Proteolytic processing of membrane-type-1 matrix metalloproteinase is associated with gelatinase A activation at the cell surface

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Human fibroblasts and HT-1080 fibrosarcoma cells express membrane-type-1 matrix metalloproteinase (MT1-MMP), the cell surface activator of gelatinase A, in separate forms of 63 kDa, 60 kDa and in some cases 43 kDa. In the present work the interrelationships between MT1-MMP processing and gelatinase A activation were analysed using HT-1080 fibrosarcoma cells as a model. It was found that MT1-MMP was synthesized as a 63 kDa protein, which was constitutively processed to a 60 kDa active enzyme with N-terminal Tyr^{112}, as shown by immunoprecipitation, immunoblotting and sequence analyses. Co-immunoprecipitation results indicated that only the active 60 kDa form of MT1-MMP bound gelatinase A at the cell surface. Both the activation of pro-MT1-MMP and the membrane binding of the tissue inhibitor of metalloproteinases type 2 (TIMP-2) and gelatinase A, and subsequent activation of gelatinase A, were inhibited by calcium ionophores. Although the active MT1-MMP was required for cell surface binding and activation of gelatinase A, it was inefficient in activating gelatinase A in fibroblasts or in control HT-1080 cells alone. Low expression levels of TIMP-2 and rapid synthesis of MT1-MMP were found to be critical for gelatinase A activation. In HT-1080 cells, MT1-MMP was further processed to an inactive, 43 kDa cell surface form when overexpressed, or when the cells were treated with PMA. Under these conditions, the activated gelatinase A was detected in the culture medium, in cell membrane extracts and in MT1-MMP-containing complexes. These results indicate that proteolytic processing (activation and degradation/inactivation) of MT1-MMP and MT1-MMP/TIMP-2 relationships at the cell surface are important regulatory levels in the control of gelatinolytic activity.

INTRODUCTION

Many physiological and pathological processes depend on tightly controlled proteolytic degradation of the extracellular matrix (ECM) [1]. Serine proteases, including the plasminogen activator/plasmin system, and matrix metalloproteinases (MMPs) are two protease families, which together catabolize all glycoprotein, proteoglycan and collagenous components of the ECM [2]. Gelatinases A and B of the MMP family (MMP-2 and MMP-9) digest, in addition to denatured collagen, type IV collagen of basement membranes [3,4]. Their proteolytic activity is proposed to play a critical role in, for example, the invasion of cancer cells and angiogenesis, processes that depend on the destruction of tissue barriers to facilitate the spread of cells to other tissues. Elevated expression of these enzymes by tumour cells has been demonstrated [5]. However, in the case of gelatinase A, the activation of the proenzyme by removal of the N-terminal prodomain and interaction with specific tissue inhibitors of metalloproteinases (TIMPs) correlate to a greater degree with the invasive phenotype [6] and are likely to be the key steps which determine total gelatinase A activity.

The activation of gelatinase A appears to differ from that of other MMPs, as this enzyme cannot be effectively activated by serine proteases [7,8], the suggested physiological activators of other MMPs [1]. Many reports have described the binding of gelatinase A to cell membranes and its subsequent processing and activation [9–14] by a mechanism reminiscent of the cell-surface-associated receptor/activator system for plasminogen activator [15]. Gelatinase A processing activity is induced by phorbol esters and concanavalin A and inhibited by retinoic acid [9,10]. The gelatinase A activator is present in plasma membrane fractions of various tumour cells, and its action is inhibited by inhibitors of metalloproteinases like 1,10-phenanthroline, EDTA and TIMP type 2 (TIMP-2) indicating a role for MMPs in the process. In addition, the activation of gelatinase A by PMA or concanavalin A-treated HT-1080 cells can be prevented by calcium ionophores [16].

Four cDNAs encoding MMPs with C-terminal transmembrane domains have been cloned. Their protein products have been named membrane-type-1, -2, -3 and -4 matrix metalloproteinases (MT-MMPs; MMP-14, -15, -16 and -17 respectively) [17–20]. Overexpression of MT1-MMP, MT2-MMP or MT3-MMP induces the processing of gelatinase A to its active 64 kDa and 62 kDa forms [17,19,21,22]. The activated form of MT1-MMP has been purified from HT-1080 membranes as a ternary complex with gelatinase A and TIMP-2 and found to function as a high affinity receptor for gelatinase A [8]. MT1-MMP was reported to be expressed in various human carcinomas, including colon, breast, gastric, head and neck, lung and cervical carcinomas, in association with gelatinase A [23–26]. These findings further suggest that MT-MMPs are related to the membrane activation system of gelatinase A.

Recently we have found that fibroblast and fibrosarcoma cell lysates contain the MT1-MMP protein in three separate forms of

Abbreviations used: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT1-MMP, membrane-type-1 MMP; TIMP, tissue inhibitor of metalloproteinases; TIMP-2, TIMP type 2; MEM, Eagle's minimal essential medium; Ab-1, antibody to a bacterially-generated recombinant fusion protein of *Schistosoma japonicum* glutathione S-transferase and amino acid residues 260–484 of MT1-MMP; Ab-2, antibody to a 26 amino acid residue synthetic peptide corresponding to the intracellular C-terminal domain of MT1-MMP.

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about 63 kDa, 60 kDa and in some cases 43 kDa [27]. On the other hand, most of the MT1-MMP protein is detected at the cell surface as the unprocessed zymogen of 63 kDa when overexpressed in COS cells, and also when analysed in human vascular endothelial cells [17,21,27–29]. The presence of processed or unprocessed MT1-MMP protein in different cell systems suggest that the processing is not always constitutive. Because the regulation of MT1-MMP processing is evidently an important step in the regulation of ECM degradation by activated gelatinase A and MT1-MMP itself, we carried out a study on MT1-MMP processing and its effects on gelatinase A activation.

The work reported here was presented, in part, at the 36th Annual Meeting of the American Society for Cell Biology, San Francisco, CA, U.S.A. in December 1996.

MATERIALS AND METHODS

Reagents

PMA, ionomycin, calcium ionophore A23187 and thapsigargin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and purified gelatinase A and TIMP-2 from Chemicon Co. (Temecula, CA, U.S.A.).

