pierce, Rockford, IL, U.S.A.). The catalytic activity of sMT1-MMP was analysed by peptide cleavage assay using a quenched fluorescent peptide (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH₂ (Bachem, Torrance, CA, U.S.A.) as a substrate [35]. The samples were incubated in 100 μl of the MT1-MMP assay buffer containing 1 μM of the peptide for 30 min at 25 °C. The reaction was stopped by the addition of 0.1 M (final concn.) sodium acetate, pH 4.0. The fluorescence was measured with a Kontron (Milan, Italy) SFM 25 fluorimeter at an excitation wavelength of 328 nm and an emission wavelength of 393 nm.

Preparation of crude membrane fraction containing MT1-MMP

Crude membrane fractions were prepared by the method of Parker et al. [36] with minor modification. All procedures were performed at 0–4 °C. The Sf9 cells at 4 days after infection with 1 m.o.i. of fMT1-MMP or wild-type baculovirus were recovered by centrifugation for 5 min at 1000 g and were resuspended at 10^7 cells/ml in 5 mM Tris/HCl, pH 7.5, containing 100 μl /ml protease inhibitor cocktail (catalogue no. P-8340; Sigma) and 1 mM PMSF. The suspension was homogenized by 20 strokes in a homogenizer (catalogue 08-414-16A; Wheaton, Millville, NJ, U.S.A.) and centrifuged for 20 min at 25000 g. The pellet was washed and resuspended with the MT1-MMP assay buffer, and the crude membrane fractions were stored at –70 °C in small aliquots. Amounts of catalytically active fMT1-MMP on the crude membrane fraction were determined by the peptide-cleavage assay described above using the purified sMT1-MMP as a control.

Processing of pro-MMP-2 by MT1-MMPs *in vitro*

A 5 ng portion of the recombinant pro-MMP-2 was incubated with the crude membrane fraction containing fMT1-MMP of which the catalytic activity is equivalent to 3 ng of sMT1-MMP (corresponding to 3.2 ng of fMT1-MMP) or 3 ng of sMT1-MMP and various amounts of TIMP-2 for 6 h at 37 °C in 20 μl of the MT1-MMP assay buffer. The reaction products were analysed by gelatin zymography.

Formation of ternary complex and processing of pro-MMP-2 in the complex

All procedures were performed at 0 °C unless otherwise mentioned. To form the MT1-MMP–TIMP-2–pro-MMP-2 ternary complex, the crude membrane fraction containing 3.2 ng of fMT1-MMP (estimated by catalytic activity) was incubated with 30 ng of TIMP-2 in 20 μl of the MT1-MMP assay buffer for 30 min. Excess TIMP-2 was washed out twice with the assay buffer by centrifugation for 5 min at 25000 g. The crude membrane fraction containing the fMT1-MMP–TIMP-2 complex was then incubated with 15 ng of pro-MMP-2 in 20 μl of the assay buffer. Excess pro-MMP-2 was washed out as described above. The crude membrane fraction containing the ternary complex was resuspended in 10 μl of the assay buffer. The crude membrane fraction was incubated in a 15 μl reaction volume at 37 °C for various time intervals in the absence or the presence of 3 ng of the recombinant sMT1-MMP as an external activator. The reaction products of MMP-2 were examined by gelatin zymography.

