# Activation of gelatinase–tissue-inhibitors-of-metalloproteinase complexes by matrilysin

Dorothea C. VON BREDOW*, Anne E. CRESS†, Eric W. HOWARD‡, G. Timothy BOWDEN† and Raymond B. NAGLE*[1]

*Department of Pathology, University of Arizona, Tucson, AZ 85724, U.S.A., †Department of Radiation Oncology, University of Arizona, Tucson, AZ 85724, U.S.A., and ‡Department of Pathology, University of Oklahoma, Oklahoma City, OK 73190, U.S.A.

Matrilysin, gelatinase A and gelatinase B are matrix metalloproteinases (MMPs) implicated in normal and pathological processes that require remodelling of the extracellular matrix. In human prostate tissue, matrilysin is synthesized in ducts surrounded by inflammatory cells, and focally in prostate carcinoma, but not in normal glands. Gelatinase B expression is restricted to inflammatory cells. Gelatinase A can be found in both benign and malignant prostate tissue. MMP activities are regulated by their transition from latent to activated forms, as well as by the presence of tissue inhibitors of metalloproteinases (TIMPs). We investigated whether matrilysin can activate progelatinases A and B in the presence of their bound inhibitors TIMP2 and TIMP1 respectively. Incubation of progelatinase B–TIMP1 complex with active matrilysin resulted in 78 and 68 kDa active forms, as measured by SDS–PAGE and enzyme activity assays. TIMP-free gelatinase B was also activated by matrilysin. In addition, activation of progelatinase B by matrilysin was demonstrated in the conditioned medium of phorbol ester-treated HT1080 cells, confirming the results obtained in the *in vitro* experiments. In contrast, matrilysin did not proteolytically cleave gelatinase A–TIMP2 complex, but led to a transient increase in gelatinolytic activity of the proenzyme. Matrilysin did not enhance the autocatalytic conversion of its own proform. The data presented here suggest that matrilysin participates in a proteolytic cascade and can activate gelatinases in the presence of TIMPs.

## INTRODUCTION

Matrix metalloproteinases (MMPs) are important enzymes in many normal and pathological processes that require degradation and remodelling of the extracellular matrix [1–3]. Many types of cancer have been associated with increased MMP activity, and their presence can contribute to tumour cell invasion and metastasis [4,5]. Gelatinases A and B, and matrilysin, are three of the at least sixteen different MMPs identified so far.

MMPs are secreted as proenzymes and are activated by proteolytic removal of the N-terminal prodomain. Experimentally, this can be achieved with mercurial compounds such as 4-aminophenyl mercuric acetate (APMA), which leads to perturbation of the enzyme conformation and to autocatalytic cleavage [6]. Identification of physiological activators for MMPs has been an important research question in recent years. Many pro-MMPs can be activated by active MMPs. Examples include the activation of progelatinase B by stromelysin [7] or gelatinase A [8], or the activation of procollagenase I or promatrilysin by stromelysin [9]. Stromelysin 3 and membrane-type (MT)-MMPs are thought to be activated by serine proteases of the furin type (reviewed in [1]). Activity of an MMP from which the prodomain has been removed can further be regulated by the presence or absence of tissue inhibitors of metalloproteinases (TIMPs) (reviewed in [10]). So far, three different TIMPs have been identified in humans. Although TIMP-1 and TIMP-2 are able to inhibit the active forms of all MMPs, TIMP-1 binds preferentially to progelatinase B [11,12], and TIMP-2 can be found in tight association with progelatinase A [13,14]. Expression of progelatinase B and TIMP1 appear to be co-regulated [15–17].

Interactions of MMPs with TIMPs are facilitated by the C-terminal haemopexin domain [18]. The only MMP identified to date that does not have this domain is matrilysin, which therefore is expected to be less susceptible to inhibition by TIMPs. Matrilysin is overexpressed in many types of cancer, such as prostate [19], colon, gastric [20] or squamous-cell carcinomas [21]. In prostate and squamous-cell carcinomas, matrilysin is synthesized very focally, with tumour cells expressing high amounts of both protein and mRNA next to cells that are completely negative [22]. However, matrilysin can also be present abundantly in non-malignant tissue, for example in inflamed ducts in prostate [22] or in human endometrium [23], in a hormonally regulated mechanism involving transforming growth factor-$\beta$ [24]. The function of this expression pattern is unclear. Taking into account the fact that matrilysin expression appears to be highly regulated, this enzyme may have a regulatory function itself. One putative regulatory mechanism of matrilysin would be the activation of other MMPs.

Gelatinase B is made by many types of inflammatory cells, such as polymorphonuclear leukocytes [25], macrophages [26,27] or neutrophils [28]. Furthermore, various transformed cell lines secrete gelatinase B [12,29,30]. Gelatinase B has also been linked to a metastatic phenotype in several systems [31,32]. Progelatinase B has a molecular mass of 92 kDa. Several active forms of this enzyme migrating at 83, 78, 68 and 67 kDa have been described [9,29,30]. In addition, higher-molecular-mass complexes consisting of two covalently linked molecules of progelatinase B, two molecules of 68 kDa gelatinase B, or progelatinase B and interstitial collagenase can be formed [33].

Progelatinase A, in complex with TIMP-2, is also secreted by several types of normal and transformed cells [13,34]. It is found in the conditioned media of fibroblasts, melanoma cells, macrophages, bronchial epithelial cells and tumour cells and has been implicated in tumour cell invasion [34,35]. Gelatinase A localizes to cell surfaces [36] by binding, as a gelatinase A–TIMP2 complex, to one of its activators, MT-MMP1 [37].

Abbreviations used: APMA, 4-aminophenyl mercuric acetate; DMEM, Dulbecco's modified Eagle medium; MMP, matrix metalloproteinase; MT, membrane-type; TIMP, tissue inhibitor of metalloproteinases.

[1] To whom correspondence should be addressed.

Although several MMPs have been shown to activate TIMP-free MMPs [8,22,38-40], only limited information is available about the activation of gelatinases when complexed with their respective inhibitors. Considering that all active MMPs can be inhibited by both TIMP1 and TIMP2 [10] in a process facilitated by the haemopexin domain present in all MMPs except matrilysin [18], the latter appears to be a good candidate enzyme for activation of gelatinases bound to TIMP. In the present study we investigated interactions of matrilysin with progelatinase–TIMP complexes both *in vitro* and in a cell-culture system. We also asked whether matrilysin can activate its own proform.

## MATERIALS AND METHODS

### Cell-culture techniques

HT1080 cells derived from a fibrosarcoma, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum, 100 $\mu$g/ml penicillin and 100 $\mu$g/ml streptomycin (all from Gibco, Long Island, NY, U.S.A.). Cells were grown to confluency at 37 °C in 5 % $CO_2$ and 95 % air. Conditioned media were generated by rinsing cells with serum-free DMEM three times and further incubation in 5 ml of serum-free DMEM per $T_{75}$ tissue-culture flask for 16 h in the presence of 100 ng of PMA/ml to increase gelatinase B and TIMP1 expression.