Cell cultures and treatment with chemicals

Human fibrosarcoma HT-1080 cells [CCL-121, American Type Culture Collection (A.T.C.C.), Rockville, MD, U.S.A.], human embryonic lung fibroblasts (CCL-137, A.T.C.C.), stably transfected HT-1080 cell clones (HT-1080 + MTpc3 and HT-1080 + ΔMTpc3) producing an approx. 10-fold excess of wild-type MT1-MMP [26], and a soluble transmembrane deletion mutant of MT1-MMP, were cultivated in Eagle's minimal essential medium (MEM) containing 10 % (v/v) heat-inactivated fetal-calf serum (Life Technologies, Gaithersburg, MD, U.S.A.), 100 units/ml penicillin and 50 μg/ml streptomycin. The cultures were incubated at 37 °C in a humidified air/CO_2 (19:1) atmosphere until confluency was reached. The cultures were rinsed twice with serum-free medium before the experiments and then incubated under serum-free conditions for 6 h. For immunoblotting and cell surface biotinylation analyses, confluent cultures of cells were treated with PMA (40 nM), ionomycin (500 nM), the calcium ionophore A23187 (500 nM) or thapsigargin (500 nM) under serum-free conditions for 24 h as indicated.

Antibodies

Polyclonal antibodies to MT1-MMP were raised in rabbits against two immunogens (see Figure 1): a bacterially generated recombinant fusion protein of *Schistosoma japonicum* glutathione S-transferase and amino acid residues 260–484 of MT1-MMP (Ab-1), and a 26 amino acid residue synthetic peptide corresponding to the C-terminal intracellular domain of MT1-MMP (Ab-2). In addition, polyclonal rabbit antibodies were raised against 26 amino acid residue synthetic peptides corresponding to the C-terminal intracellular domains of MT2-MMP and MT3-MMP. The antibodies were affinity-purified using the respective immunogens coupled to Sepharose 4B [27]. Polyclonal rabbit antibodies against TIMP-2 were from Chemicon.

SDS/PAGE and immunoblotting

The cells, on ice, were lysed with Triton lysis buffer (50 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1 % (v/v) Triton X-100, 0.02 % (w/v) sodium azide, 10 mM EDTA,

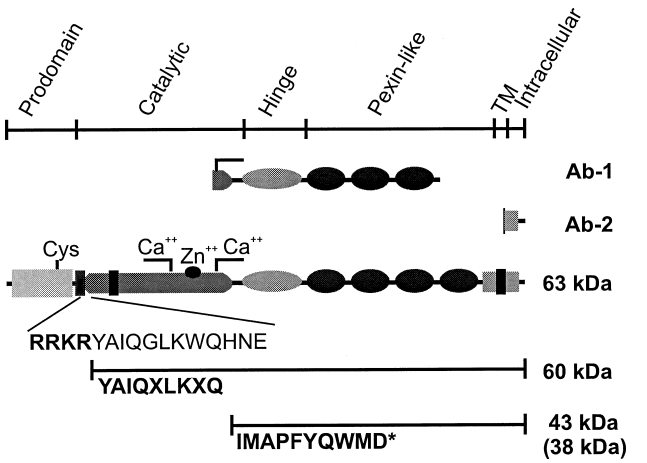


Figure 1 Different cell surface forms of MT1-MMP and the peptide sequences used for immunization

The general domain structure is according to Sato et al. [17]. The immunogens for rabbit antibodies Ab-1 and Ab-2 are shown. The RRKR recognition sequence for furin-like proteases is indicated below the 63 kDa form of MT1-MMP and the N-terminal sequence with N-terminal Tyr^{112}, obtained by peptide sequencing, is shown below the 60 kDa form. The N-terminal sequence (*) of the 43 kDa form was obtained from the soluble mutant MT1-MMP (see legend to Figure 5). The calculated molecular mass of wild-type MT1-MMP with N-terminal Ile^{256} is 37761 Da. TM, trans-membrane domain.

10 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml aminoethylbenzenesulphonyl fluoride (Calbiochem, San Diego, CA, U.S.A.) and the lysates were clarified by centrifugation. For immunoblotting, aliquots of conditioned medium (corresponding to approx. 2×10^4 cells), cell lysates (25 μg protein) and membrane extracts (6 μg protein) were subjected to SDS/PAGE as described previously [27]. Proteins were then electrophoretically transferred to nitrocellulose membranes (Gelman Sciences, Ann Arbor, ME, U.S.A.). Immunodetection of the proteins was performed as described [30].

Pulse–chase studies and immunoprecipitation

For pulse–chase studies, confluent cultures of HT-1080 and CCL-137 cells were treated with PMA (40 nM) and ionomycin (500 nM) under serum-free conditions for 8 h, incubated in methionine-free MEM containing PMA and ionomycin for 30 min, followed by metabolic labelling for 10 min with 250 μCi/ml [^{35}S]methionine (< 1000 Ci/mmol) in methionine-free MEM containing PMA and ionomycin as indicated. The medium was then removed and the cells were chased with MEM (over 500-fold excess of unlabelled methionine) containing PMA and ionomycin. At the indicated time points the cells were rinsed with PBS and lysed as described above for the immunoblotting assay.

For immunoprecipitation analysis, cell lysates were preabsorbed by incubation on ice for 1 h with preimmune serum, followed by the addition of Protein A-Sepharose beads. After incubation at 4 °C for 1 h, the beads were removed by centrifugation and the supernatants were transferred to fresh tubes. Subsequently, the samples were incubated on ice with MT1-MMP antibodies (1 μg/ml of each) for 1 h, followed by incubation with Protein A-Sepharose, with agitation, at 4 °C for 1 h. The beads were collected by centrifucation and washed three times with Triton X-100 lysis buffer and once with PBS. Bound material was eluted by Laemmli sample buffer containing 10 % (v/v) 2-mercaptoethanol and incubation at 95 °C for 5 min. The

immune complexes were resolved by SDS/PAGE and detected by fluorography.

Preparation of cell membrane extracts

Cells were washed with PBS and centrifuged at 1000 ***g*** for 5 min. The cells were then resuspended in 25 mM Tris/HCl buffer (pH 7.4) containing 8.5 % (w/v) sucrose, 50 mM NaCl, 10 mM EDTA, 10 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml aminoethylbenzenesulphonyl fluoride and homogenized by trituration with a 22-G needle. Whole cells, nuclei and heavy organelles were removed by centrifugation at 5500 ***g*** for 10 min. The supernatant was centrifuged at 100000 ***g*** for 60 min, and the pellet containing plasma membranes, Golgi and ribosomes was resuspended in 50 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1 % (v/v) Triton X-100, 0.02 % (w/v) sodium azide, 10 mM EDTA, 10 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 μg/ml aminoethylbenzenesulphonyl fluoride. The membrane extracts were then clarified by centrifugation at 14000 ***g*** for 5 min.

Gelatin zymography and reverse zymography

To analyse the gelatinolytic proteins, aliquots of cell-conditioned medium (corresponding to about 5×10^3 cells for zymography and 2×10^4 cells for reverse zymography), cell membrane extracts (HT-1080 cells, 8 μg; CCL-137 cells, 4 μg) or immunoprecipitated MT1-MMP immune complexes (from about 3×10^6 cells as starting material) were analysed by gelatin zymography essentially as described previously [31,32]. For reverse zymography 25 ng/ml gelatinase A (Chemicon) was added to the 15 % acrylamide gels containing 1 mg/ml gelatin. The washes and digestion were performed as described for gelatin zymography [31,32].