RESULTS

Expression of the recombinant fMT1-MMP, sMT1-MMP and TIMP-2

We have expressed the full-size human fMT1-MMP, a TM-deleted sMT1-MMP and human TIMP-2 in a baculovirus/Sf9 insect-cell system. To detect the expressed recombinant proteins, cell lysate and medium samples from the Sf9 cells infected with fMT1-MMP, sMT1-MMP, or TIMP-2 baculovirus were examined by Western-blot analysis (Figure 1). Using an antibody against the catalytic domain of MT1-MMP, recombinant fMT1-MMP was detected as a 66 kDa band only in cell lysate, and the sMT1-MMP was detected as 62.5 kDa and 62 kDa bands in cell lysate and as a 62 kDa band in medium (Figure 1A, top). The sizes of the expressed MT-MMPs were slightly larger than the mature MT1-MMPs expressed in mammalian cells. When the same blot was allowed to react with a polyclonal antibody against the pro-domain of MT1-MMP, none of these bands except a positive control were detected (Figure 1A, bottom). We thus confirmed that both the fMT1-MMP in the cell lysate and the sMT1-MMP in the medium did not contain their pro-domains. In the cell lysate sample of the Sf9 cells infected with sMT1-MMP virus, a 62.5 kDa band was detected in addition to a 62 kDa band that is the size of the secreted sMT1-MMP. In experiments using the COS-1 expression system, others reported that the TM-deleted MT1-MMPs in cell lysates which were 3–6 kDa larger than those in media would be the TM-deleted pro-MT1-MMP [7,23]. Because the 62.5 kDa form found in the cell lysate was a little larger than the secreted sMT1-MMP and was not detected with the pro-domain antibody, the size variation may have resulted from differences in post-translational modification such as glycosylation.

The recombinant TIMP-2 was detected as a 23 kDa band similar to the TIMP-2 from HT-1080 cells (Figure 1B). The quenched-fluorescent-peptide cleavage assay demonstrated that

Figure 1 Western-blot analyses of fMT1-MMP, sMT1-MMP and TIMP-2 expressed in Sf9 insect cells

(A) Western blot analyses were done using a mouse monoclonal antibody recognizing the catalytic domain of the human MT1-MMP (top panel) and using a rabbit polyclonal antibody recognizing the pro-domain of the human MT1-MMP (bottom panel) respectively. Sf9 cells were infected with 1 m.o.i. of wild-type AcMNPV baculovirus ('Wild Type'), fMT1-MMP baculovirus (fMT1-MMP) or sMT1-MMP baculovirus (sMT1-MMP). At 4 days after infection, medium samples (lanes M) and cell layer samples (lanes C) were prepared as described in the Experimental section. Samples (10 μ l) of the medium samples and the cell-layer samples corresponding to the fraction of the medium samples were subjected to SDS/PAGE and blotted on to nitrocellulose membrane. The pcMT1-MMP containing the pro-, catalytic and hinge domains of the human MT1-MMP (lane pcMT1-MMP), which was expressed in *E. coli*, were used as a positive control. The numbers on the left show the positions of molecular size markers. (B) Western-blot analysis of the culture medium of Sf9 cells infected with TIMP-2 baculovirus was performed using a mouse monoclonal antibody recognizing the human TIMP-2. Samples (10 μ l) of the culture medium of the TIMP-2 virus-infected Sf9 cells (lane TIMP-2) and 10 μ l of the 20 $\times$ concentrated culture medium of HT-1080 cells (lane HT-1080), which are known to express 22-kDa TIMP-2, were applied to the gel.

Figure 2 Activation of pro-MMP-2 expressed in the HT-1080 cells by the Sf9 cells infected with the fMT1-MMP and sMT1-MMP

Sf9 cells were infected with 1 m.o.i. of wild-type virus (A), fMT1-MMP virus (B) or sMT1-MMP virus (C). At 1 h after infection, the medium was replaced with the HT-1080 culture medium containing 68 kDa pro-MMP-2 and 88 kDa pro-MMP-9. The medium samples were withdrawn at given time intervals and analysed by gelatin zymography. The HT-1080 culture medium was used as a molecular marker (lane M). The numbers on the left represent molecular masses in kDa.

the recombinant TIMP-2 inhibited catalytic activity of the MMP-2 activated by 4-aminophenylmercuric acetate (results not shown).

