TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP

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The matrix metalloproteinase (MMP)-2 has a crucial role in extracellular matrix degradation associated with cancer metastasis and angiogenesis. The latent form, pro-MMP-2, is activated on the cell surface by the membrane-tethered membrane type 1 (MT1)-MMP, in a process regulated by the tissue inhibitor of metalloproteinase (TIMP)-2. A complex of active MT1-MMP and TIMP-2 binds pro-MMP-2 forming a ternary complex, which permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP. It remains unclear how MMP-2 activity in the pericellular space is regulated in the presence of TIMP-2. To address this question, the effect of TIMP-2 on MMP-2 activity in the extracellular space was investigated in live cells, and their isolated plasma membrane fractions, engineered to control the relative levels of MT1-MMP and TIMP-2 expression. We show that both free and inhibited MMP-2 is detected in the medium, and that the net MMP-2 activity correlates with the level of TIMP-2 expression. Studies to displace MT1-MMP-bound TIMP-2 in a purified system with active MMP-2 show minimal displacement of inhibitor, under the experimental conditions, due to the high affinity interaction between TIMP-2 and MT1-MMP. Thus inhibition of MMP-2 activity in the extracellular space is unlikely to result solely as a result of TIMP-2 dissociation from its complex with MT1-MMP. Consistently, immunoblot analyses of plasma membranes, and surface biotinylation experiments show that the level of surface association of TIMP-2 is independent of MT1-MMP expression. Thus low-affinity binding of TIMP-2 to sites distinct to MT1-MMP may have a role in regulating MMP-2 activity in the extracellular space generated by the ternary complex.

Key words: gelatinase, matrix metalloproteinase, protease, protease inhibitor, zymogen.

INTRODUCTION

Matrix metalloproteinase (MMP)-2 (also called gelatinase A) is a key member of the MMP family of zinc-dependent endopeptidases which has been associated with many pathological conditions, particularly cancer metastasis and angiogenesis [1–5]. The activation of the zymogen form of MMP-2 (pro-MMP-2) is a cell-surface event that is mediated by members of the membrane-type (MT) subfamily of MMPs [6], and MT1-MMP (MMP-14) was the first MT-MMP identified as a major physiological activator of pro-MMP-2 [7]. The activation of pro-MMP-2 by MT1-MMP evolved to incorporate in its process the tissue inhibitor of metalloproteinase (TIMP)-2, a member of the TIMP family of MMP inhibitors. While TIMP-2 inhibits active MT1-MMP and MMP-2 by binding to the active site of the enzymes via its N-terminal inhibitory region, it can also form a non-covalent complex with pro-MMP-2 by binding to the haemopexin-like domain of the zymogen via its C-terminal domain [8–14]. It has been shown that the simultaneous binding of TIMP-2 to active MT1-MMP via its inhibitory N-terminal domain, and to pro-MMP-2 via its C-terminal region, yields ternary complexes on the cell surface that permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP molecule, which hydrolyses the Asn^{37}–Leu^{38} peptide bond of pro-MMP-2 [12,15,16]. This process generates an inactive intermediate MMP-2 form that is subsequently cleaved at the Asn^{80}–Tyr^{81} peptide bond by a fully active MMP-2, in an intermolecular autocatalytic event, which leads to full activation [17]. In cultured cells, active MMP-2 (62 kDa) is detected in the supernatant and in the cell layer, indicating that the 62 kDa species dissociates from the ternary complex upon activation. However, it is unclear whether the 62-kDa species of MMP-2 detected in the medium is free or inhibited (in complex) by TIMP-2. A previous study, using isolated plasma membranes (PMs) of concanavalin A (ConA)-treated human uterine cervical fibroblasts expressing natural MT1-MMP, showed that the active MMP-2 in the medium was in a complex with TIMP-2, and was thus devoid of catalytic activity [18]. In another study, conducted on breast carcinoma cells stably transfected to express human MT1-MMP, MMP-2 was shown to dissociate from the cell surface free of TIMP-2 [19]. Thus the effects of TIMP-2 on the MMP-2 activity generated by the ternary complex system of pro-MMP-2 activation remain unclear. To investigate the interaction of MMP-2 with TIMP-2 after activation by MT1-MMP, we determined the net proteolytic activity of MMP-2 in a cellular system designed to control the relative levels of TIMP-2 and MT1-MMP expression.

MATERIALS AND METHODS

Cells and recombinant viruses

Non-malignant monkey kidney epithelial BS-C-1 (CCL-26) cells were obtained from the American Type Culture Collection

Abbreviations used: APMA, *p*-aminophenylmercuric acetate; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; HRP, horseradish peroxidase; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MOCAcPLGLA_2pr(Dnp)AR-NH_2, (7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-glycyl-L-leucyl-[N_3-(2,4-dinitrophenol)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide; MT1-MMP, membrane type 1-MMP; MT1-MMP_{cat}, catalytic domain of MT1-MMP; NP40, Nonidet P40; pAb, polyclonal antibody; pfu, plaque-forming unit(s); PM, plasma membrane; TIMP, tissue inhibitor of metalloproteinase.