### Proteases

Recombinant matrilysin was generously given by Dr. Paul Cannon (Syntex Research, Palo Alto, CA, U.S.A.). Purification occurred from a transfected CHO-cell system as described previously [41]. Active matrilysin was obtained by treating purified promatrilysin with 1 mM APMA (Aldrich Chemical Company, Milwaukee, WI, U.S.A.) and subsequent dialysis to eliminate APMA. Progelatinase A–TIMP-2 complexes and progelatinase B–TIMP-1 complexes were purified as described previously [42,43]. Briefly, gelatinase B–TIMP-1 complexes were purified from medium conditioned by cultured U937 cells. To confirm that gelatinase B was fully complexed with TIMP-1, purified material was incubated with polyclonal anti-TIMP-1 IgG [44], followed by precipitation with protein G–Sepharose. Immobilized material and supernates were then assayed for the presence of gelatinase B by gelatin zymography, which revealed that at least 90 % of the gelatinase B was bound to TIMP-1 (results not shown). This binding has been previously reported to occur on the carboxyl domain of gelatinase B [33]. TIMP-free progelatinase B was generated by cloning full-length gelatinase B cDNA (obtained from I. Collier, Washington University, St. Louis, MO, U.S.A.) into pcDNAI–Ne0 (Invitrogen) and co-transfected with RSV-NEO plasmid (from Dr. Z. Werb, University of California, San Francisco, CA, U.S.A.) into mouse NSO myeloma cells from the European Collection of Animal Cell Cultures (ECACC) by precipitation with $Ca_2PO_4$. Stable transfectants were selected by growth in G418. After several weeks under selective pressure, isolated clones were analysed for gelatinase secretion by zymography. Gelatinase B free of TIMP-1 was purified by gelatin–Agarose chromatography and lentil-lectin–Sepharose, as previously described for the gelatinase B–TIMP-1 complex [42].

### Antibodies

Specific antibodies for gelatinase A and B were generated by immunizing chickens with free gelatinase A isolated by reverse-phase-HPLC or with gelatinase B electroeluted from gel slices respectively. Chicken antibodies were developed in conjunction with Ferrell Farms (McLoud, OK, U.S.A.). Chickens were injected with up to 50 $\mu$g of antigen in complete Freund's adjuvant. IgY was purified from eggs by precipitation with polyethylene glycol, as described previously [44a]. Binding of primary antibodies was revealed using appropriate secondary antibodies coupled to horseradish peroxidase (Pierce, Rockville, IL, U.S.A.) for immunoblotting (Cappel, Durham, NC, U.S.A.). For the detection of matrilysin, a rabbit polyclonal antibody (Rb-2) was used [45].

### Activation of MMPs with matrilysin or APMA

Purified gelatinase A–TIMP-2 complex, gelatinase B–TIMP-1 complex, TIMP-free gelatinase B and promatrilysin were incubated with or without active matrilysin in buffer containing 10 mM $CaCl_2$ and 50 mM Tris, pH 7.5, at 37 °C. Control activations were performed in 1 mM APMA in the same buffer. The reactions were stopped by dissolving the samples in the respective buffers for regular and substrate gel electrophoresis (see below).

### Treatment of conditioned medium with matrilysin

Serum-free conditioned media collected from confluent cells was centrifuged for 5 min at 900 ***g*** at 4 °C to remove cell debris. The resulting supernatant was used as the starting material for incubation with matrilysin.

### Degradation of [$^3$H]gelatin

Gelatin-degradation assays were performed with $^3$H-labelled rat type I collagen (DuPont NEN) that had been diluted with unlabelled rat collagen I (isolated as described previously [46]) to a final specific activity of $1.5 \times 10^6$ d.p.m. per mg of collagen. The collagen was dialysed extensively into 50 mM Tris, pH 7.5, and 10 mM $CaCl_2$, boiled for 5 min, and incubated with the different proteinase samples for 3 h at 37 °C. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 20 %. The assay tubes were stored at −70 °C for 8 min, and after centrifugation for 8 min, the supernatants were analysed for acid-soluble gelatin peptides by liquid scintillation spectrometry.

### SDS/PAGE

Proteins were separated by one-dimensional SDS/PAGE as described previously [47]. Proteins from conditioned medium were recovered by precipitation with 2.5 % (v/v) trichloroacetic acid/ml at −70 °C for 15 min, centrifuged, washed with 70 % ethanol, dissolved in sample buffer and loaded on to the gel. The gels were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.; pore size 0.45 $\mu$m).

### Immunoblotting

For immunoblotting, the membranes were blocked in 2.5 % (w/v) non-fat dried milk in a buffer containing 10 mM Tris/HCl, pH 8, 150 mM NaCl and 0.05 % (v/v) Tween 20 (TBST) for 30 min and incubated with specific antibodies at 4 °C overnight. After washing the membranes three times for 15 min each in TBST, they were incubated for 1 h with a horseradish peroxidase-coupled secondary antibody (Pierce), washed again three times in TBST and subjected to enhanced chemiluminescence–Western blotting detection reagent (Amersham, Arlington Heights, IL, U.S.A.). Antibody binding was revealed by exposure to Kodak X-OMAT AR film.

### Zymography

Protease activity was analysed by substrate gel electrophoresis (zymography) in polyacrylamide gels containing 2 mg/ml gelatin or 1.5 mg/ml casein. Samples were dissolved in modified Laemmli sample buffer [containing 2.5 % (v/v) SDS without 2-mercaptoethanol] [47] and electrophoresed, without prior boiling, at 4 °C. After removal of the SDS by washing in 2.5 % (v/v) Triton X-100 in 50 mM Tris/HCl, pH 7.5, for 1 h, the gels were incubated overnight at 37 °C in a buffer containing 40 mM Tris/HCl, pH 7.5, and 10 mM $CaCl_2$. Staining with 0.5 % (w/v) Coomassie Brilliant Blue (Bio-Rad, Richmond, CA, U.S.A.) in 30 % (v/v) isopropanol/10 % (v/v) acetic acid followed by destaining with 30 % isopropanol/10 % acetic acid allowed identification of gelatinolytic or caseinolytic activity as clear zones in a blue background.

## RESULTS

### Activation of gelatinase B–TIMP1 complexes by matrilysin

Incubation of the gelatinase B–TIMP1 complex with active, APMA-free, matrilysin in a 2:1 molar ratio led to a shift in molecular mass from 92 kDa to 78 kDa evident after 1 h (Figures 1A and 1B, lanes 2) and complete by 20 h (Figures 1A and 1B, lanes 6). APMA-activated gelatinase B migrated at 83 kDa. Activity at about 68 kDa was also detectable after 4 h (Figure 1B, lanes 4–6). Incubation with matrilysin allowed a stepwise reduction in size of the higher-molecular-mass complexes detectable in the zymogram over a period of 20 h (Figure 1B, lanes 1–6).