Activation of pro-MMP-2 by fMT1-MMP and sMT1-MMP

To examine the role of the TM domain of MT1-MMP on activation of pro-MMP-2 in the Sf9 insect-cell system, we analysed processing of gelatinolytic MMPs in the HT-1080 culture medium overlaid on the Sf9 cells which had been infected

Figure 3 Activation of pro-MMP-2 expressed in Sf9 cells by the Sf9 cells infected with the fMT1-MMP and sMT1-MMP

Sf9 cells were infected with 1 m.o.i. of fMT1-MMP virus (A) or sMT1-MMP virus (B). At 1 h after infection, the medium was replaced with the culture medium of Sf9 cells at 4 days after infection with 1 m.o.i. of pro-MMP-2 baculovirus. The medium samples were withdrawn at given time intervals and analysed by the same procedures as those described in Figure 2.

with wild-type, fMT1-MMP or sMT1-MMP baculovirus. Western-blot analysis showed that sMT1-MMP started to be detected in the culture medium at 2 days after infection and reached 1 $\mu\text{g}/\text{ml}$ at 4 days after infection. Although it was difficult to measure the amount of fMT1-MMP on the cell surface, fMT1-MMP also started to be detected in the lysate of the Sf9 cells at 2 days after infection. In addition, we confirmed that the amount of fMT1-MMP in the lysate was very similar to the total amounts of sMT1-MMP in the culture medium and the lysate at each given period after infection. Pro-MMP-2 in the HT-1080 culture medium was not processed during 4 days after infection of the Sf9 cells with wild-type baculovirus (Figure 2A), demonstrating no MT1-MMP activity in Sf9 cells. In the Sf9 cells infected with fMT1-MMP baculovirus, pro-MMP-2 was processed to the 64 kDa intermediate form and 62 kDa mature form from 2 days after infection (Figure 2B). However, in the Sf9 cells infected with sMT1-MMP baculovirus, pro-MMP-2 was not processed to the 64 kDa intermediate form until 3 days after infection (Figure 2C). These results suggest that activation of pro-MMP-2 in the HT-1080 culture medium can occur at low concentrations of fMT1-MMP but requires higher concentrations of sMT1-MMP.

Pro-MMP-9 in the overlaid HT-1080 culture medium was processed to the 80 kDa activated form in the Sf9 cells infected with either fMT1-MMP or sMT1-MMP (Figures 2B and 2C). The amount of the activated MMP-9 was correlated with that of the activated MMP-2. Considering that pro-MMP-9 was reported to be a substrate for MMP-2 [37], it is likely that the

activated MMP-9 was processed by MMP-2 species activated by MT1-MMP.

It has been reported that activation of pro-MMP-2 by MT1-MMP can be enhanced by TIMP-2 [21,24]. As shown in Figure 1(B), HT-1080 cells secrete low levels of TIMP-2: approx. 0.1 $\mu\text{g}/\text{ml}$ at 1 day after changing with serum-free medium. Thus it is likely that TIMP-2 present in the HT-1080 culture medium may facilitate activation of pro-MMP-2 by fMT1-MMP, but not by sMT1-MMP. To test this possibility, we examined activation of the TIMP-2-free pro-MMP-2 by either fMT1-MMP or sMT1-MMP in the Sf9 cell system. As a source of the TIMP-2-free pro-MMP-2, the culture medium of the Sf9 cells infected with pro-MMP-2 baculovirus was used [30]. Western-blot analysis showed that the amount of fMT1-MMP in the cell lysate was very similar to the total amounts of sMT1-MMP in the culture medium and the lysate at each given period after infection. Interestingly, activation of pro-MMP-2 was detected at 2 days after infection in the Sf9 cells infected both with fMT1-MMP virus and with sMT1-MMP virus (Figure 3). Amounts of the species of pro-MMP-2 activated either by fMT1-MMP or by sMT1-MMP were very similar under TIMP-2-free conditions (Figure 3). These results demonstrate that the TM domain of MT1-MMP does not affect activation of pro-MMP-2 in the absence of TIMP-2.