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(A.T.C.C., Manassas, VA, U.S.A.), and were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % foetal bovine serum (FBS) and antibiotics. HeLa S3 cells were obtained from the ATCC (CCL-2.2), and were grown in suspension in minimal essential medium (Spinner modification; Quality Biologicals, Gaithersburg, MD, U.S.A.), supplemented with 5 % (v/v) horse serum. All other tissue-culture reagents were purchased from Invitrogen (Carlsbad, CA, U.S.A.). The production of the recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase has been described by Fuerst et al. [20]. Recombinant vaccinia viruses expressing either pro-MT1-MMP (vT7-MT1) or TIMP-2 (vSC59-T2) were obtained by homologous recombination, as previously described [10,21].

Recombinant proteins and antibodies

Human recombinant pro-MMP-2 and TIMP-2 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses, and were purified to homogeneity, as described in [22]. Pro-MMP-2 was activated by incubation with *p*-aminophenylmercuric acetate (APMA), as described in [23], and the MMP-2 concentration was determined by titration against a TIMP-2 solution of known concentration. The catalytic domain of MT1-MMP (MT1-MMP_{cat}), expressed in bacteria, was obtained from Calbiochem (La Jolla, CA, U.S.A.). The anti-MMP-2 mouse monoclonal antibody (mAb) CA-801, the anti-TIMP-2 mAb CA-101 and the rabbit polyclonal antibodies (pAb) 437 and 160, against the haemopexin-like and catalytic domains of MT1-MMP respectively, have been described previously [21,24,25].

Cell infection and PM isolation

BS-C-1 cells in six-well plates were co-infected with vTF7-3 vaccinia virus [5 plaque-forming units (pfu)/cell], vT7-MT1 (5 pfu/cell), and increasing pfu/cell (0–5) of vSC59-T2 virus, in 0.5 ml/well of infection medium (DMEM supplemented with 2.5 % FBS and antibiotics), for 45 min at 37 °C. The virus-containing medium was aspirated and replaced with fresh infection medium for an additional incubation of 16 h, at 37 °C. To isolate PM fractions, BS-C-1 cells in 150 mm diameter tissue-culture dishes were co-infected with vTF7-3 vaccinia virus (5 pfu/cell) and either constant vT7-MT1 vaccinia virus (5 pfu/cell) and increasing vSC59-T2 vaccinia virus (0–5 pfu/cell) or increasing vT7-MT1 virus (0–5 pfu/cell) and constant vSC59-T2 virus (0.05 pfu/cell), in 25 ml/dish of infection medium, for 45 min at 37 °C. After 16 h incubation in fresh infection medium, the cells were subjected to subcellular fractionation to isolate the PM fraction, as described in [21].

Pro-MMP-2 activation by cells and PM fractions

BS-C-1 cells in six-well plates were infected to co-express MT1-MMP and TIMP-2, as described above. At 16 h after infection, the medium was aspirated and replaced with serum-free Opti-MEM (modified Eagle's medium), without Phenol Red, supplemented with 50 nM of purified human recombinant pro-MMP-2. At various times ($\leqslant$ 6.5 h), medium was removed for analysis of MMP-2 activation by gelatin zymography, immunoblotting, and by a proteolytic activity assay, using the fluorescence-quenched substrate MOCAcPLGLA_2pr(Dnp)AR-NH_2 {(7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-glycyl-L-leucyl-[N_3-(2,4-dinitrophenol)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide; Peptides International, Louisville, KY, U.S.A.} [26], as described in [27]. Briefly, 20 μl medium aliquots were added to 2 ml of a 7 μM solution of the fluorogenic substrate in a buffer consisting of 50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 0.01 % (v/v) Brij-35 and 1 % (v/v) DMSO (buffer R). Substrate hydrolysis was monitored at 25 °C, using a Photon Technology International (PTI) spectrofluorometer, at excitation and emission wavelengths of 328 and 393 nm respectively. After approx. 6.5 h, the remaining medium was collected, the cells were rinsed with cold PBS, and were lysed with cold lysis buffer [25 mM Tris/HCl (pH 7.5), 1 % Igepal CA-630, a non-ionic detergent from Sigma, and 100 mM NaCl] containing protease inhibitors (one pellet of Complete Mini, EDTA-free protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany, in 10 ml of buffer). The medium samples were centrifuged at 500 ***g***, for 5 min at 4 °C, to remove cell debris. The lysates were centrifuged at 9500 ***g*** for 15 min at 4 °C. To ascertain the extent of TIMP-2–MMP-2 complex formation, the medium was incubated overnight at 4 °C with gelatin immobilized on crossed-linked 4 % beaded agarose [50 μl of a 50 % slurry in collagenase buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 0.02 % (v/v) Brij-35]. After centrifugation at 9500 ***g*** for 5 min, the supernatants were collected, and the beads (pellet) were subjected to three washing/centrifugation steps with collagenase buffer (200 μl). The beads were mixed with reducing Laemmli sample buffer and were boiled for 5 min. The supernatants were subjected to immunoblot analysis for TIMP-2 and pro-MMP-2/MMP-2. Lysate fractions were analysed for pro-MMP-2 activation by gelatin zymography, and for MT1-MMP and TIMP-2 expression by immunoblot analysis. Pro-MMP-2 activation by PM fractions was monitored in reaction mixtures containing pro-MMP-2 (50 nM) and PM (0.150 μg/ml) in collagenase buffer. At various times ($\leqslant$ 7 h), aliquots of the reaction mixtures were subjected to gelatin zymography and immunoblot analysis, and MMP-2 proteolytic activity was assayed with the fluorogenic substrate MOCAcPLGLA_2pr(Dnp)AR-NH_2, as described above.