Previous studies have shown that after removal of the prodomain of gelatinase B, TIMP1 remains attached and therefore may interfere with enzyme activity [30]. Therefore we tested whether the gelatinase B samples that had been preincubated with matrilysin displayed any proteolytic activity. As demonstrated in Figure 1(C), preincubation with matrilysin led to proteolytic activity corresponding to 75.8 % of the maximum activity observed with TIMP-free gelatinase B treated with APMA. In contrast, gelatinase B–TIMP1 complex that had been treated with APMA for 1 h displayed proteolytic activity corresponding to 54.9 % of the maximum.

### Activation of TIMP-free gelatinase B by matrilysin

TIMP-free progelatinase B was also converted to 78 and 68 kDa forms by matrilysin, as shown by immunoblotting (Figure 2A) and zymography (Figure 2B) at a rate comparable with that observed with gelatinase B–TIMP1 complexes (Figure 1). Activation of TIMP-free progelatinase B was also demonstrated in the protease activity assay (Figure 2C). In the absence of TIMP1, [$^3$H]gelatin degradation increased to 95 % of the maximum activity obtained by activation with APMA, when progelatinase B had been preincubated with matrilysin for 1 h. However, the activity decreased to 46.3 % of the maximum in the samples that had been preincubated with matrilysin for 4 h.

### Interactions of gelatinase A–TIMP2 complexes with matrilysin

As a comparison, we investigated whether matrilysin was also able to activate the progelatinase A–TIMP2 complex. Addition of active matrilysin did not increase the amount of gelatinase A migrating at 59 kDa (Figures 3A and 3B, lanes 6) as compared with the untreated sample (lanes 7), suggesting that the gelatinase A–TIMP2 complex is not a substrate for matrilysin. However, in the activity assay, a strong increase of [$^3$H]gelatin degradation

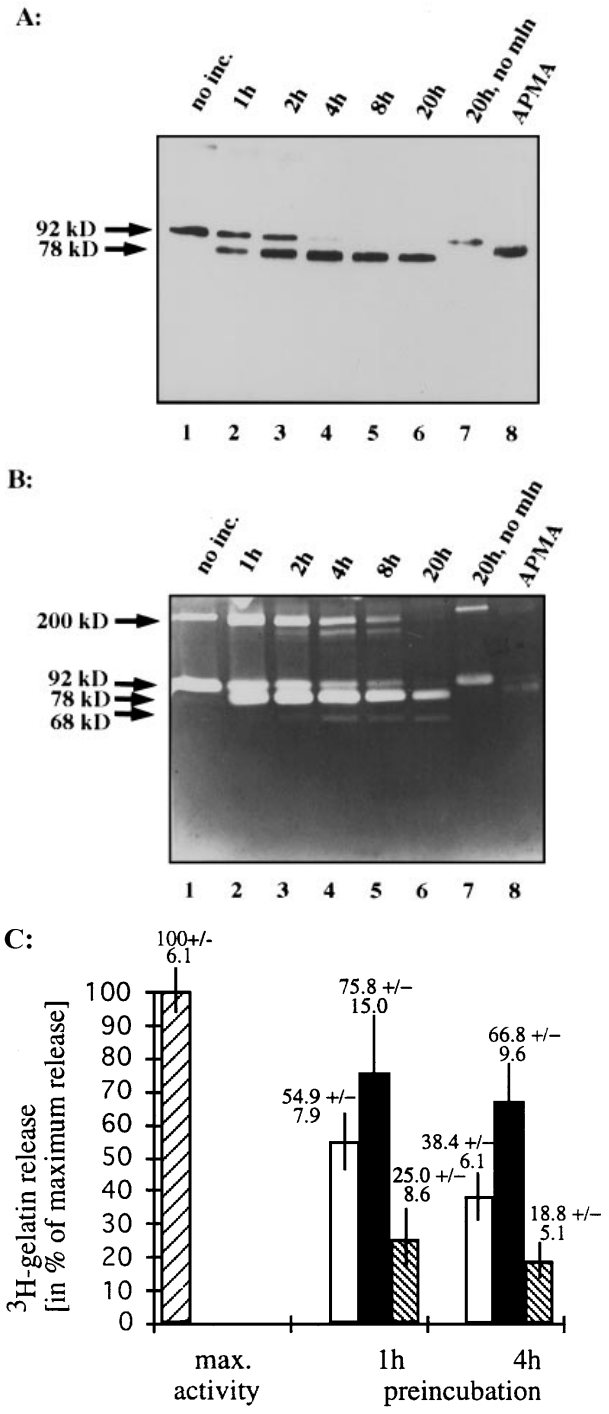


**Figure 1 Activation of the gelatinase B–TIMP-1 complex by matrilysin**

Purified gelatinase B–TIMP-1 complex was incubated with active matrilysin (mln) at a 1:2 molar ratio for 1–20 h, or with 1 mM APMA for 4 h, as indicated. (**A**) Immunoblotting with a monoclonal chicken antibody recognizing human gelatinase B. (**B**) Gelatinolytic activity of gelatinase B–TIMP1 complex incubated with matrilysin. Samples containing 20 ng of gelatinase B each were electrophoresed on a gel containing 2 mg/ml gelatin. Molecular masses are indicated at the left. Abbreviations: no inc., no incubation; kD, kDa. (**C**) Degradation of [$^3$H]gelatin by matrilysin-activated gelatinase B–TIMP1 complex. *y*-axis, release of soluble gelatin-degradation products as a percentage of the maximum (max.) activity. ▨, free gelatinase B + APMA; □, gelatinase B–TIMP1 complex − APMA; ■, gelatinase B–TIMP1 complex + matrilysin; ▧, gelatinase B–TIMP1 complex. The values presented are the means ± S.E. of at least three independent experiments. The values obtained have been corrected by subtracting the background values resulting from [$^3$H]gelatin degradation by matrilysin.