Purification and N-terminal sequencing of MT1-MMP

The 60 kDa form of wild-type MT1-MMP was immune affinity purified from HT-1080 + MTpc3 cells using MT1-MMP antibodies as described above for the immunoprecipitation analyses. The 43 kDa form of soluble mutant MT1-MMP with six additional C-terminal histidine residues was purified from HT-1080 + ΔMTpc3 cells using TALON metal affinity resin (Clontech, Palo Alto, CA, U.S.A.), according to the manufacturer's protocol. Proteins were dissolved with non-reducing Laemmli sample buffer, resolved by SDS/PAGE and electrotransferred to a PVDF membrane (Millipore Corporation, Bedford, MA, U.S.A.). After staining with Coomassie Blue R-250, the bands were excised from the membrane and peptide sequenced (Ariad Pharmaceuticals, Cambridge, MA, U.S.A.).

Cell surface biotinylation

Cells were rinsed at 4 °C with PBS and incubated in PBS containing 0.5 mg/ml of Sulfo-NHS-biotin (Pierce, Rockford, IL, U.S.A.) on ice for 1 h. The reaction was terminated by washing with 10 mM Tris/HCl buffer (pH 7.4) containing 10 mM glycine, 0.4 M sucrose, 1.5 mM $CaCl_2$ and 5 mM $MgCl_2$ for 10 min. The cells were scraped from the dish, lysed and subjected to immunoprecipitation analysis with MT1-MMP antibodies as described above. The immune complexes were resolved by SDS/PAGE, electrotransferred to nitrocellulose and detected with horseradish-peroxidase-conjugated streptavidin (Dako A/S, Copenhagen, Denmark) and an enhanced chemiluminescence detection system (Pierce).

RESULTS

Inhibition of plasma-membrane association and activation of gelatinase A by ionomycin

It was found previously that the activation of gelatinase A by HT-1080 cells treated with PMA was completely blocked by calcium ionophores, e.g. ionomycin [16]. To analyse the effects of ionomycin on the activation of gelatinase A in an MT1-MMP overexpression system, stably transfected HT-1080 cells (HT-1080 + MTpc3) producing a 10-fold excess of MT1-MMP were treated with PMA and ionomycin, and aliquots of the conditioned medium and membrane extracts were analysed by gelatin zymography. Wild-type HT-1080 cells and CCL-137 fibroblasts were used as controls. Zymograms of the conditioned media indicated that MT1-MMP overexpression in HT-1080 cells resulted in the ability of these cells to process gelatinase A to the activated 64 kDa and 62 kDa forms, and that the activation was slightly enhanced by PMA treatment (Figure 2A). Treatment of the cells with ionomycin, which has no notable effect on MT1-MMP mRNA levels [16], prevented gelatinase A activation induced by MT1-MMP overexpression, in both the presence and absence of PMA, as efficiently as it prevented PMA-induced gelatinase A activation in wild-type HT-1080 cells (Figure 2A). Conditioned medium of normal fibroblasts (CCL-137) contained high levels of gelatinase A, which remained in the latent form under all conditions analysed (Figure 2A).

Analysis by gelatin zymography of the respective cell membrane extracts (Figure 2B) indicated that control HT-1080 cells contained cell-membrane-associated gelatinase A, mainly in the latent form, whereas gelatinase B was not membrane associated. Membranes of HT-1080 + MTpc3 cells and PMA-treated HT1080 cells contained gelatinase A, which was mainly activated to the 64 kDa and 62 kDa forms. Membranes of ionomycin-treated cells contained, on the contrary, only very low or negligible levels of the membrane-bound latent gelatinase A (Figure 2B). Membranes of normal fibroblasts contained significantly higher levels of gelatinase A than HT-1080 cell membranes, mainly in the latent form. PMA treatment increased and ionomycin decreased the levels of membrane-bound gelatinase A in fibroblasts.

To study the interactions of MT1-MMP with gelatinase A we carried out co-immunoprecipitation analyses of cell lysates. After concentration by immunoprecipitation with MT1-MMP antibodies (Ab-1 and Ab-2), MT1-MMP was also observed to digest gelatin in zymograms, unlike cell-conditioned medium or membrane extracts. This lower-molecular-mass gelatinolytic protein in the immune complexes (MT1-MMP in Figure 2C) was detected, by metabolic labelling, as the 60 kDa form of MT1-MMP (see Figure 4) with N-terminal Tyr^{112} as demonstrated below. No gelatinolytic bands corresponding to the unprocessed 63 kDa or degraded 43 kDa forms of MT1-MMP were detected under any conditions, suggesting that SDS is ineffective in inducing autoactivation of latent MT1-MMP. Ionomycin treatment appeared to decrease the levels of active MT1-MMP.

Gelatin zymograms of the immune complexes indicated that gelatinase A co-precipitated with MT1-MMP in HT-1080 + MTpc3 and CCL-137 cells (Figure 2C). The levels of latent and activated gelatinase A in the immune complexes of control and PMA-treated cells correlated with their levels in the culture medium and membrane extracts (Figures 2A and 2B). Only low levels of latent gelatinase A were detected in the immune complexes from ionomycin-treated cells. Thus ionomycin prevented the membrane binding of gelatinase A, its association with MT1-MMP and subsequent activation. Gelatin zymography of the proteins immunoprecipitated from the conditioned me-