Displacement of MT1-MMP-bound TIMP-2

To examine the displacement of membrane-bound TIMP-2 by MMP-2, increasing concentrations of PM fractions, derived from BS-C-1 cells infected to co-express varying MT1-MMP/TIMP-2 ratios, were incubated (1 h, 37 °C) with 5 nM MMP-2 in collagenase buffer. The remaining MMP-2 activity was measured with the synthetic peptide substrate MOCAcPLGLA_2pr(Dnp)AR-NH_2, as described above. To follow the ability of MMP-2 to displace TIMP-2 bound to MT1-MMP in a purified system, 130 nM MT1-MMP_{cat} was incubated (1 h, 37 °C) with 100 nM TIMP-2, to ensure that all the TIMP-2 was complexed with MT1-MMP. Complete MT1-MMP_{cat} inhibition by TIMP-2 was confirmed with the fluorogenic substrate MOCAcPLGLA_2pr-(Dnp)AR-NH_2. Displacement experiments were carried out either by adding MMP-2 (0.8 nM) to 2 ml of buffer R, containing the pre-formed MT1-MMP_{cat}–TIMP-2 complex (0.6 nM) and the fluorogenic substrate (7 μM), and monitoring substrate hydrolysis over 20 min, or by pre-incubating MMP-2 (5 nM) with increasing MT1-MMP_{cat}–TIMP-2 complex (0–20 nM) for 1 h, at 37 °C, in a total volume of 100 μl in buffer R, and measuring the remaining MMP-2 activity with the fluorogenic substrate MOCAcPLGLA_2pr(Dnp)AR-NH_2, as described above. The activity due to MT1-MMP_{cat} added in excess of TIMP-2 was measured in a reaction mixture, run in parallel, containing the same complex concentration but no MMP-2, and subtracted from that obtained in the presence of the enzyme.

Gelatin zymography and immunoblot analysis

Gelatin zymography was performed as previously described in [28]. The samples for immunoblot analysis were subjected to reducing SDS/PAGE, followed by transfer to nitrocellulose membranes. The transferred proteins were developed with pAb 437 and pAb 160 against MT1-MMP, mAb CA-101 against TIMP-2, or mAb CA-801 against pro-MMP-2/MMP-2. Horseradish peroxidase (HRP)-labelled anti-(rabbit MT1-MMP), and anti-(mouse pro-MMP-2/MMP-2 and TIMP-2) IgG (ImmunoPure® from Pierce, Rockford, IL, U.S.A.) were the secondary antibodies used. Detection was performed using SuperSignal®, West Pico or Femto sensitivity, enhanced chemiluminescent substrate for HRP according to the manufacturer's (Pierce) instructions.

Surface biotinylation

BS-C-1 cells, in 150 mm diameter dishes, were co-infected with vTF7-3 (5 pfu/cell), vSC59-T2 (0.05 pfu/cell) and increasing vT7-MT1 (0–5 pfu/cell) viruses, as described above. After 18 h of incubation, the cells were washed extensively with cold PBS-CM (PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$). To each dish, 10 ml of freshly prepared 0.5 mg/ml sulpho-*N*-hydroxysuccinimide-biotin (Pierce), in PBS-CM were added. After incubation for 30 min at 4 °C, the cells were rinsed with cold PBS-CM and were incubated for 10 min at 4 °C with 50 mM NH_4Cl in PBS-CM. The cells were rinsed with PBS-CM and lysed with NP40 (Nonidet P40) buffer [25 mM Tris (pH 7.5), 100 mM NaCl and 1 % (v/v) NP40] containing protease inhibitors, as described above. The lysates were centrifuged at 9500 ***g*** at 4 °C for 13 min. The supernatants were incubated with 120 μl of a 1:1 slurry of ImmunoPure® immobilized streptavidin on agarose beads (Pierce) for 10 min at 4 °C, followed by centrifugation for 10 min at 9500 ***g***. The beads were extensively washed with NP40 buffer, treated with 4× reducing Laemmli sample buffer, and boiled. After centrifugation, the samples were subjected to immunoblot analysis.

RESULTS

TIMP-2 modulates MMP-2 activity in cells

We followed the activity of MMP-2 generated after pro-MMP-2 activation, in cells infected to express constant amounts of MT1-MMP, and increasing amounts of TIMP-2, by using a mammalian cell expression system and recombinant vaccinia viruses. Generation of active MMP-2 was monitored in the medium by zymography, immunoblot analysis, and by a proteolytic activity assay, as a function of time, after addition of exogenous pro-MMP-2 to the MT1-MMP–TIMP-2-expressing cells. As shown in Figure 1(A), the peptide-substrate assay demonstrated that the net activity of MMP-2 increased with time, and with the level (pfu/cell) of TIMP-2 virus > 0.1 pfu/cell. Higher levels of TIMP-2 virus (0.5 and 1 pfu/cell), and consequently TIMP-2 protein (Figure 1D), caused a significant inhibition of MMP-2 activity in the supernatant (Figure 1A). This inhibition of activity did not correlate with the zymographic and immunoblot analyses of MMP-2 (Figures 1B and 1C), which revealed the presence of active MMP-2 species, even at the highest levels of TIMP-2, suggesting that the decrease in activity was likely to be due to inhibition by TIMP-2. In fact, increasing concentrations of TIMP-2 were detected in the medium by immunoblot analysis (Figure 1D), and no significant amount of free TIMP-2 was measured by titration with exogenous MMP-2, at all levels of TIMP-2 virus infection (results not shown).