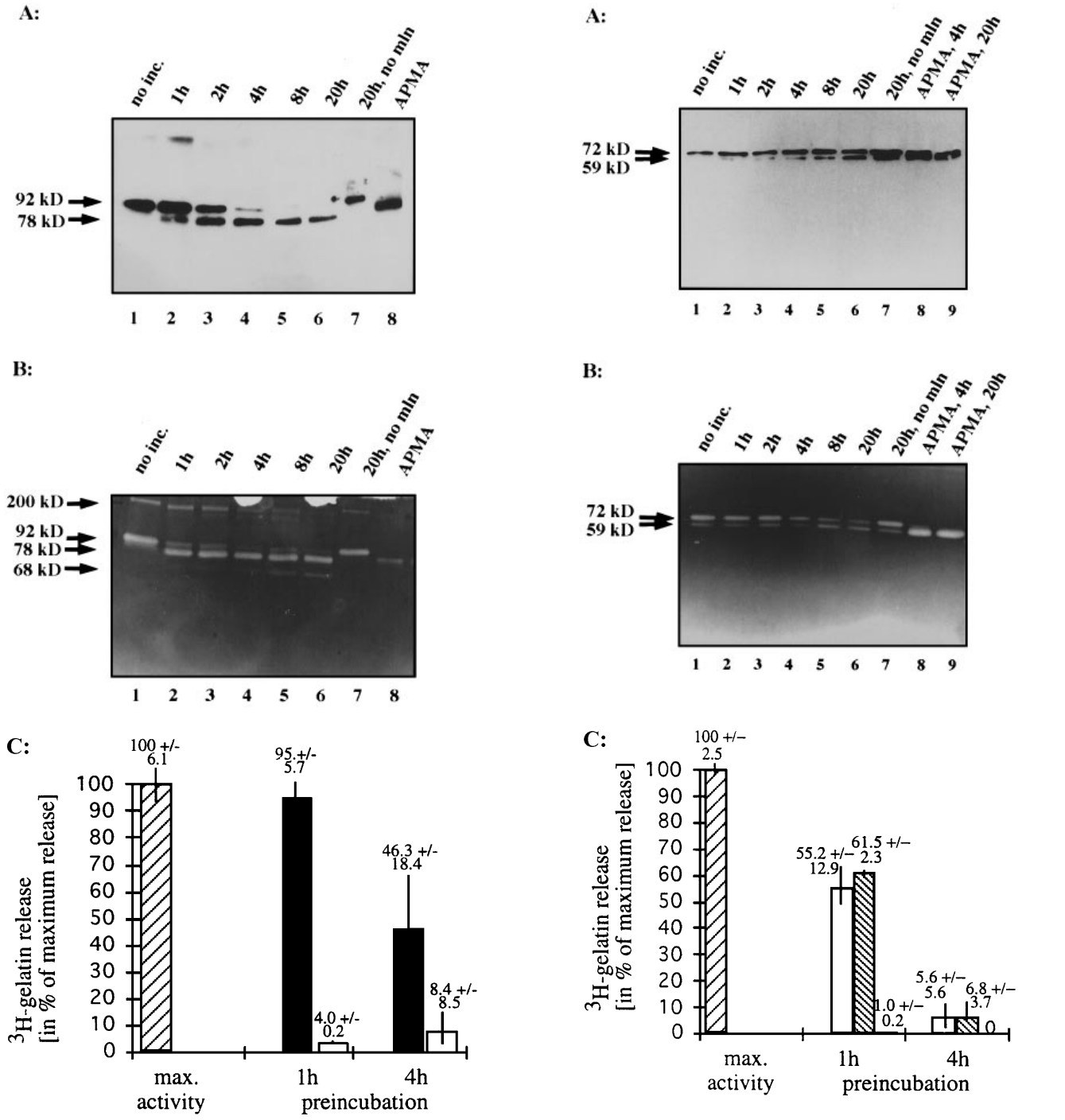


**Figure 2 Activation of TIMP-free gelatinase B by matrilysin**

Active matrilysin (mln) in a 2:1 molar ratio, or APMA, was added to TIMP-free gelatinase B and incubated for up to 20 h, as indicated. (**A**) Immunoblot using a chicken anti-(gelatinase B) monoclonal antibody. (**B**) Gelatin zymography of the samples containing 20 ng TIMP-free gelatinase B. Molecular masses are indicated at the left. Abbreviations: no inc., no incubation; kD, kDa. (**C**) Protease activity assay using [$^3$H]gelatin as a substrate, similar to Figure 1(C). *y*-axis, release of soluble gelatin-degradation products as a percentage of the maximum (max.) activity. ▨, free gelatinase B + APMA; ■, free gelatinase B + matrilysin; □, free gelatinase B. The values presented are the means ± S.E. of at least three independent experiments. The values obtained have been corrected by subtracting the background values resulting from [$^3$H]gelatin degradation by matrilysin.

**Figure 3 Interactions of the gelatinase A–TIMP-2 complex with matrilysin**

Purified gelatinase A–TIMP2 complex was incubated with active matrilysin (mln) in a 1:2 molar ratio, or in 1 mM APMA for up to 20 h, as indicated. (**A**) Immunoblot using a chicken monoclonal antibody against gelatinase A. (**B**) Zymography of corresponding samples run on a SDS/10% polyacrylamide gel containing 2 mg/ml gelatin. Molecular masses are indicated at the left. Abbreviations: no inc., no incubation; kD, kDa. (**C**) Degradation of [$^3$H]gelatin by matrilysin-activated gelatinase A–TIMP2 complex. *y*-axis, release of soluble gelatin-degradation products as a percentage of the maximum (max.) activity. ▨, free gelatinase A + APMA; □, gelatinase A–TIMP1 complex + APMA; ▧, gelatinase A–TIMP1 complex + matrilysin; ■, gelatinase A–TIMP1 complex. The values presented are the means ± S.E. of at least three independent experiments. The values obtained have been corrected by subtracting the background values resulting from [$^3$H]gelatin degradation by matrilysin.

**Table 1 Time course of [$^3$H]gelatin release (as a percentage of maximum release) by gelatinase A–TIMP-2 complex preincubated with or without matrilysin**

| | [$^3$H]Gelatin release | | | | | |
|---|---|---|---|---|---|---|
| Preincubation time (h) ... | 0 | 1 | 2 | 4 | 8 | 20 |
| Gelatinase A–TIMP-2 only | 3.3 ± 1.0 | 1.0 ± 0.2 | 0 | 0 | 2.3 ± 0.5 | 3.3 ± 1.8 |
| Gelatinase A–TIMP-2 + matrilysin | 57.0 ± 3.3 | 61.5 ± 2.3 | 26.6 ± 4.1 | 6.8 ± 3.7 | 0.6 ± 7.4 | 1.6 ± 3.0 |