TIMP-2 effect on pro-MMP-2 activation by fMT1-MMP and sMT1-MMP in Sf9-cell system

Our results have shown that the TM domain of MT1-MMP is not essential for activation of pro-MMP-2 by MT1-MMP in the absence of TIMP-2 (Figure 3). However, in the presence of small amounts of TIMP-2, fMT1-MMP activates pro-MMP-2 more efficiently than the TM-deleted sMT1-MMP does (Figure 2). In order to understand the role of the TM domain of MT1-MMP on pro-MMP-2 activation by MT1-MMP in function of TIMP-2 concentration, we have examined activation of pro-MMP-2 with increasing ratios of TIMP-2 relative to either fMT1-MMP or sMT1-MMP in the Sf9 cell system. Increasing ratios of TIMP-2 to MT1-MMPs were achieved by co-infection of Sf9 cells with a fixed m.o.i. of either fMT1-MMP or sMT1-MMP baculovirus while increasing m.o.i. of TIMP-2 baculovirus. Then, the culture medium from the Sf9 cells infected with pro-MMP-2 baculovirus was overlaid to provide the TIMP-2-free pro-MMP-2. In the experiment using fMT-MMP baculovirus, activation of pro-MMP-2 by fMT1-MMP was enhanced at 0.3 to 0.6 m.o.i. of TIMP-2 baculovirus, compared with that of no TIMP-2 baculovirus, and sharply decreased at 6 m.o.i. (Figure 4A). In contrast, when using sMT1-MMP baculovirus, activation of pro-MMP-2 was slowly decreased with increasing m.o.i. of TIMP-2 baculovirus, without any enhancing effect by TIMP-2 (Figure 4B). These results demonstrate that activation of pro-MMP-2 by fMT1-MMP was enhanced at low concentrations of TIMP-2, but was inhibited at higher concentrations. In contrast, activation of pro-MMP-2 by sMT1-MMP was inhibited by TIMP-2 in a dose-dependent manner.

TIMP-2 effect on pro-MMP-2 activation by fMT1-MMP and sMT1-MMP *in vitro*

To confirm the enhancing effect of TIMP-2 on activation of pro-MMP-2 in the Sf9 cell system, we examined the TIMP-2 effect on activation of pro-MMP-2 by fMT1-MMP and sMT1-MMP *in vitro*. The recombinant pro-MMP-2 was incubated with the recombinant fMT1-MMP or sMT1-MMP and various amounts of the recombinant TIMP-2. As enzyme sources for fMT1-MMP and sMT1-MMP, the crude membrane fraction of the Sf9 cells

Figure 4 Effect of TIMP-2 on activation of the TIMP-2-free pro-MMP-2 by fMT1-MMP and sMT1-MMP in the Sf9-cell system

The Sf9 cells were co-infected with 1 m.o.i. of fMT1-MMP virus (A) or sMT1-MMP virus (B) and various m.o.i. (lanes 0, 0.3, 0.6 and 6 m.o.i.) of TIMP-2 virus. At 1 h after infection, the medium was replaced with the culture medium of Sf9 cells at 4 days after infection with 1 m.o.i. of pro-MMP-2 baculovirus (lane 'ProMMP-2'). The medium samples were recovered at 4-day incubation and analysed by gelatin zymography. Lane M, the HT-1080 culture medium. The numbers on the left represent molecular masses in kDa.