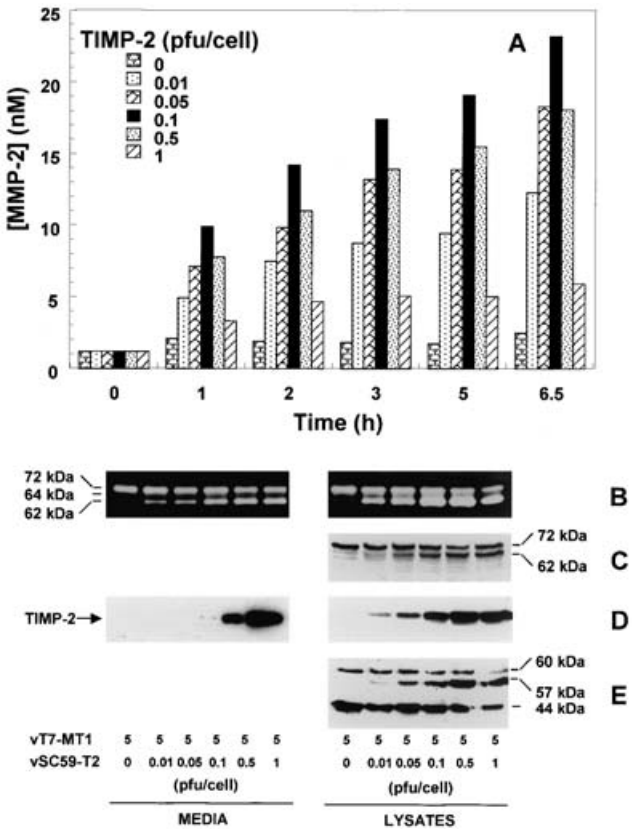

Figure 1 Effect of TIMP-2 levels on MMP-2 activity in cells

(**A**) BS-C-1 cells were co-infected to express MT1-MMP (5 pfu/cell), and increasing amounts of TIMP-2 (0–1 pfu/cell), as described in the Materials and methods section. At 16 h post-infection, the medium was aspirated and was replaced with Opti-MEM (modified Eagle's medium) containing 50 nM of purified human recombinant pro-MMP-2. At various times ($\leqslant$ 6.5 h), aliquots of the medium were assayed for MMP-2 proteolytic activity, using the fluorogenic substrate $MOCAcPLGLA_2pr(Dnp)AR\text{-}NH_2$, as described in the Materials and methods section. (**B**) After 6.5 h of incubation, medium and lysates were subjected to gelatin zymography, as described in the Materials and methods section. (**C**) Immunoblot analysis of the lysates using the mAb CA-801 against pro-MMP-2/MMP-2. (**D**) Immunoblot analysis of the medium and lysates, after 6.5 h incubation, using the mAb Ca-101 against TIMP-2. (**E**) Immunoblot of the lysates using pAb 437 against MT1-MMP.

The expression of TIMP-2 also modulated the profile of MT1-MMP forms in the cells. As shown in Figure 1(E), in the absence of TIMP-2, MT1-MMP exhibited mostly 60 and 44 kDa forms, representing the zymogen and a membrane-tethered form lacking the catalytic domain respectively [21]. Expression of TIMP-2 correlated with the presence of the 57 kDa active species of MT1-MMP, and the concomitant reduction in the 44 kDa form, consistent with the ability of TIMP-2 to prevent the autocatalytic turnover of MT1-MMP, as previously described in [21]. Thus the sustained level of pro-MMP-2 activation detected by zymography (Figures 1B and 1C) correlated with a reduced autocatalytic processing of MT1-MMP.

To ascertain whether the TIMP-2 found in the medium was free or bound to MMP-2, the medium was incubated with gelatin–agarose beads. Gelatin beads are known to bind both latent and active gelatinases, free or complexed with TIMPs. The bound fraction, comprising all free and complexed MMP-2 forms, was subjected to immunoblot analysis, whereas the unbound fraction, comprising free TIMP-2, was analysed for its ability to inhibit MMP-2 activity. As shown in Figure 2(B), increasing amounts of TIMP-2 were detected in the bound fraction, which

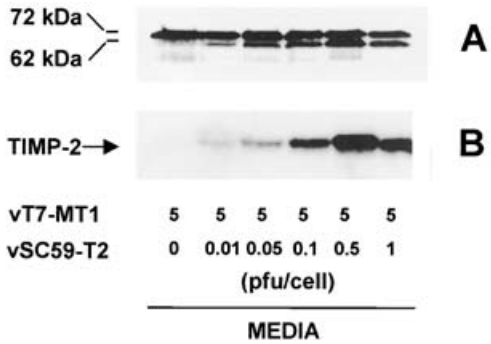

Figure 2 Effect of TIMP-2 levels in MMP-2–TIMP-2 complex formation

(**A**) After 6.5 h of incubation, the MMP-2-containing medium of BS-C-1 cells co-infected to express constant MT1-MMP and increasing TIMP-2, as described in Figure 1, was treated with gelatin–agarose beads, as described in the Materials and methods section, and the bound fraction was subjected to immunoblot analysis using mAb CA-801 against pro-MMP-2–MMP-2. (**B**) The blot was reprobed using mAb CA-101 against TIMP-2.

correlated with the extent of pro-MMP-2 activation, as revealed by the increased intensity of the 62 kDa MMP-2 form (Figure 2A). In contrast, the unbound fraction revealed no inhibitory activity after titration against active MMP-2, suggesting that the TIMP-2 released into the medium is bound to the enzyme (results not shown). Taken together, these results indicate that, although the highly regulated mechanism of pro-MMP-2 activation by MT1-MMP seems to confine proteolytic activity to the cell surface, both active and inhibited MMP-2 are found in the medium, and the level of proteolytic activity is a function of the MT1-MMP/TIMP-2 ratio expressed by the cells.