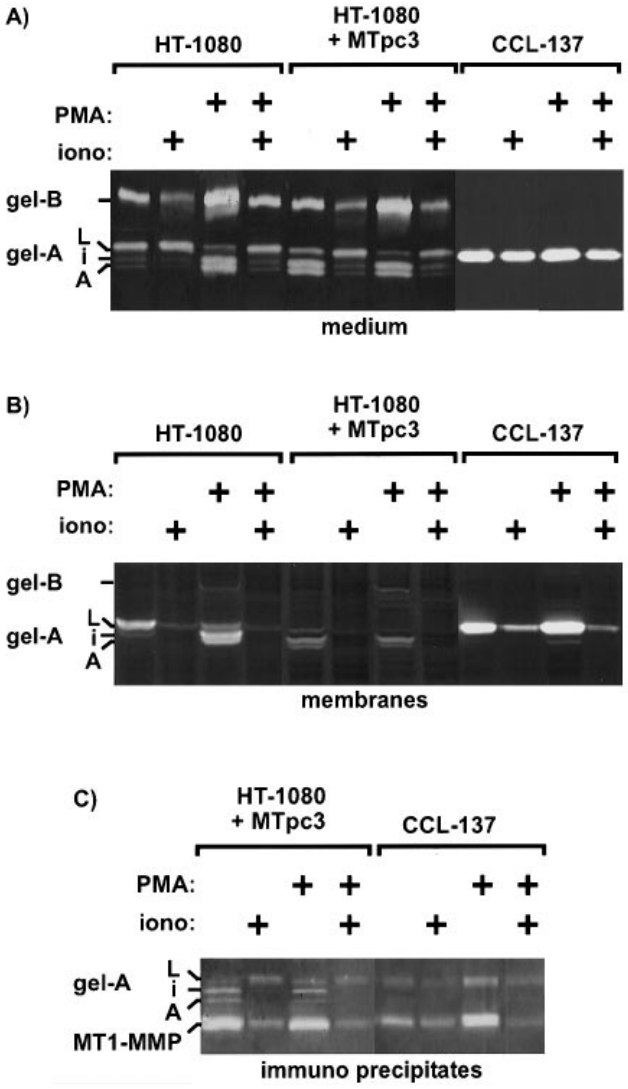


Figure 2 Gelatin zymograph showing ionomycin inhibition of cell-surface association and activation of gelatinase A in cells overexpressing MT1-MMP

Confluent cultures of wild-type HT-1080 fibrosarcoma cells, transfected HT-1080 cell clone producing 10-fold excess of MT1-MMP (HT-1080 + MTpc3) and normal fibroblasts (CCL-137) were treated with PMA (40 nM), ionomycin (iono, 500 nM) or both for 24 h as shown. (**A**) Conditioned media. (**B**) Membrane extracts. (**C**) MT1-MMP immunoprecipitates of the cell lysates (Ab-1 and Ab-2). gel-B, PMA-inducible 92 kDa gelatinase B; gel-A, gelatinase A; L, latent form of gelatinase A; i, intermediate form of gelatinase A; A, active form of gelatinase A.

dium with MT1-MMP antibodies did not yield any gelatinolytic bands (results not shown), indicating that the antibodies did not cross-react with gelatinase A.

Prevention of processing of the latent MT1-MMP by calcium ionophores

It was found previously that HT-1080 and CCL-137 cell lysates contained MT1-MMP in three forms of approx. 63 kDa, 60 kDa and, in some cases, 43 kDa in size [27]. To analyse the effects of ionomycin on the proteolytic processing of MT1-MMP to its lower-molecular-mass forms, HT-1080, HT-1080 + MTpc3 and CCL-137 cells were treated with PMA and/or ionomycin for 24 h. Subsequently the cells were lysed and the levels of different

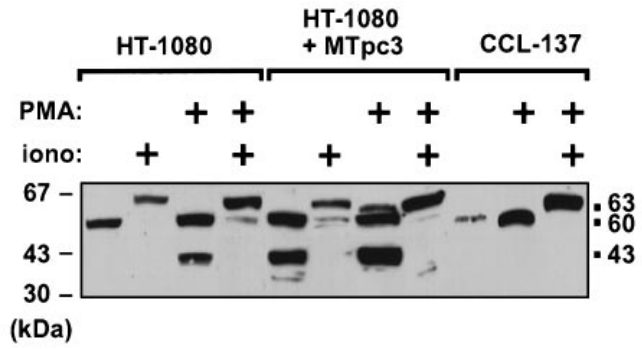


Figure 3 Immunoblot showing stimulation by PMA and inhibition by ionomycin of the processing of the 63 kDa MT1-MMP to the 60 kDa and 43 kDa forms

Confluent cultures of HT-1080 fibrosarcoma cells, HT-1080 + MTpc3 cells (transfected HT-1080 cell clone producing 10-fold excess of MT1-MMP) and CCL-137 human lung fibroblasts were treated with PMA (40 nM), ionomycin (iono, 500 nM) or both in serum-free conditions for 24 h. Cells were then lysed and aliquots of cell lysates were subjected to SDS/PAGE. The proteins were transferred to nitrocellulose and immunostained for MT1-MMP protein using antibodies against the intracellular part of MT1-MMP (Ab-2). Molecular-mass markers (kDa) are shown on the left and the molecular masses of the MT1-MMP immunoreactive proteins are indicated on the right (63, 60, 43).

MT1-MMP protein forms were analysed by immunoblotting using Ab-2. Inspection of the immunoblots indicated that in control HT-1080 and CCL-137 cells, MT1-MMP was present mainly in a 60 kDa form, whereas in PMA-treated HT-1080 cells, and to a greater extent in HT-1080 + MTpc3 cells, the N-terminally processed 43 kDa form was also detected (Figure 3). PMA treatment of CCL-137 cells caused an increase (approx. 5-fold, based on scanning) in the levels of the 60 kDa MT1-MMP protein, but no 43 kDa form was detected. Treatment of all cell types with ionomycin, either alone or in the presence of PMA, resulted in the appearance of a major MT1-MMP protein band of 63 kDa and a decrease in the 60 kDa protein band. In addition, the generation of the 43 kDa processing product was inhibited (Figure 3). Similar results (not shown) on the processing of MT1-MMP were obtained with the calcium ionophore A23187 and thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPase, all of which inhibit the activation of gelatinase A [16].

To study the contribution of other MT-MMPs in gelatinase A association and activation, HT-1080 cell lysates were analysed by immunoblotting using antibodies against the intracellular domains of MT2-MMP and MT3-MMP. The results indicated that the cells expressed no detectable levels of these enzymes (results not shown).

Effects of MT1-MMP overexpression, PMA and ionomycin on MT1-MMP synthesis and processing

To analyse the temporal profiles of MT1-MMP processing and to discover if the 60 kDa and 43 kDa proteins were processing products of the 63 kDa protein, pulse–chase experiments were performed. HT-1080, HT-1080 + MTpc3 and CCL-137 cells were treated with PMA and ionomycin, pulse labelled with [^{35}S]-methionine and chased with MEM containing PMA and ionomycin. The cells were lysed at intervals between 0 and 16 h and the lysates were subjected to immunoprecipitation with MT1-MMP antibodies (Ab-1 and Ab-2).