infected with fMT1-MMP baculovirus and sMT1-MMP purified from the culture medium of the Sf9 cells infected with sMT1-MMP baculovirus were used respectively. In the absence of TIMP-2, both fMT1-MMP and sMT1-MMP activated pro-MMP-2. However, the crude membrane fraction containing fMT1-MMP (the catalytic activity of which was identical with that of the sMT1-MMP) activated more pro-MMP-2 than did sMT1-MMP (Figures 5A, 5B and 5C; no TIMP-2). This difference was statistically significant in pairwise comparisons by two-tailed Student *t* test ($P = 0.003$). In the presence of TIMP-2, the results *in vitro* showed the same patterns as those in the Sf9 cell system. Activation of pro-MMP-2 by fMT1-MMP was enhanced at low concentrations of TIMP-2, but inhibited at higher concentrations of TIMP-2 (Figures 5A and 5C). In contrast, activation of pro-MMP-2 by sMT1-MMP was inhibited in a TIMP-2-dependent manner (Figures 5B and 5C). Therefore we conclude that the TM domain of MT1-MMP is required in enhancing the effect of TIMP-2 on activation of pro-MMP-2 by MT1-MMP.

Under our conditions, 3.2 ng of fMT1-MMP (2.42 nM) or 3 ng of sMT1-MMP (2.42 nM) and 5 ng of pro-MMP-2

Figure 5 Effect of TIMP-2 on pro-MMP-2 activation by fMT1-MMP and sMT1-MMP *in vitro*

Either the crude membrane fraction containing 3.2 ng of fMT1-MMP (A) or 3 ng of sMT1-MMP (B) was incubated with 5 ng of pro-MMP-2 in the presence of various amounts of TIMP-2 (lanes 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 5 and 10 ng) at 37 °C for 6 h. The reaction products were analysed by gelatin zymography. Lane 'M', the HT-1080 culture medium; lane 'ProMMP-2', pro-MMP-2. The numbers on the left represent molecular masses in kDa. The band intensities in (A) and (B) were determined by densitometry and the ratio of the 64 kDa intermediate-form band/the 68 kDa pro-form band versus the amount of TIMP-2 was plotted in (C). Each value in (C) is the mean $\pm$ S.D. for three to five independent experiments.

(3.68 nM) were included. The highest enhancing effect of TIMP-2 on pro-MMP-2 activation by fMT1-MMP was detected at 0.05 ng of TIMP-2 (0.114 nM) (Figure 5C), that is, at 0.05 molar fraction of TIMP-2/fMT1-MMP in the presence of 3.68 nM pro-MMP-2. Complete inhibition of pro-MMP-2 activation by either fMT1-MMP or sMT1-MMP was achieved at approx. 10 ng of TIMP-2 (Figure 5C), more than the 2.7 ng of TIMP-2 (6.10 nM) which would be required for titration of both MT1-MMP and pro-MMP-2. However, TIMP-2 can inhibit MT1-MMP or bind pro-MMP-2 in a 1 : 1 stoichiometry providing that TIMP-2 concentrations are sufficiently higher than inhibition constants (K_i) for MT1-MMP or equilibrium constant (K_d) for pro-MMP-2. The K_i for MT1-MMP would be much higher than

Figure 6 Activation of the pro-MMP-2 within ternary complexes on the crude membrane fraction in the absence or presence of sMT1-MMP

Sf9 cells were infected with 1 m.o.i. of the fMT1-MMP virus or wild-type virus. The crude membrane fraction containing 3.2 ng of fMT1-MMP or the same amount of the crude membrane fraction infected with wild-type virus was incubated in the presence of TIMP-2 (30 ng) for 30 min at 0 °C. The unbound TIMP-2 was removed by centrifugation. The resuspended pellet was incubated with pro-MMP-2 (15 ng) for 30 min at 0 °C. The unbound pro-MMP-2 was removed by centrifugation and the crude membrane fraction containing ternary complexes was resuspended with the reaction buffer. The crude membrane fraction was incubated in the absence (–) or the presence (+) of sMT1-MMP (3 ng) for 0, 3, 6 and 12 h at 37 °C. The reaction mixtures were analysed by gelatin zymography. Lane 'M', the HT-1080 culture medium; lane 'ProMMP-2', pro-MMP-2. The numbers on the left represent molecular masses in kDa.

the K_i (7.2 nM) for MMP-2 [38], because MT1-MMP was reported to bind TIMP-2 13 times more slowly than MMP-2 [24]. The K_d values for the N-terminal and C-terminal domains of pro-MMP-2 were reported to be 190 nM and 5.2 nM respectively [38]. Because 6.10 nM TIMP-2 was lower or not sufficiently higher than these constants, it is thought that pro-MMP-2 activation by either fMT1-MMP or sMT1-MMP was not completely inhibited by the TIMP-2 concentration required for titration of these MMPs.