Activation of pro-MMP-2 by PM fractions

Since the infected cells secrete TIMP-2 constitutively, it was difficult to establish whether the inhibition of MMP-2 was due to TIMP-2 secreted into the medium over the time period of the experiment, or due to the dissociation of TIMP-2 originally bound to the cell surface. Therefore, to measure MMP-2 activity in the absence of secreted TIMP-2, we used, instead of live cells, the PM fraction derived from cells infected to express MT1-MMP and increasing amounts of TIMP-2. The isolated PM fractions were washed thoroughly to remove all unbound TIMP-2 and thus all the inhibitor present was expected to be bound to the PM. Immunoblot analyses of the PM fractions demonstrated the presence of MT1-MMP (Figure 3C) and TIMP-2 (Figure 3D), as expected. The PM fractions were incubated for various times with exogenous pro-MMP-2, and activation was monitored as described above. As shown in Figure 3(A), the MMP-2 activity generated by the PM fractions correlated with the amount of TIMP-2 virus used to infect the cells > 0.1 pfu/cell. However, higher levels of TIMP-2 virus caused a sharp decline in activity. In terms of MT1-MMP, pro-MMP-2 activation correlated with accumulation of active MT1-MMP (57 kDa), and with the concomitant decrease of the 44 kDa species with increasing TIMP-2 expression (Figure 3C). These results did not differ significantly from the results obtained with the live cells, suggesting that MMP-2 inhibition is likely to be mediated by TIMP-2 associated with the PM.

MMP-2 displaces surface-bound TIMP-2

The experiments described above suggested that, once activated, MMP-2 could displace cell associated TIMP-2 resulting in MMP-

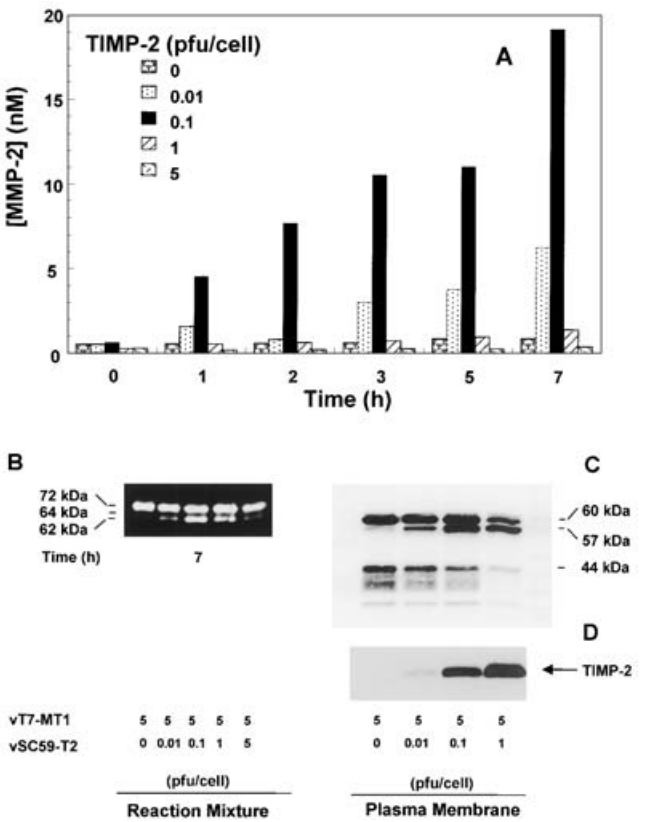

Figure 3 Effect of TIMP-2 levels in MMP-2 activity in PM fraction

(**A**) PM (0.15 μg/μl) isolated from BS-C-1 cells co-infected to express constant levels of MT1-MMP (5 pfu/cell) and increasing levels of TIMP-2, indicated by the pfu/cell of the TIMP-2-expressing virus (vSC59-T2) (0–5 pfu/cell), were incubated at 37 °C with 50 nM of pro-MMP-2, in a total volume of 200 μl of collagenase buffer. At various times ($\leqslant$ 7 h), MMP-2 activity was assayed with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$ in buffer R. (**B**) Zymographic analysis of the reaction mixtures after 7 h of incubation. (**C**) Immunoblot analysis of MT1-MMP in PM fractions with pAb 437. (**D**) Immunoblot analysis of TIMP-2 in PM fractions with mAb CA-101.