Analysis of the immunoprecipitates indicated that MT1-MMP is synthesized as a 63 kDa zymogen, which was the major form

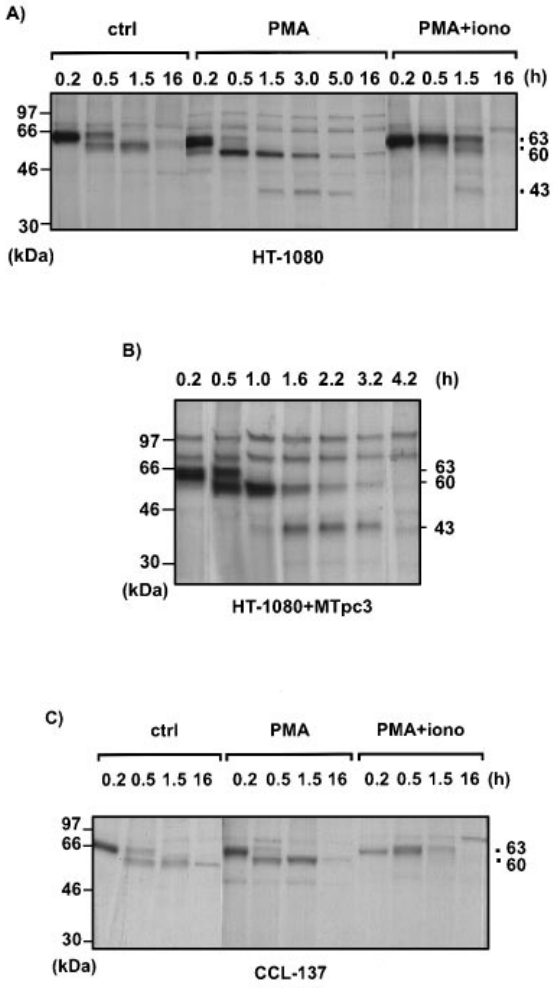


Figure 4 Immunoprecipitation and pulse–chase analysis of HT-1080 cells, HT-1080 + MTpc3 cells and CCL-137 fibroblasts

Confluent cultures of (**A**) HT-1080 cells and (**C**) CCL-137 fibroblasts were treated with PMA (40 nM) or ionomycin (500 nM) (iono) in serum-free conditions for 8 h; ctrl, control cells. (**B**) Confluent cultures of HT-1080 + MTpc3 cells were incubated in serum-free conditions for 8 h. Subsequently, the cells were labelled with 250 μCi/ml [^{35}S]methionine in methionine-free conditions for 10 min and chased with MEM containing > 500-fold excess of unlabelled methionine. The cells were lysed at the times indicated above the gels and the cell lysates were immunoprecipitated with both Ab-1 and Ab-2. The precipitates were subjected to SDS/PAGE (4–15% gradient gel) followed by fluorography. The migration of the immunoprecipitated MT1-MMP protein bands (63 kDa, 60 kDa and 43 kDa) is indicated on the right. Molecular-mass markers are shown on the left.

present after 0.2 h of chase in all cell types regardless of treatment (Figures 4A–4C). In control HT-1080 and CCL-137 cells, about half of the labelled MT1-MMP protein was processed to the 60 kDa form within 0.5 h of chase and almost total processing was seen after 1.5 h of chase (Figures 4A and 4C). The 60 kDa form corresponds to the major form present in the immunoblots of control and PMA-treated HT-1080 and CCL-137 cells (see Figure 3). In PMA-treated wild-type HT-1080 cells and HT-1080 + MTpc3 cells the processing of the 63 kDa form to the 60 kDa form was accelerated to some extent during the first 0.5 h of chase, but the main differences were the faster disappearance of the 60 kDa form and the appearance of a 43 kDa processed form of MT1-MMP, which was detectable after 1.5 h of chase (Figures 4A and 4B). In CCL-137 cells PMA did not induce any further processing of the 60 kDa form, and the processing profiles of control and PMA-treated cells were similar (Figure 4C). Under conditions where processing to the 43 kDa form was not detected, the radioactivity corresponding to MT1-MMP protein was gradually decreased from the level observed at 1.5 h of chase to the barely detectable levels present after 16 h of chase (Figure 4). Non-specific bands of approx. 46 kDa and approx.75–95 kDa were occasionally detected and the levels of these did not change over the time of the chase procedure.

Pulse–chase analysis also indicated that ionomycin, when added together with PMA, almost totally prevented the processing of labelled MT1-MMP in HT-1080 cells during the first 0.5 h of chase. After 1.5 h of chase, minor radioactive MT1-MMP bands of 60 kDa and 43 kDa in size appeared (Figure 4A). The treatment of HT-1080 cells with ionomycin alone totally prevented this processing, and thapsigargin alone inhibited the processing to about the extent seen in cells treated with both ionomycin and PMA, except that no 43 kDa form was seen (results not shown). In CCL-137 fibroblasts, ionomycin almost totally prevented the processing of the 63 kDa zymogen, even in the presence of PMA (Figure 4C).

Correspondence of the 60 kDa form to an active and the 43 kDa form to an inactive form of MT1-MMP

To localize the cleavage site, the 60 kDa protein was immune affinity purified from HT-1080 + MTpc3 cells for N-terminal sequencing using Ab-2. After 9 cycles of sequence analysis the 60 kDa band was identified as MT1-MMP with N-terminal Tyr^{112} (sequence: YAIQXLKXQ) (Figure 1). This form corresponded to the active MT1-MMP, where the approx. 10 kDa propeptide domain had been cleaved after the RRKR recognition sequence for proprotein convertases [33]. In repeat experiments, we were unsuccessful in obtaining the N-terminal sequence of the 43 kDa form with wild-type enzyme.

To study the molecular forms of a soluble ΔMT1-MMP mutant in cell culture, HT-1080 cells were transfected with an expression vector containing the coding sequence for amino acids 1–539 of MT1-MMP and six additional C-terminal histidines. Stably transfected cell clones were then selected (HT-1080 + ΔMTpc3). Immunoblotting analysis of the conditioned media, using Ab-1 against the extracellular part of MT1-MMP, indicated that ΔMT1-MMP protein was present mainly as forms that migrated less than the 60 kDa and 43 kDa forms of wild-type MT1-MMP (Figure 5). The cell lysates of HT-1080 + MTpc3 were used as controls. The immunoreactive 43 kDa protein from HT-1080 + ΔMTpc3-conditioned medium was purified using a metal-affinity resin that binds to the C-terminal histidines and analysed by N-terminal sequencing. It was identified as soluble MT1-MMP with N-terminal Ile^{256} (sequence: IMAPFYQW-MD), thus lacking the zinc-binding catalytic site. The calculated molecular mass of wild-type MT1-MMP with N-terminal Ile^{256} was 37761 Da. We, however, prefer to call it the 43 kDa form because this reflects its mobility on SDS/PAGE.