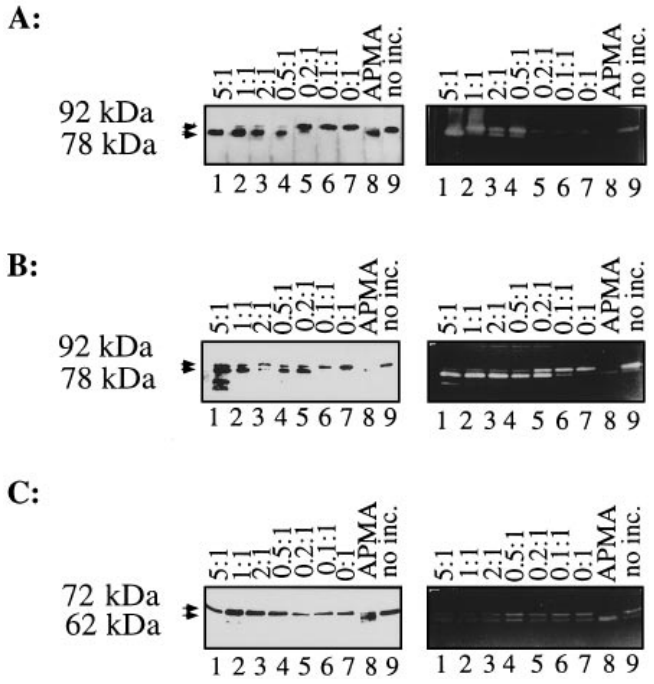


**Figure 4 Activation of the gelatinase B–TIMP-1 complex and of free gelatinase B depends on the concentration of matrilysin**

Gelatinase B–TIMP1 complex, free gelatinase B, and gelatinase A–TIMP 1 complex were incubated with different concentrations of active matrilysin for 4 h and analysed by immunoblotting (left panels) and gelatin zymography (right panels). The molar ratios of matrilysin/gelatinase are between 5:1 and 0:1, as indicated; lane 8, gelatinase in 1 mM APMA; lane 9, gelatinase only, no incubation (inc.). (**A**) Gelatinase B–TIMP1 complex incubated with active matrilysin. (**B**) Effects of different concentrations of active matrilysin on free gelatinase B. (**C**) Gelatinase A–TIMP2 complex incubated with up to 5 mol of active matrilysin per mol of gelatinase. Molecular masses (kDa) are indicated at the left.

corresponding to 61.5% of the maximum activity could be observed when the progelatinase A–TIMP2 complex had been preincubated with matrilysin for 1 h (Figure 3C). After preincubation with matrilysin for 4 h, [$^3$H]gelatin degradation was reduced to background levels. Comparable results in the activity assay were obtained when the gelatinase A–TIMP2 complex was treated with APMA (Figure 3C), consistent with the binding of the TIMP2 inhibitory domain to the active site of gelatinase A once the prodomain has been removed [48]. A time course confirmed the transient increase of [$^3$H]gelatin degradation by gelatinase A–TIMP2 complex incubated with matrilysin (Table 1).

In summary, the data presented in Figure 3 indicate that matrilysin can lead to a transient increase in proteolytic activity of the gelatinase A–TIMP2 complex in a mechanism independent of the removal of the prodomain by matrilysin.

### Activation of gelatinase B depends on the concentration of matrilysin

As shown in Figure 4(A) (lanes 1), a 5:1 molar excess of matrilysin to gelatinase B–TIMP1 complex allowed almost complete conversion into the lower-molecular-mass form of the gelatinase within 4 h. Activation was still possible with a 0.5:1 ratio of matrilysin to gelatinase B (Figure 4A, lanes 4). TIMP-free gelatinase B could be activated with matrilysin/gelatinase B ratios down to 0.2:1, as judged by immunoblotting and zymography (Figure 4B). Almost no activation of the gelatinase A–TIMP2 complex could be observed (Figure 4C). Some activity co-migrating with the APMA-treated sample (Figure 4C, right panel, lane 8) was detectable in all samples. However, since this band was also present in the samples that had not been incubated with matrilysin (Figure 4C, right panel, lanes 7 and 9), these data suggest that matrilysin cannot convert progelatinase A –TIMP2 complex into its lower-molecular-mass form.

### Matrilysin does not enhance autoactivation of promatrilysin

Recently, activation of promatrilysin by stromelysin 1 has been reported [9]. Yet stromelysin and matrilysin are frequently not co-expressed [49]. Stromelysin 1 and matrilysin degrade similar substrates, and, at least *in vitro*, promatrilysin autoactivates rather quickly (R. B. Nagle, unpublished work). Therefore, we hypothesized that trace amounts of active matrilysin present in a promatrilysin preparation may start a cascade of promatrilysin activation. However, when we incubated promatrilysin with APMA-free active matrilysin, no enhanced conversion of the 29 kDa form (promatrilysin) to the 18 kDa form (active matrilysin) could be observed. Nevertheless, prolonged incubation of the 29 kDa form led to the conversion into the 18 kDa form (compare lanes 1 and 7 in Figure 5), suggesting that the activation of promatrilysin *in vitro* can occur by an intramolecular mechanism of autoactivation.

### Matrilysin activates progelatinase B, but not progelatinase A, in the conditioned medium of HT1080 cells

HT1080 fibrosarcoma cells secrete both gelatinases A and B. Both gelatinase B and TIMP1 expression can be up-regulated selectively by the phorbol ester PMA in these cells [17,30]. Therefore, these cells represent a suitable system to test whether the activation of gelatinase B–TIMP1 complex observed with purified proteases can also take place under the conditions of cell culture. Conditioned medium from HT1080 cells, which had been treated with PMA for 16 h, was incubated with 18, 80 and 170 nM active matrilysin for 20 h. The amount of matrilysin used in this experiment corresponds to the concentration of the enzyme expected in tissues, since concentrations in the nanomolar range have been reported for other MMPs *in vivo* [50,51].

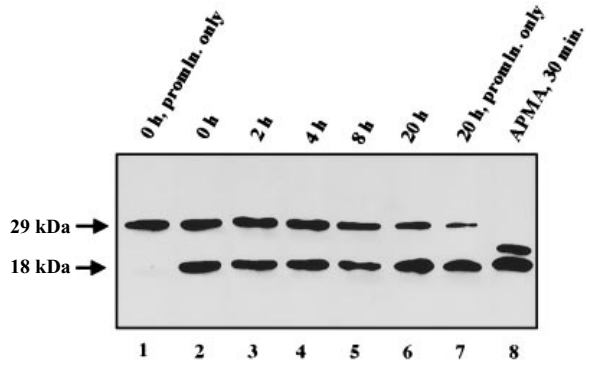


**Figure 5 Matrilysin (mln.) does not enhance autoactivation of promatrilysin**

Promatrilysin was incubated with active matrilysin at a 1:2 molar ratio, or in 1 mM APMA, for the amounts of time indicated. An immunoblot with a polyclonal antibody recognizing pro- and active matrilysin (Rb-2) is shown. Molecular masses (kDa) are indicated at the left.