2 inhibition. To test this hypothesis, APMA-activated MMP-2 was incubated for 1 h at 37 °C with various amounts of PM fractions isolated from cells infected to express either constant levels of MT1-MMP and increasing TIMP-2 amounts, or increasing levels of MT1-MMP and constant amounts of TIMP-2. Then the remaining MMP-2 activity was measured with the fluorogenic peptide substrate. As shown in Figure 4(A), the inhibition of MMP-2 activity correlated with the level of TIMP-2 detected in the PM fractions isolated from cells expressing increasing levels of TIMP-2 (shown in the immunoblot of Figure 3D). Since MT1-MMP is known to function as a high-affinity receptor for TIMP-2 [15,29], this result suggests that MMP-2 may be displacing MT1-MMP-bound TIMP-2. To test this hypothesis, MMP-2 was added to a solution of a pre-formed MT1-MMP$_{cat}$–TIMP-2 complex in low equimolar concentrations (approx. 0.8 nM). Although both MT1-MMP$_{cat}$ and MMP-2 react with the fluorogenic substrate, MMP-2 reacts with higher catalytic efficiency, as revealed by the respective k_{cat}/K_m values (1×10^5 and 10×10^5 M$^{-1} \cdot$ s^{-1} respectively [22,25]). Thus TIMP-2 displacement from the MT1-MMP$_{cat}$–TIMP-2 complex by MMP-2 should result in a net decrease of proteolytic activity. However, no decay of activity was detected over a 20 min period, suggesting that the MT1-MMP–TIMP-2 complex cannot easily be dissociated by MMP-2 at low equimolar enzyme concentrations

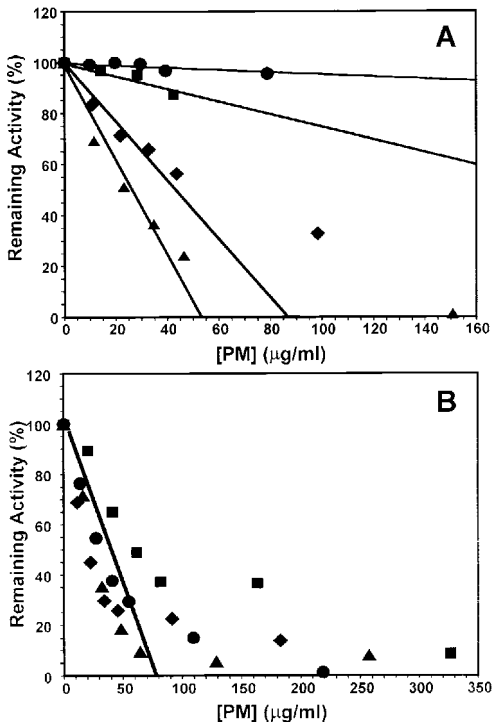

Figure 4 MMP-2 displaces TIMP-2 from PM

Recombinant active MMP-2 (5 nM) was incubated with increasing concentrations of PM fractions isolated from BS-C-1 cells co-infected with vTF7-3 (5 pfu/cell) and (**A**) vT7-MT1 (5 pfu/cell) viruses and various vSC59-T2 virus [0 (●), 0.01 (■), 0.1 (◆) and 1 (▲) pfu/cell] or (**B**) increasing vT7-MT1 [0 (●), 0.01 (■), 0.1 (◆) and 5 (▲) pfu/cell] and constant vSC59-T2 (0.05 pfu/cell) in a total volume of 50 μl, in collagenase buffer, for 1 h, at 37 °C. The remaining MMP-2 proteolytic activity after incubation was measured using the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, as described in the Materials and methods section.

(results not shown), indicating that MMP-2 displacement of MT1-MMP-bound TIMP-2 would require high concentrations of MMP-2 and/or long incubation periods. In fact, at higher MMP-2 concentrations (5 nM) and longer incubation periods (1 h at 37 °C), a 40 % decay in MMP-2 activity with increasing MT1-MMP$_{cat}$–TIMP-2 concentrations (0–20 nM) was observed (results not shown). Of note, the decrease in proteolytic activity is likely to be larger than 40 %, since it includes the contribution of the dissociated MT1-MMP$_{cat}$. To confirm that TIMP-2 was displaced, the reaction mixtures were incubated with gelatin–agarose beads, and the free and bound fractions were subjected to immunoblot analysis. MMP-2, but not MT1-MMP$_{cat}$, was detected in the bound fraction, as expected, given the low gelatinolytic activity of MT1-MMP$_{cat}$. Increasing TIMP-2 amounts were also found in the bound fraction, indicating that MMP-2 can displace MT1-MMP-bound TIMP-2. Thus the ability of the PM fractions with increasing TIMP-2 levels to inhibit MMP-2 may not be caused solely by displacement of MT1-MMP-bound TIMP-2, but also by TIMP-2 associated with sites that are distinct from MT1-MMP. Consistently, titration of MMP-2 against increasing amounts of PM fractions derived from cells infected to express constant levels of TIMP-2 and increasing levels of MT1-MMP revealed that MMP-2 inhibition correlated with the level of TIMP-2, rather than that of MT1-MMP (Figure 4B). As shown in Figure 5(A), immunoblot analysis of the PM fractions showed that the relative amount of TIMP-2 present was independent of the level of MT1-MMP expression. Furthermore, cell-surface biotinylation of BS-C-1 cells infected to express the same levels of MT1-MMP and TIMP-2 showed similar amounts of surface-associated TIMP-2, regardless of the level of MT1-MMP expression, as shown in Figure 5(B), suggesting that TIMP-2 binds to sites other than MT1-MMP on the cell surface. Taken together, these results indicate that, although MT1-MMP has been identified as a major high-affinity TIMP-2 receptor, presumably weaker specific or non-specific TIMP-2 binding to the cell surface may modulate MMP-2 proteolytic activity in the pericellular environment.