Detection of MT1-MMP at the cell surface

To analyse the cell-surface exposure of the different forms of MT1-MMP, confluent cultures of HT-1080 and CCL-137 cells were treated with ionomycin and PMA and surface-labelled with biotin. Subsequently, the cell lysates were subjected to immunoprecipitation with MT1-MMP antibodies (Ab-1 and Ab-2). Analysis of the immunoprecipitates revealed the presence of

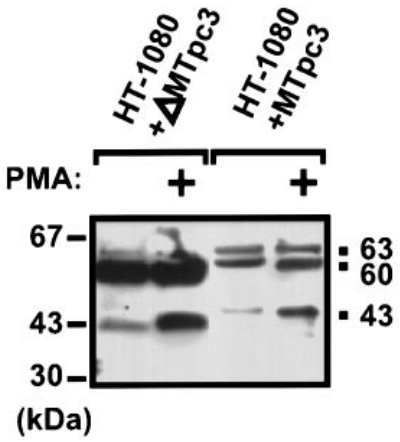


Figure 5 Immunoblot of HT-1080 + MTpc3 and HT-1080 + ΔMTpc3 cells treated with PMA

Confluent cultures of HT-1080 + MTpc3 and HT-1080 + ΔMTpc3 cells (transfected HT-1080 cell clones producing wild-type and soluble mutant MT1-MMP proteins) were treated with PMA (40 nM) under serum-free conditions for 24 h. Aliquots of cell lysates (HT-1080 + MTpc3) or conditioned medium (HT-1080 + ΔMTpc3) were subjected to SDS/PAGE (10% gel). The proteins were transferred to nitrocellulose and immunostained for MT1-MMP protein using Ab-1. The N-terminal sequence of the 43 kDa protein shown in Figure 1 was obtained from the soluble 43 kDa protein shown here. Migration of the MT1-MMP immunoreactive proteins is indicated on the right (63, 60 and 43). The molecular-mass markers are shown on the left.

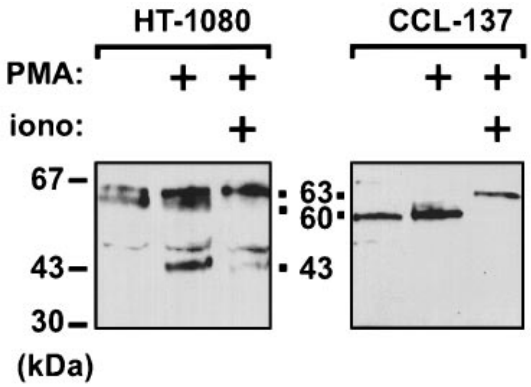


Figure 6 Cell-surface labelling of MT1-MMP to show that the 63 kDa, 60 kDa and 43 kDa forms are exposed at the cell surface

Confluent cultures of HT-1080 fibrosarcoma cells and CCL-137 fibroblasts were treated with PMA (40 nM) and ionomycin (iono, 500 nM) under serum-free conditions for 24 h. Cells were surface labelled with Sulfo-NHS-biotin. The cell lysates were immunoprecipitated with Ab-1 and Ab-2. The immune complexes were separated by SDS/PAGE (4-15% gradient gel), transferred to nitrocellulose and detected with horseradish peroxidase-conjugated streptavidin followed by enhanced chemiluminescence. Migration of the MT1-MMP protein bands (63, 60, 43) is indicated between the panels. Molecular-mass markers are shown on the left.

biotinylated 60 kDa and 63 kDa forms of MT1-MMP at the surface of both the control and PMA-treated HT-1080 cells and the presence of the 60 kDa form at the surface of control and PMA-treated CCL-137 cells (Figure 6). The treatment of HT-1080 cells with PMA induced the appearance of a biotinylated 43 kDa form of the enzyme. The unprocessed 63 kDa form was the main form in both fibroblasts and HT-1080 cells treated with both PMA and ionomycin. In addition, some of the 43 kDa form was detected at the surface of ionomycin-treated HT-1080 cells.

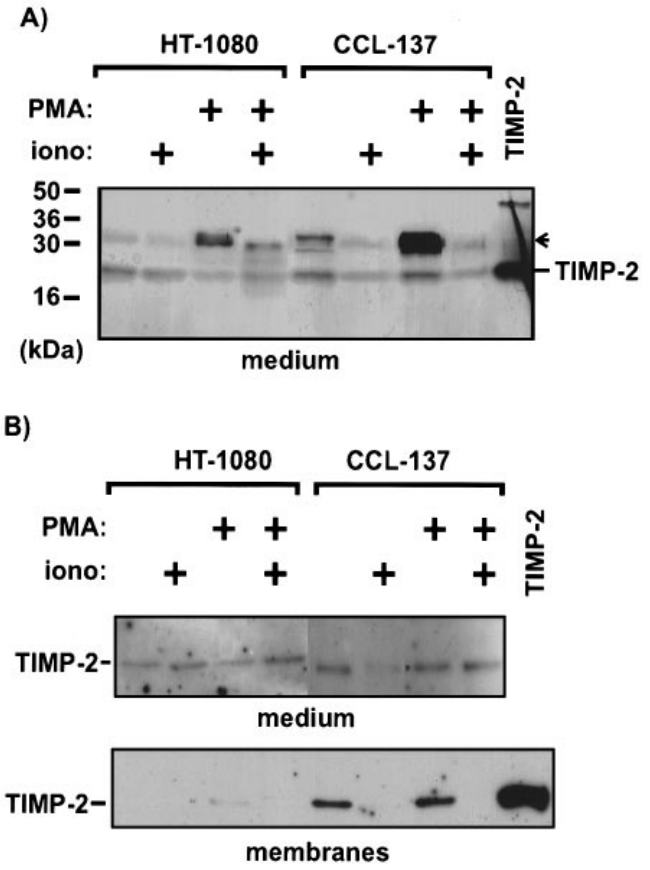


Figure 7 Reverse zymography and immunoblot showing that ionomycin inhibits the cell-surface association of TIMP-2

Confluent cultures of HT-1080 fibrosarcoma cells and CCL-137 fibroblasts were treated with PMA (40 nM), ionomycin (iono, 500 nM) or both in serum-free conditions for 24 h. (**A**) Reverse zymography of conditioned media. The 30 kDa bands with gelatinase A inhibitory activity, which most probably correspond to TIMP-1, are indicated with an arrowhead. The minor bands between 21 and 30 kDa correspond probably to other TIMPs. (**B**) Immunoblot of conditioned medium and membrane extracts with TIMP-2 antibodies. Purified TIMP-2 protein was used as a control.

Analysis of TIMP-2 expression

To investigate the expression levels of 21 kDa TIMP-2 protein, aliquots of conditioned medium and membrane extracts from HT-1080 cells and fibroblasts were analysed by reverse zymography and immunoblotting with anti-TIMP-2 antibodies. Reverse zymograms of the conditioned media indicated that in HT-1080 cells TIMP-2 levels were slightly decreased by PMA treatment (Figure 7A). Ionomycin had no notable effect on the TIMP-2 levels. In the conditioned media of normal fibroblasts (CCL-137), TIMP-2 levels were slightly decreased after ionomycin treatment (Figure 7A). The most prominent effect of PMA was the induction of a 30 kDa protein with gelatinolysis inhibitory activity. This band was most likely TIMP-1, and it was not analysed further.