Catalytic activity of MT1-MMP within ternary complex

It was known that catalytic activity of MT1-MMP is inhibited by TIMP-2 [22,26]. In addition, TIMP-2 inhibited activation of pro-MMP-2 by sMT1-MMP in a dose-dependent manner as well as by fMT1-MMP at high molar fractions of TIMP-2/fMT1-MMP. These results suggest that MT1-MMP in a ternary complex may not activate pro-MMP-2. To test this possibility, ternary complexes were made by serial binding and washing of TIMP-2 and pro-MMP-2 to the crude membrane fraction containing fMT1-MMP. Then the crude membrane fraction containing the ternary complexes was incubated in the MT1-MMP assay buffer. As expected, pro-MMP-2 within ternary complexes was not activated by fMT1-MMP within the complexes up to 12 h incubation (Figure 6, lane fMT1-MMP/–/12).

The enhancing effect of TIMP-2 on pro-MMP-2 activation by fMT1-MMP was observed only at very low molar fractions of TIMP-2/fMT1-MMP, in which the TIMP-2-free fMT1-MMP is superior in number to the TIMP-2-bound fMT1-MMP. It suggests that MT1-MMP free of TIMP-2 would be the molecule activating pro-MMP-2 within the ternary complex. To prove this possibility, we incubated the ternary complexes in the presence of the recombinant sMT1-MMP as a TIMP-2-free MT1-MMP source. It was found that the sMT1-MMP activated the pro-MMP-2 within the ternary complex and that the activated MMP-2 was further processed to lower-molecular-mass forms (Figure 6). These results demonstrate that MT1-MMP free of TIMP-2 can activate pro-MMP-2 within ternary complexes, but MT1-MMP within the complexes cannot.

We found that some of the pro-MMP-2 was also bound to the crude membrane fraction prepared from the Sf9 cells infected with wild-type baculovirus. The pro-MMP-2 bound to the membrane was not activated without external activator but with addition of sMT1-MMP (Figure 6). This result suggests the presence of other pro-MMP-2 receptor(s) on plasma membrane, in addition to the MT1-MMP–TIMP-2 complex.

DISCUSSION

We expressed human fMT1-MMP and sMT1-MMP in a baculovirus/insect-cell system where neither gelatinolytic MMPs nor TIMP-2 were expressed [30], and compared their effects on activation of pro-MMP-2 in the presence or absence of TIMP-2. We further analysed activation of pro-MMP-2 within the MT1-MMP–TIMP-2–pro-MMP-2 ternary complex.

Cao et al. [28] reported that pro-MMP-2 activation by MT1-MMP was abolished by deletion of the TM domain in a transient expression system using COS-1 cells and proposed that the TM domain is essential for activation of pro-MMP-2 by MT1-MMP. When the HT-1080 culture medium was used as a pro-MMP-2 source, we also found that the pro-MMP-2 was not activated until large amounts of sMT1-MMP were accumulated (Figure 2). In contrast, other studies suggested that the purified soluble forms of MT1-MMPs expressed in mammalian cells [6] and in *Escherichia coli* [25–27,39] could activate pro-MMP-2 *in vitro*. In agreement with this, we found that sMT1-MMP was able to activate TIMP-2-free pro-MMP-2 in a Sf9 cell system and *in vitro*. However, activation of TIMP-2-free pro-MMP-2 by sMT1-MMP was inhibited by TIMP-2. Considering that both HT-1080 and COS-1 cells secreted TIMP-2, these results explain that poor activation of the pro-MMP-2 in the HT-1080 or COS-1 culture medium by soluble forms of MT1-MMP resulted from the endogenous TIMP-2.