DISCUSSION

MT1-MMP activation of pro-MMP-2 requires the participation of TIMP-2, which acts as a linker between MT1-MMP and pro-MMP-2, to generate a ternary complex at the cell surface. It is postulated that, while in the ternary complex, pro-MMP-2 is fully activated by two sequential cleavages at the pro-domain, which are mediated first by a neighbouring TIMP-2-free MT1-MMP and then, presumably, by a closely associated fully active MMP-2 acting in an intermolecular autocatalytic manner [17]. After activation, the newly activated MMP-2 must dissociate from the ternary complex, since the active enzyme can be detected in the supernatant of cultured cells induced to activate pro-MMP-2 by various agents [30–34], or by overexpression of MT1-MMP [21]. Since gelatin zymography, the most common method to follow pro-MMP-2 activation, cannot assess net proteolytic activity [35], the interplay between MMP-2 and TIMP-2 after activation has been overlooked. The present study was designed to measure the net activity of MMP-2 generated after activation, and to assess whether TIMP-2 affects the level of activity of the released MMP-2. To address this issue, we used a mammalian cell expression system, in which the level of MT1-MMP and TIMP-2 could be controlled by using defined amounts of vaccinia viruses expressing these proteins. We have previously shown that infection of cells with vaccinia viruses minimizes any possible influence of the endogenous MMPs and TIMPs [10], because vaccinia virus significantly inhibits host-protein synthesis. Therefore, the effects of TIMP-2 on MMP-2 activity could be reliably assessed, as opposed to cellular systems expressing natural MMPs and TIMPs or stable/transient transfection systems in which the involvement of endogenous TIMPs cannot be excluded. Furthermore, such cellular systems usually express other MMPs and are thus unsuitable for measurement of MMP enzymic activity because of the lack of MMP-2 specificity. In the present study, we have found that measurable MMP-2 activity was generated in the supernatant of cells expressing MT1-MMP and TIMP-2, and that this activity was modulated by the level of TIMP-2 expression in the system. At the highest levels, TIMP-2 inhibited MMP-2 net proteolytic activity, in spite of the presence of fully active forms, as determined by gelatin zymography. Thus, even at the highest level of TIMP-2, there was considerable cleavage of the pro-domain of pro-MMP-2, and yet MMP-2 activity was undetectable. Furthermore, the increase in pro-MMP-2 activation correlated, to an extent, with the accumulation of active MT1-MMP (57 kDa) on the cell surface and its autocatalytic processing to the inactive membrane-tethered 44-kDa species lacking the catalytic domain [21]. These results demonstrate the complexity of the pro-MMP-2 activation machinery that tightly regulates the proteolytic activity of both MT1-MMP and MMP-2 by TIMP-2. The delicate and dynamic balance between pro-MMP-2, MT1-MMP and TIMP-2 within, as well as outside, the ternary complex determines the amount of active MT1-MMP on the cell surface by controlling its rate of autocatalytic processing and the level of active MMP-2.

In the present study, there was a gradual loss of net MMP-2 activity as a function of TIMP-2 level, which could not be