The results of immunoblotting of the conditioned-media TIMP-2 protein were in accord with the results of the reverse zymography (Figure 7B). Membrane-associated TIMP-2 protein was detectable only as a very faint band after PMA treatment in HT-1080 cells by immunoblotting. The same quantities of HT-1080 + MTpc3 cell membrane extracts contained no detectable

levels of TIMP-2 (results not shown). In contrast, membranes of control and PMA-treated CCL-137 cells contained significantly higher levels of TIMP-2 protein. Ionomycin treatment decreased the levels of membrane-associated TIMP-2 in CCL-137 cells, in accordance with the levels of latent gelatinase A detected in membrane extracts by zymography (Figure 2B).

DISCUSSION

The present study was carried out to aid understanding of the interrelationships between MT1-MMP processing and gelatinase A activation at the cell surface. HT-1080 fibrosarcoma cells were used as a cell-culture model for gelatinase A activation. They produce mainly latent gelatinase A but can be induced to activate it by treatment with PMA, concanavalin A or by MT1-MMP overexpression. In a previous study we found evidence for proteolytic processing of MT1-MMP, in association with gelatinase A activation [27]. Human embryonic lung fibroblasts, which produce high levels of latent gelatinase A that is not activated even when MT1-MMP expression levels are increased by PMA or concanavalin A [27], were used as a control.

In the light of the recent studies, active MT1-MMP seems to have a dual role in the cell-membrane-associated activation of gelatinase A: first, it functions, together with TIMP-2, as a receptor for gelatinase A [8] and, secondly, it is a proteolytically active processing enzyme [33–35]. The results of the present work support the receptor role of MT1-MMP because (i) the activation/processing of MT1-MMP is required for its association with TIMP-2 and gelatinase A and (ii) these associations are required for gelatinase A activation in HT-1080 cells. The protease role of MT1-MMP is evident because the balance between MT1-MMP, TIMP-2 and gelatinase A expression in our cell models suggests a requirement for rapidly synthesized, uncomplexed MT1-MMP, which can proteolytically activate gelatinase A in adjacent complexes.

Prevention of gelatinase A activation by ionomycin

Gelatinase A is purported to bind to the cell surface through a mechanism that involves the formation of a complex consisting of activated MT1-MMP, TIMP-2 and gelatinase A [8,36–40]. In this complex the N-terminal inhibitory domain of TIMP-2 binds to the catalytic centre of activated MT1-MMP [40], whereas the C-terminal domain of TIMP-2 interacts with the C-terminal domain of progelatinase A [38]. The processing of MT1-MMP to expose its catalytic centre to allow interactions (activated form) would thus be a prerequisite for the formation of this kind of complex.

We found by pulse–chase experiments that MT1-MMP was synthesized as a 63 kDa protein, which was constitutively processed to the activated 60 kDa MT1-MMP with N-terminal Tyr^{112}. This processing/activation of MT1-MMP was prevented by ionomycin. Concomitantly, the cell-surface binding of TIMP-2 and gelatinase A and activation of gelatinase A were inhibited. These findings are suggestive of a requirement for MT1-MMP activation and subsequent association with TIMP-2 and gelatinase A for gelatinase A activation. In ionomycin-treated cells the 63 kDa zymogen was present at the cell surface, which excludes the possibility that ionomycin might prevent the transport of MT1-MMP to the cell surface. In the surface labelling analyses of HT-1080 cells, the 63 kDa form was disproportionally highly labelled when compared with the labelling of the major 60 kDa form. A probable explanation is the association of TIMP-2 and gelatinase A with MT1-MMP soon after its activation and the less effective biotinylation of the 60 kDa MT1-MMP after complex formation.

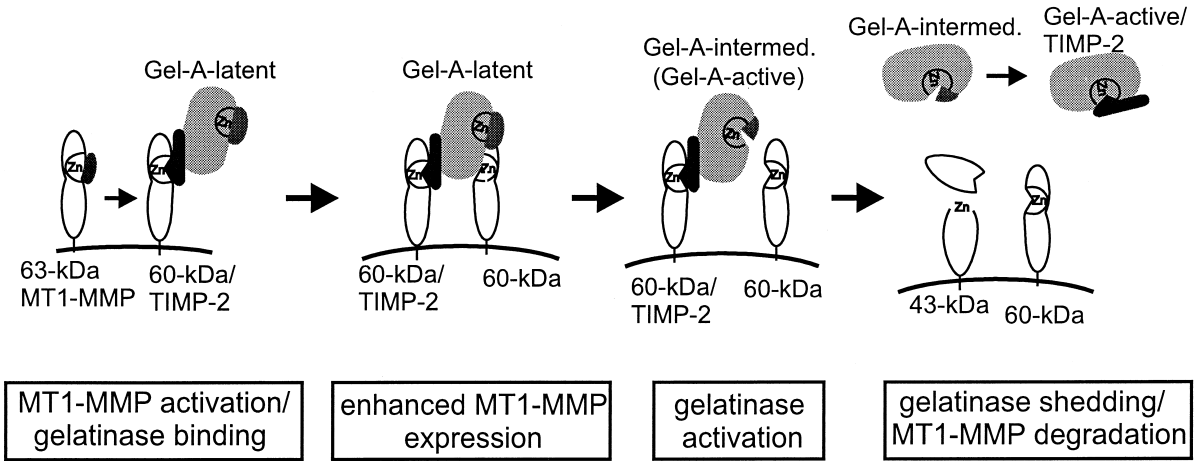
Reliance on MT1-MMP/TIMP-2 relationships at the cell surface for gelatinase A activation

Several studies have indicated that MT1-MMP is able to catalyse the initial cleavage in the propeptide part of progelatinase A [33–35]. The intermediate form of gelatinase A is then autoproteolytically cleaved to the fully active form [28,34,35]. However, several cell types, such as normal fibroblasts, MDA-MB-231 breast cancer cells and hepatic stellate cells, which contain high levels of MT1-MMP mRNA and activated MT1-MMP and MT1-MMP–gelatinase A complexes at the cell surface, are unable to activate gelatinase A [14,27,41]. In the present work, it was found that neither the presence nor levels of active MT1-MMP alone could explain gelatinase A activation. The induction of MT1-MMP expression produced gelatinase A activation in fibrosarcoma cells, whereas in normal fibroblasts, a comparable level of active MT1-MMP was inefficient in gelatinase A activation. These differences suggest that gelatinase A activation is dependent on the balance of many components at the cell surface.