In the Sf9 cell system, as well as in the cell membrane system *in vitro*, we have demonstrated that TIMP-2 enhanced activation of pro-MMP-2 by fMT1-MMP at low concentrations, but inhibited it at higher concentrations. Kinoshita et al. [40] recently recovered the similar TIMP-2 effect in a non-membrane system in which soluble forms of MT1-MMP were immobilized on agarose beads. With these and other findings, the TIMP-2 effect on pro-MMP-2 activation by MT1-MMP would be explained as follows. The MT1-MMP–TIMP-2 complex that is formed by interaction of the N-terminal domain of MT1-MMP with the N-terminal domain of TIMP-2 would function as a cell-surface receptor of pro-MMP-2 [7,21,24]. Then the C-terminal domain of pro-MMP-2 binds the C-terminal domain of TIMP-2 in the MT1-MMP–TIMP-2 complex, forming a ternary complex, MT1-MMP–TIMP-2–pro-MMP-2, on the cell surface. If TIMP-2-free MT1-MMP is an enzyme that can activate the pro-MMP-2 within the ternary complex (see below), MT1-MMP plays dual roles on pro-MMP-2 activation on the cell surface, as a component of pro-MMP-2 receptor and an activator of pro-MMP-2. To be a pro-MMP-2 receptor, MT1-MMP needs to bind TIMP-2 but lose its catalytic activity. On the other hand, to be an activator of pro-MMP-2, MT1-MMP should be free of TIMP-2. Thus a delicate balance between TIMP-2-free MT1-MMP and MT1-MMP bound to TIMP-2 would be required for the maximal activation of pro-MMP-2 on the cell surface.

In our experiment using the crude membrane containing fMT1-MMP, an enhancing effect of TIMP-2 was detected at molar fractions of TIMP-2/fMT1-MMP lower than 0.7, and the maximal effect, at its 0.05 molar fraction (Figure 5C). Therefore it seems that excess TIMP-2-free fMT1-MMP molecules are required for the maximal activation of the pro-MMP-2 molecules

bound to fMT1-MMP-TIMP-2 complexes. At molar fractions lower than 0.05, although TIMP-2-free MT1-MMP molecules as pro-MMP-2 activator are present in sufficiently high numbers, the amount of the pro-MMP-2 molecules recruited on the cell surface would be a limiting factor to get the maximal effect. At molar fractions higher than 0.05, although the pro-MMP-2 molecules recruited on the cell surface are present in sufficiently high numbers, their activation would be limited, owing to a decrease of TIMP-2-free MT1-MMP molecules.

It is noteworthy that TIMP-2 at low molar fractions of TIMP-2/fMT1-MMP enhances pro-MMP-2 activation by fMT1-MMP in spite of a decrease in the numbers of catalytically active TIMP-2-free MT1-MMP molecules. This result demonstrates that the pro-MMP-2 within a ternary complex should be activated by MT1-MMP more efficiently than pro-MMP-2 in solution. To explain this, two hypotheses can be presented. First, binding of pro-MMP-2 to the MT1-MMP-TIMP-2 complex may give rise to conformational change of the pro-MMP-2 to become a better substrate for TIMP-2-free MT1-MMP. Secondly, collision between the pro-MMP-2 in a ternary complex and TIMP-2-free MT1-MMP by lateral movement on a membrane may be more efficient than that between pro-MMP-2 in solution and MT1-MMP on a membrane (in solution in the case of sMT1-MMP). As found for fMT1-MMP, addition of TIMP-2 to the sMT1-MMP and pro-MMP-2 mixture will form sMT1-MMP-TIMP-2 complexes and ternary complexes of sMT1-MMP, TIMP-2 and pro-MMP-2, and thus decrease concentration of TIMP-2-free sMT1-MMP molecules. If the first hypothesis is correct, the pro-MMP-2 bound to sMT1-MMP-TIMP-2 complex would be a better substrate for TIMP-2-free sMT1-MMP. Then we can expect an enhancing effect of TIMP-2 in the pro-MMP-2 activation by sMT1-MMP. If the second hypothesis is correct, collision efficiency between the pro-MMP-2 bound to the sMT1-MMP-TIMP-2 complex with TIMP-2-free sMT1-MMP would not be different from that between TIMP-2-free pro-MMP-2 and TIMP-2-free sMT1-MMP, both of which occur in solution. Then pro-MMP-2 activation by sMT1-MMP will simply be decreased by decrease of TIMP-2-free sMT1-MMP molecules. Since we found that pro-MMP-2 activation by sMT1-MMP was dose-dependently inhibited by TIMP-2 without an enhancing effect, we propose that the enhancing effect of TIMP-2 results from efficient collision between the pro-MMP-2 in the ternary complex and TIMP-2-free MT1-MMP by lateral movement on cell membrane.