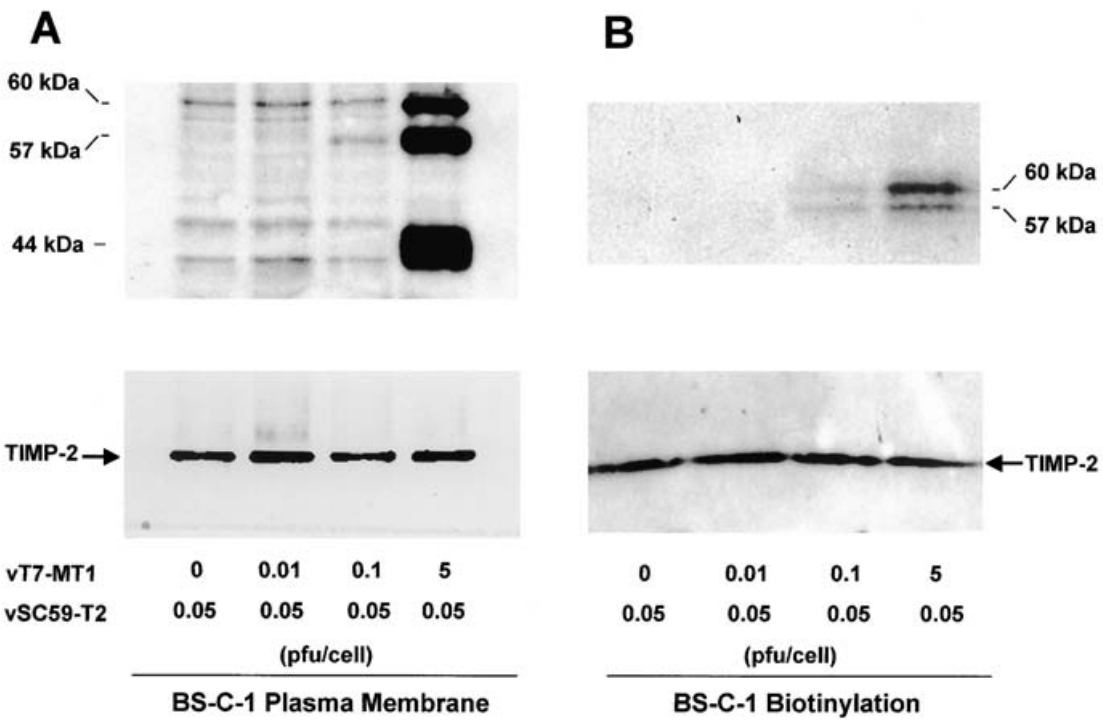

Figure 5 Effect of MT1-MMP expression in TIMP-2 binding to the cell membrane

(**A**) Immunoblot analysis of MT1-MMP and TIMP-2 of PM fractions of BS-C-1 cells infected to express increasing MT1-MMP (indicated by the increasing pfu/cell of vT7-MT1, 0–5 pfu/cell) and constant TIMP-2-expressing vSC59-T2 virus (0.05 pfu/cell) with pAb 437 and mAb CA 101 respectively. (**B**) BS-C-1 cells were co-infected with vTF7-3 (5 pfu/cell), vSC59-T2 (0.05 pfu/cell) and increasing vT7-MT1 virus (0–5 pfu/cell), biotinylated and lysed, as described in the Materials and methods section. The lysates were centrifuged, and the supernatants were incubated with streptavidin–agarose beads. The beads were washed, treated with reducing Laemmli sample buffer and boiled. After centrifugation, the supernatants were subjected to immunoblot analysis with pAb 160 and mAb CA-101, against MT1-MMP$_{cat}$ and TIMP-2 respectively.

identified by zymography, and by the accumulation of active MT1-MMP observed by immunoblot analysis. Inhibition was probably mediated by TIMP-2 binding to the active MMP-2, which was either released from the ternary complex with MMP-2 or secreted by the cells independently of the MT1-MMP/TIMP-2 axis, or both. To address the source of the inhibitory TIMP-2, we used the PM fraction of the infected cells, thus avoiding TIMP-2 secretion. These studies showed a pattern of MMP-2 activity and inhibition analogous to that observed with live cells. Although in the cells, the importance of the secreted TIMP-2 cannot be ignored, the studies with the PM fractions suggest a critical role for cell-surface-associated TIMP-2 in mediating the inhibition of the activated MMP-2. Indeed, our results indicate that MMP-2 can displace PM-associated TIMP-2, resulting in inhibition of enzymic activity. However, the source of the TIMP-2 in the PM that is displaced by MMP-2 is unlikely to be the TIMP-2 bound to MT1-MMP. First, MMP-2 was not efficient in displacing TIMP-2 from a pre-formed complex of the inhibitor with a recombinant MT1-MMP$_{cat}$. Because TIMP-2 is a slow, tight-binding inhibitor of both MMP-2 and MT1-MMP [22,25], MMP-2 displacement of TIMP-2 from its binding to MT1-MMP$_{cat}$ is not prompt and requires high concentrations of MMP-2 and long incubation periods. Although this process cannot be completely ruled out, as we have shown in the displacement experiments, it is unlikely that active MMP-2 can displace MT1-MMP-bound TIMP-2 under physiological conditions, where the enzyme levels tend to be low. Secondly, whereas TIMP-2 binds to active MT1-MMP with K_d values in the nanomolar range [15,29], the inhibitor may also associate with the PM via sites that are distinct from MT1-MMP. This is suggested by the observation that the levels of TIMP-2 in the PM fractions were independent of the levels of MT1-MMP expression. Furthermore, surface biotinylation of the cells demonstrated surface-associated TIMP-2, regardless of the levels of MT1-MMP. Moreover, MT1-MMP autocatalytic processing, as determined by the presence of the 44 kDa species, could still be observed at the highest level of TIMP-2 expression suggesting that not all the TIMP-2 present in the cells interacts with MT1-MMP. Taken together, these results suggest that TIMP-2, occupying presumably weaker binding sites other than MT1-MMP on the cell surface, may be recruited by the active MMP-2 resulting in inhibition of enzymic activity. This conclusion is in partial agreement with a previous study [18], which showed that cell-surface-activated MMP-2 is inhibited by TIMP-2 bound to the PM of ConA-treated human uterine cervical fibroblasts at sites distinct from MT1-MMP. This study provided evidence for two populations of TIMP-2 binding sites on the membranes, one sensitive (MT1-MMP), and a second one (40–50 % of the sites) insensitive to synthetic hydroxamate inhibitors. In agreement with this possibility, two TIMP-2 binding sites with different affinities (K_d values of 15 and 35 nM) have also been detected in a lymphoma cell line [36]. The existence of alternate TIMP-2-binding sites on the cell surface, which can affect MMP-2 activity, may depend on the nature of the cellular background and on the levels of MT1-MMP and TIMP-2 expression. Although the nature of the low-affinity TIMP-2 binding sites remains to be determined, it is known that TIMP-2 can bind to heparin [37,38] and, therefore, may also interact with surface-associated heparan sulphate moieties.

In summary, we have shown that pro-MMP-2 activation on the cell surface yields MMP-2 free and in complex with TIMP-2 in solution. The relative amount of both forms is regulated by the MT1-MMP/TIMP-2 ratio expressed by the cells, and also by

TIMP-2 bound at sites distinct from MT1-MMP. Conversely, the accumulation of MMP-2 in solution may lead to displacement of TIMP-2 from the cell surface, in a self-regulatory mechanism. Taken together, the results of the present study emphasize the crucial role played by TIMP-2 in the regulation of MMP-2 activity, not only on the cell surface, but also in the extracellular environment, which may have important consequences for extracellular matrix remodelling *in vivo*.

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