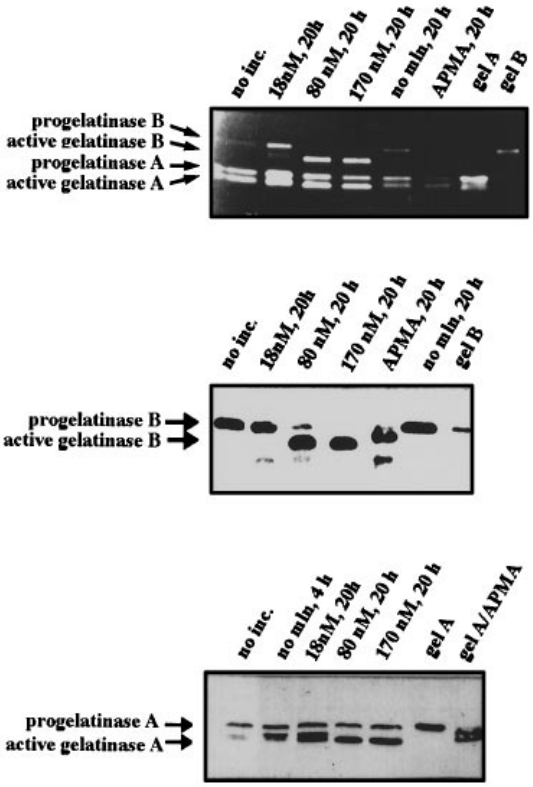


**Figure 6 Matrilysin (mln) activates gelatinase (gel) B, but not gelatinase A, in the conditioned medium of HT1080 cells**

Aliquots (0.4 ml each) were incubated with 18, 80 and 170 nM active matrilysin, 1 mM APMA or no additions for 20 h, as indicated. Aliquots (20 $\mu$l each) of the 0 and 20 h samples were dissolved in zymogram buffer and subjected to PAGE on a 10% gel containing 2 mg/ml gelatin (**A**). The remainders of the samples were precipitated with trichloroacetic acid, analysed by SDS/PAGE under reducing conditions and immunoblotting with chicken anti-gelatinase B (**B**) or chicken anti-gelatinase A (**C**) antibodies. Abbreviation: no inc., no incubation.

Treatment of conditioned medium with 170 nM matrilysin resulted in complete conversion of progelatinase B into its 78 kDa form after 20 h of incubation (Figures 6A and 6B). Similar to the results obtained with purified enzymes, the lower-molecular-mass forms generated by APMA treatment (Figure 6B) migrated at 83 kDa, indicating that the products resulting from activation with matrilysin and with APMA are not completely identical.

Consistent with the data from the *in vitro* experiments with purified gelatinase A, no significant decrease in the amount of progelatinase A due to treatment with matrilysin could be detected in the conditioned medium of the HT1080 cells (Figures 6A and 6C) or of human foreskin fibroblasts (results not shown). The bands migrating at 62 and 59 kDa in the immunoblot for gelatinase A (Figure 6C), present in all samples, represent active gelatinase A that has been observed previously in HT1080 cells due to treatment with phorbol esters [52]. The 62 kDa form was undetectable in the samples that had been incubated with matrilysin, indicating that, once the prodomain of gelatinase A is removed, the 62 kDa form can be proteolytically processed by matrilysin.

## DISCUSSION

Matrilysin is an MMP focally expressed in human prostate carcinoma [22]. but not in normal prostate glands. It is also present in non-malignant tissue in ducts surrounded by inflammatory cells [22]. Considering that gelatinase B is synthesized by inflammatory cells, interactions of matrilysin with gelatinase B in prostate tissue are expected. In prostate carcinoma, expression of matrilysin allows interactions of this MMP with gelatinase A, which is expressed in both benign and malignant prostate tissue [53].

Degradation of the extracellular matrix by MMPs depends on the presence of activators of the latent proenzymes. Many proteases, such as trypsin [7], cathepsin G [7], gelatinase A [8], matrilysin and stromelysin [39,54], have been shown to activate progelatinase B. However, the presence of TIMPs prevented activation of progelatinase B by gelatinase A [8] and stromelysin [33]. In other studies, TIMP-free enzyme preparations had been used [7,9]. We investigated here for the first time the effects of matrilysin on the proteolytic activity of progelatinase B–TIMP1 and progelatinase A–TIMP2 complexes. Results obtained are summarized in the model presented in Figure 7.

Specifically, we show that active, APMA-free, matrilysin is able to activate progelatinase B both in the presence and the absence of TIMP1. Both forms gave rise to gelatinolytic activities migrating at 78 and 68 kDa, whereas APMA activation resulted in an 83 kDa active form, consistent with the published molecular mass reported for active gelatinase B [29]. Proteinase activity assays demonstrated an increase in gelatin degradation dependent on the preincubation of the gelatinase with matrilysin both in the samples containing TIMP1 and in the TIMP-free samples. Gelatinase B–TIMP1 complexes activated with matrilysin displayed approx. 50% more proteolytic activity than the samples that had been activated with APMA. This suggests that TIMP1 may have bound to the active site of gelatinase B after removal of the prodomain in the APMA-treated samples, consistent with previous observations [30]. Matrilysin, however, if present, may compete for the TIMP1 molecules available and therefore reduce the number of TIMPs that bind to active gelatinase B. This would lead to a shift in enzymic activity from matrilysin, which has a very broad substrate specificity, to a very specific MMP (gelatinase B). While our study was in progress, conversion of TIMP-free progelatinase B by matrilysin into an active 78 kDa form with the N-terminal sequence of $Leu^{16}$-Arg-Thr-(Asn)-Leu and a truncation in the C-terminus was reported [9]. In our experiments, both progelatinase B–TIMP1 complexes and TIMP-

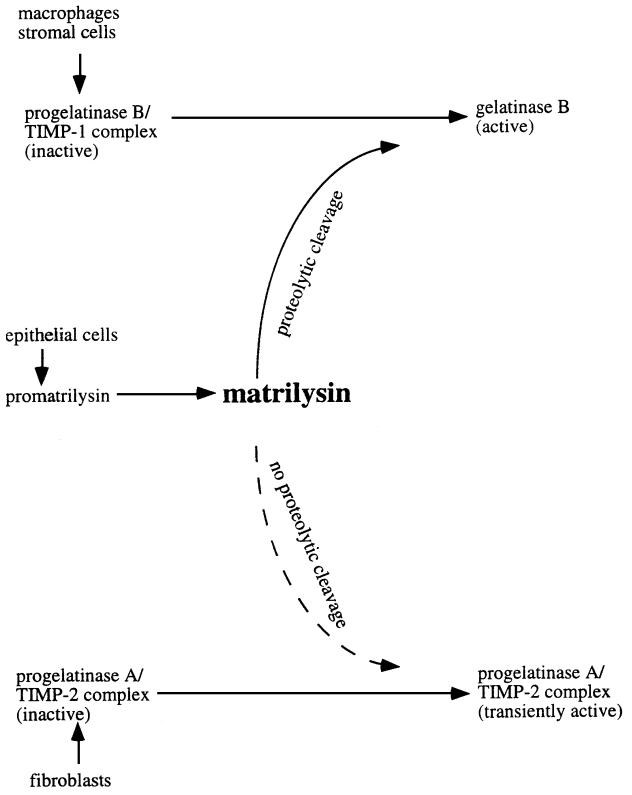


**Figure 7 Summary of the activation of gelatinase–TIMP complexes by matrilysin**

Promatrilysin is secreted by epithelial cells [59] and converted into active matrilysin, possibly by stromelysin [9] or an intramolecular autoactivation mechanism. Active matrilysin activates progelatinase–TIMP1 complexes, which have been synthesized by macrophages and stromal cells [60]. Progelatinase A–TIMP2 complexes derived from fibroblasts [35] can transiently be activated by matrilysin without proteolytic removal of the prodomain.

free progelatinase B also gave rise to a 78 kDa form, presumably with Leu[16]-Arg-Thr-(Asn)-Leu at the N-terminus.