Pulse–chase experiments indicated that MT1-MMP was more rapidly synthesized in fibrosarcoma cells than in normal fibroblasts, and that its turnover was also accelerated under overexpressing conditions in fibrosarcoma cells. With immunoblotting, the steady-state levels of active MT1-MMP were, however, more constant. Conversely, although the TIMP-2 mRNA levels were similar in fibroblasts and fibrosarcoma cells and were not changed by PMA, concanavalin A or ionomycin [27], TIMP-2 protein levels were greater in fibroblasts, especially in the cell membranes. Likewise, the amount of membrane-bound gelatinase A was higher in fibroblasts than in HT-1080 cells. It thus appears that, in a situation where the slowly synthesized MT1-MMP is immediately complexed with TIMP-2 and gelatinase A in fibroblasts, there is no active MT1-MMP available for proteolytic activation of gelatinase A. TIMP-1 was found to be strongly induced by PMA in fibroblasts. However, TIMP-1 is a poor inhibitor of MT1-MMP [34] and can bind only to active gelatinase A [42]. Thus TIMP-1 is of little if any significance in the gelatinase A activation process. Overall, in the cell models used in the present work, gelatinase A was activated when MT1-MMP was rapidly synthesized and the expression levels of TIMP-2 and gelatinase A were relatively low.

Association of MT1-MMP degradation with gelatinase A activation

The one direct correlation between MT1-MMP protein expression and gelatinase A activation is the generation of the 43 kDa form of MT1-MMP [27]. The pulse–chase experiments of the present study indicated that the 60 kDa MT1-MMP is N-terminally processed to the 43 kDa form within 1–4 h of translation and disappears relatively soon thereafter. The accessibility of this form to cell surface labelling excludes the possibility that it could be an intracellular degradation product. The 43 kDa form was proteolytically inactive in zymographic analyses of MT1-MMP immune complexes. In addition, the N-terminal sequence of the 43 kDa form of ΔMT1-MMP indicated that this form lacks the zinc- and calcium-binding catalytic region. The results of immunoblotting suggest that the soluble mutant form is proteolytically processed in a manner similar to that of the wild-type MT1-MMP. Assuming that the cleavage site is the same in the wild-type MT1-MMP as in the soluble mutant, the 43 kDa form does not contain the catalytic site for TIMP-2 binding. These results suggest that the 43 kDa form represents an inactive by-product, reflecting the consumption of MT1-MMP in the activation and release of gelatinase A. An MMP-mediated processing to a 43 kDa form is suggested by the finding that the



Scheme 1 Proposed events in MT1-MMP-mediated activation of gelatinase A at fibrosarcoma cell surface

MT1-MMP is synthesized as a 63-kDa zymogen, which is constitutively processed to the active 60-kDa enzyme lacking the propeptide. Latent gelatinase A and TIMP-2 can associate with the active 60-kDa form of MT1-MMP to generate a complex, where TIMP-2 presumably interacts with the catalytic site of MT1-MMP and with the C-terminal domain of gelatinase A (gelatinase binding). MT1-MMP overexpression and PMA treatment enhance local MT1-MMP levels and lead to a gelatinase A activating state. Free MT1-MMP may then activate the latent gelatinase A by proteolysis. This step occurs through an initial cleavage of gelatinase A to an intermediate 64-kDa form by MT1-MMP, followed by autoproteolytic activation of gelatinase A to the 62-kDa form [28] (gelatinase activation). The membrane-bound gelatinase A is likely to retain its activity, since the interaction of TIMP-2 with the C-terminal domain of gelatinase A interferes with the binding of another TIMP molecule to the active site of gelatinase A [44]. The complex is, however, unstable. Gelatinase A dissociates from MT1-MMP to a soluble form, presumably by cleaving MT1-MMP to the observed inactive 43-kDa form, which disappears from the cell surface relatively soon thereafter (gelatinase shedding/MT1-MMP degradation). Alternatively, the affinity of activated gelatinase A to MT1-MMP is lower, and gelatinase A dissociates from the complex and free MT1-MMPs undergo autoinactivation. It is not known whether the newly released activated gelatinase A retains its activity for some time or whether it is immediately inactivated by TIMP-2. In general, all of the gelatinase A in the culture medium of fibrosarcoma cells is complexed with TIMP-2 and is thus inactive [16]. The fate of the TIMP-2 molecule from the activation complex is not clear, but it might bind to the newly released gelatinase A thus inactivating it. The Scheme excludes the role of the cell-surface plasminogen-activator system, which is known to contribute to gelatinase A activation [47]. The catalytic sites in the enzymes are indicated by Zn, light and dark shading illustrates the propeptides and TIMP-2 is in black.

metalloproteinase inhibitor Ro-31-9790 prevented both the activation of gelatinase A and processing of MT1-MMP to the 43 kDa form, whereas processing to the 60 kDa form occurred as usual (H. Valtanen, K. Lehti, J. Lohi and J. Keski-Oja, unpublished work). A possible explanation for the correlation between the accelerated turnover of MT1-MMP via a 43 kDa form and the release of active gelatinase A to the culture medium is that, upon or after activation, gelatinase A releases itself from MT1-MMP complexes by cleaving MT1-MMP to the 43 kDa form. However, it is also possible that MT1-MMP inactivates itself or adjacent MT1-MMPs to the 43 kDa form (see Scheme 1). Similar MMP-dependent inactivation has recently been described for stromelysin-3 [43].

Regulation of molecular interactions and gelatinolytic activity

It has been found previously that, in addition to gelatinase A activation, the pericellular gelatinolytic activity in HT-1080 fibrosarcoma cell culture is induced by PMA and inhibited by ionomycin [16]. Ionomycin also inhibits, to some extent, the expression of latent gelatinase B in HT-1080 cell culture [16]. Activated gelatinase A is relatively insensitive to inhibition by TIMP-2 in the complexes, because its C-terminal domain is still bound by TIMP-2 to MT1-MMP [44]. This interaction interferes sterically with the binding of another TIMP-2 molecule. It is not known whether the newly released gelatinase A retains its activity locally in the pericellular space, or whether it is instantly inactivated by TIMP-2 from the complex or by free TIMPs. However, the correlation between gelatinase A activation and pericellular gelatinolytic activity suggests that local gelatinase A activity can be generated in HT-1080 cell culture without immediate inhibition by TIMPs. The binding of gelatinase A to other cell-surface receptors such as $\alpha_V\beta_3$ integrins [45] upon or after MT1-MMP degradation could be a secondary event to target the activity.

The results of experiments *in vitro*, where MT1-MMP was either overexpressed or where purified gelatinase A and MT1-MMP were mixed, could be explained *in vivo* by the binding of enzymes at specific regions of the cell membrane to increase their focal concentrations. During gelatinase A activating conditions, gelatinase A has been found, together with MT1-MMP, to be concentrated at invadopodia (unique surface structures in invading cells containing ECM-degrading proteolytic enzymes) [28,46]. During physiological regulation of gelatinolytic activity, high local MT1-MMP concentrations could be achieved by oligomerization of MT1-MMP in invadopodia. An activated MT1-MMP molecule with a TIMP-free catalytic site would be able to activate gelatinase A in a neighbouring complex.

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