Kinoshita et al. [40] recently suggested involvement of TIMP-2-free MT1-MMP in the activation of pro-MMP-2 in MT1-MMP-TIMP-2-pro-MMP-2 ternary complexes. They generated a mixture of ternary complex and TIMP-2-free MT1-MMP on agarose beads at 0 °C. When the temperature was shifted to 37 °C, the pro-MMP-2 bound to the beads was activated. However, these results did not exclude a possibility that molecules other than TIMP-2-free MT1-MMP might activate pro-MMP-2. To exclude this possibility, we made the ternary complexes saturated with TIMP-2 and pro-MMP-2 to MT1-MMP in the crude membrane fraction. Whereas the pro-MMP-2 in the ternary complexes was not activated for up to 12 h incubation without sMT1-MMP, it was readily activated by addition of sMT1-MMP (Figure 6). From this result we could expect that TIMP-2-free MT1-MMP would be the enzyme responsible for activation of the pro-MMP-2 within ternary complexes under physiological conditions.

In the absence of TIMP-2, we found that the crude membrane fraction containing fMT1-MMP activated more pro-MMP-2 molecules than did sMT1-MMP (Figure 5). We indeed found that pro-MMP-2 bound to the Sf9 membrane free of MT1-

MMP, up to approx. 20 % of the pro-MMP-2 binding to the Sf9 membrane containing MT1-MMP, and was activated by addition of sMT1-MMP (Figure 6). These results demonstrate the presence of pro-MMP-2 receptors, additional to the MT1-MMP-TIMP-2 complex, on the cell surface. Since it was reported that the C-terminal domain of pro-MMP-2 could bind heparin [14,24] and the $\alpha v \beta 3$ integrin [41,42], it would be interesting to determine which molecule is involved in interaction of pro-MMP-2 to the Sf9 membrane.

The pro-domain of MT1-MMP was reported to be important for binding to TIMP-2 and for activation of pro-MMP-2 by MT1-MMP [23,29]. However, both fMT1-MMP and sMT1-MMP expressed by baculovirus/insect-cell system did not contain the pro-domain (Figure 1). Processing at the furin cleavage site of our MT1-MMPs is supported by the finding that the pro-protein convertase furin in Sf9 cells was identical in substrate specificity and inhibitor profiles with that in mammalian cells [31]. Nevertheless, our fMT1-MMP was able to bind to TIMP-2 and to enhance activation of pro-MMP-2 in the presence of small amounts of TIMP-2. In addition, when the pro-domain-deleted soluble MT1-MMPs were immobilized on agarose beads, Kinoshita et al. [40] demonstrated a similar enhancing effect of TIMP-2. We thus believe that the mature MT1-MMP without the pro-domain can bind TIMP-2 and activate pro-MMP-2 bound to MT1-MMP-TIMP-2 complex as well as pro-MMP-2 free of TIMP-2.

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