Although relatively high amounts of matrilysin are required to activate progelatinase B and progelatinase B–TIMP1 complexes, several lines of evidence indicate that this type of activation may occur *in vivo*. The 78 kDa form of gelatinase B is detectable in the conditioned medium of phorbol ester-treated HT1080 cells that have been incubated with matrilysin. Therefore, the amount of matrilysin required is similar to concentrations of stromelysin found in plasma of arthritis patients [51]. High local concentrations of matrilysin are present in ducts surrounded by inflammatory cells in prostate tissue [22]. Furthermore, matrilysin can hydrolyse the progelatinase B–TIMP1 complex, but not the progelatinase A–TIMP2 complex. This specificity of the reaction also supports the hypothesis of its physiological relevance.

However, in addition to playing a role in carcinogenesis and inflammatory processes, other effects caused by the activation of gelatinase B–TIMP1 can be proposed. Considering that matrilysin appears to cleave off parts of the C-terminal domain of gelatinase B [9], interactions with TIMP1, which depend on this domain [10], may be disturbed, leading to the release of TIMP1. Both TIMP1 and TIMP2 have been shown to act as growth factors in several different cell lines [55,56]. The growth-promoting function of the TIMPs has not been observed with the inhibitor bound to its respective metalloproteinase [56], and was shown to be independent from the metalloproteinase-inhibiting activity [56,57]. Therefore a release of TIMP1 from gelatinase B by matrilysin may allow the inhibitor to have an alternate function and contribute to cell proliferation and tumour growth.

The gelatinase A–TIMP2 complex, which previously has been shown to be a substrate for stromelysin [58], is not converted into its lower-molecular-mass form by active matrilysin, even if a fivefold molar excess of matrilysin is used. Nevertheless, activation of TIMP-free gelatinase A by matrilysin has been reported [40], suggesting that the presence of the inhibitor prevented matrilysin from cleaving off the N-terminal domain of the proenzyme. Despite these findings, in the protease activity assays, a high amount of [$^3$H]gelatin degradation products was released with gelatinase A samples that had been preincubated with matrilysin for 1 h in our experiments, indicating that a temporary activation had occurred independently of the removal of the prodomain. The reason for this is unknown, but a temporary conformational change of the gelatinase A–TIMP-2 complex induced by matrilysin or APMA can be imagined.

Although proteolytic cleavage of the progelatinase A–TIMP2 complex by matrilysin could be observed neither *in vitro* nor in the conditioned medium of cells, processing of the 62 kDa active form of the gelatinase in the matrilysin-treated samples was detected in the conditioned medium of phorbol ester-treated HT1080 cells. Previously, the conversion of the 62 kDa form into the 59 kDa form has been shown to be an autocatalytic event [43]. Matrilysin may enhance this process either directly by proteolytically cleaving the 62 kDa form, or by interacting with TIMP and thereby preventing it from inhibiting the autocatalytic cleavage of the 62 kDa gelatinase A.

Taken together, the findings presented here demonstrate that matrilysin may contribute to the regulation of gelatinase activities. Matrilysin can proteolytically cleave and activate both free progelatinase B and progelatinase B in the presence of TIMP1. Furthermore, besides allowing a transient activation of the progelatinase A–TIMP2 complex, matrilysin can participate in the processing of the 62 kDa form of gelatinase A. These results demonstrate that matrilysin may be an important enzyme within a proteolytic cascade.

We thank Dr. Paul Cannon for the generous gift of purified matrilysin. This work was supported by grants PO1-CA 56666, CA 23067 and ADCRC 822701.

## REFERENCES

1 Nagase, H. (1997) Biol. Chem. **378**, 151–160
2 Coussens, L. M. and Werb, Z. (1996) Chem. Biol. **3**, 895–904
3 Birkedahl-Hansen, H. (1995) Curr. Opin. Cell Biol. **7**, 728–735
4 Basset, P., Wolf, C. and Chambon, P. (1993) Breast Cancer Res. Treat. **24**, 185–193
5 Dean, D. D., Martel-Pelletier, J., Pelletier, J. P., Howell, D. S. and Woessner, Jr., J. F. (1989) J. Clin. Invest. **84**, 678–685
6 Park, A. J., Matrisian, L. M., Kells, A. F., Pearson, R., Yuan, Z. and Navre, M. (1991) J. Biol. Chem. **266**, 1584–1590
7 Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K. and Hayakawa, T. (1992) J. Biol. Chem. **267**, 21712–21719
8 Fridman, R., Toth, M., Pena, D. and Mobashery, S. (1995) Cancer Res. **55**, 2548–2555
9 Imai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N. and Okada, Y. (1995) J. Biol. Chem. **270**, 6691–6697
10 Willenbrock, F. and Murphy, G. (1994) Am. J. Resp. Critical Care Med. **150**, S165–S170
11 Wilson, C. L., Heppner, K. J., Rudolph, L. A. and Matrisian, L. M. (1995) Mol. Biol. Cell **6**, 851–869
12 Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989) J. Biol. Chem. **264**, 17213–17221
13 Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S. and He, C. S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8207–8211
14 Howard, E. W. and Banda, M. J. (1991) J. Biol. Chem. **266**, 17972–17977
15 MacNaul, K. L., Chartrain, N., Lark, M., Tocci, M. J. and Hutchinson, N. I. (1990) J. Biol. Chem. **265**, 17238–17245

16 Campbell, C. E., Flenniken, A. M., Skup, D. and Williams, B. R. (1991) J. Biol. Chem. **266**, 7199–7206
17 Huhtala, P., Tuuttila, A., Chow, L. T., Lohi, J., Keski-Oja, J. and Tryggvason, K. (1991) J. Biol. Chem. **266**, 16485–16490
18 Baragi, V. M., Fliszar, C. J., Conroy, M. C., Ye, Q. Z., Shipley, J. M. and Welgus, H. G. (1994) J. Biol. Chem. **269**, 12692–12697
19 Pajouh, M. S., Nagle, R. B., Breathnach, R., Finch, J. S., Brawer, M. K. and Bowden, G. T. (1991) J. Cancer Res. Clin. Oncol. **117**, 144–150
20 McDonnell, S., Navre, M., Coffey, Jr., R. J. and Matrisian, L. M. (1991) Mol. Carcin. **4**, 527–533
21 Karelina, T. V., Goldberg, G. I. and Eisen, A. Z. (1995) J. Investig. Dermatol. **105**, 411–417
22 Knox, J. D., Wolf, C., McDaniel, K., Clark, V., Loriot, M., Bowden, G. T. and Nagle, R. B. (1996) Mol. Carcin. **15**, 57–63
23 Rodgers, W. H., Matrisian, L. M., Giudice, L. C., Dsupin, B., Cannon, P., Svitek, C., Gorstein, F. and Osteen, K. G. (1994) J. Clin. Invest. **94**, 946–953
24 Bruner, K. L., Rodgers, W. H., Gold, L. I., Korc, M., Hargrove, J. T., Matrisian, L. M. and Osteen, K. G. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7362–7366
25 Sopata, I. and Dancewicz, A. M. (1974) Biochim. Biophys. Acta **370**, 510–523
26 Potts, J. R. and Campbell, I. D. (1994) Curr. Opin. Cell Biol. **6**, 648–655
27 Mainardi, C. L., Hibbs, M. S., Hasty, K. A. and Seyer, J. M. (1984) Collagen Rel. Res. **4**, 479–492
28 Hibbs, M. S. (1992) Matrix Suppl. **1**, 51–57
29 Moll, U. M., Youngleib, G. L., Rosinski, K. B. and Quigley, J. P. (1990) Cancer Res. **50**, 6162–6170
30 Morodomi, T., Ogata, Y., Sasaguri, Y., Morimatsu, M. and Nagase, H. (1992) Biochem. J. **285**, 603–611
31 Sehgal, I., Baley, I. A. and Thompson, T. C. (1996) Cancer Res. **56**, 3359–3365
32 Bernhard, E. J., Gruber, S. B. and Muschel, A. J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 4293–4297
33 Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T. and Marmer, B. L. (1992) J. Biol. Chem. **267**, 4583–4591
34 Stetler-Stevenson, W. G., Krutzsch, H. C. and Liotta, L. A. (1992) Matrix Suppl. **1**, 299–306
35 Stetler-Stevenson, W. G. (1990) Cancer Metastasis Rev. **9**, 289–303
36 Emonard, H. P., Remacle, A. G., Noel, A. C., Grimaud, J. A., Stetler-Stevenson, W. G. and Foidart, J. M. (1992) Cancer Res. **52**, 5845–5848
37 Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) J. Biol. Chem. **270**, 5331–5338
38 Nguyen, Q., Willenbrock, F., Cockett, M. I., O'Shea, M., Docherty, A. J. and Murphy, G. (1994) Biochemistry **33**, 2089–2095
39 Bergmann, U., Tuuttila, A., Stetler-Stevenson, W. G. and Tryggvason, K. (1995) Biochemistry **34**, 2819–2825
40 Crabbe, T., Smith, B., O'Connell, J. and Docherty, A. (1994) FEBS Lett. **345**, 14–16
41 Barnett, J., Straub, K., Nguyen, B., Chow, J., Suttman, R., Thompson, K., Tsing, S., Benton, P., Schatzman, R., Chen, M. et al. (1994) Protein Exp. Purif. **5**, 27–36
42 Howard, E. W., Bullen, E. C. and Banda, M. J. (1991) J. Biol. Chem. **266**, 13070–13075
43 Howard, E. W., Bullen, E. C. and Banda, M. J. (1991) J. Biol. Chem. **266**, 13064–13069
44 Bullen, E. C., Longaker, M. T., Updike, D. L., Benton, R., Ladin, D., Hou, Z. and Howard, E. W. (1995) J. Invest. Dermatol. **104**, 236–240
44a Gassman, M., Thömmes, P., Weiser, T. and Hubscher, U. (1990) FASEB J. **4**, 2528–2532
45 von Bredow, D. C., Nagle, R. B., Bowden, G. T. and Cress, A. E. (1995) Exp. Cell Res. **221**, 83–91
46 Scharffetter-Kochanek, K., Klein, C. E., Heinen, G., Manch, C., Schaefer, T., Adelmann-Grill, B. C., Goerz, G., Fusenig, N. E., Krieg, T. M. and Plewig, G. (1992) J. Invest. Derm. **98**, 3–11
47 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
48 Stetler-Stevenson, W. G. (1994) Invasion Metastasis **14**, 259–268
49 McDonnell, S., Wright, J. H., Gaire, M. and Matrisian, L. M. (1994) Biochem. Soc. Trans. **22**, 58–63
50 Freestone, T., Turner, R. J., Coady, A., Higman, D. J., Greenhalgh, R. M. and Powell, J. T. (1995) Arteriosclerosis, Thrombosis Vasc. Biol. **15**, 1145–1151
51 Zucker, S., Lysik, R. M., Zarrabi, M. H., Greenwald, R. A., Gruber, B., Tickle, S. P., Baker, T. S. and Docherty, A. J. (1994) J. Rheumatol. **21**, 2329–2333
52 Brown, P. D., Levy, A. T., Margulies, I. M., Liotta, L. A. and Stetler-Stevenson, W. G. (1990) Cancer Res. **50**, 6184–6191
53 Lokeshwar, B. L., Selzer, M. G., Block, N. L. and Gunja-Smith, Z. (1993) Cancer Res. **53**, 4493–4498
54 O'Connell, J. P., Willenbrock, F., Docherty, A. J., Eaton, D. and Murphy, G. (1994) J. Biol. Chem. **269**, 14967–14973
55 Hayakawa, T., Yamashita, K., Tanzawa, K., Uchijima, E. and Iwata, K. (1992) FEBS Lett. **298**, 29–32
56 Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. (1994) J. Cell Sci. **107**, 2373–2379
57 Turck, J., Pollock, A. S., Lee, L. K., Marti, H.-P. and Lovett, D. L. (1996) J. Biol. Chem. **271**, 15074–15083
58 Miyazaki, K., Umenishi, F., Funahashi, K., Koshikawa, N., Yasumitsu, H. and Umeda, M. (1992) Biochem. Biophys. Res. Commun. **185**, 852–859
59 Newell, K. J., Witty, J. P., Rodgers, W. H. and Matrisian, L. M. (1994) Mol. Carcin. **10**, 199–206
60 Karelina, T. V., Hruza, G. J., Goldberg, G. I. and Eisen, A. Z. (1993) J. Invest. Dermatol. **100**, 159–165

Received 18 December 1997; accepted 6 February 1998