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Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT1080 fibrosarcoma cells

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The precursor of matrix metalloproteinase 9 (proMMP-9), also known as '92 kDa progelatinase/type IV procollagenase', was purified from the conditioned medium of U937 monocytic leukaemia and HT1080 fibrosarcoma cell lines stimulated with phorbol 12-myristate 13-acetate. ProMMP-9 in these culture media is non-covalently complexed with the 29 kDa tissue inhibitor of metalloproteinases (TIMP), but free proMMP-9 was separated from the TIMP–proMMP-9 complex by chromatography on Green A Dyematrex gel. The final product was homogeneous on SDS/PAGE, with a molecular mass of 88 kDa without reduction and 92 kDa with reduction. Treatment of proMMP-9 with 4-aminophenylmercuric acetate converted the 88 kDa precursor into 80 kDa and 68 kDa forms. Gelatin-containing zymographic analysis showed zones of lysis associated with all three species. However, only the 68 kDa species was shown to be catalytically active by its ability to bind to α_2-macroglobulin. In the presence of an equimolar amount of TIMP, only the 80 kDa species was generated by treatment with 4-aminophenylmercuric acetate, but no enzyme activity was detected. This indicates that TIMP binds to the 80 kDa intermediate and inhibits the generation of the active 68 kDa species. Eight endopeptidases (trypsin, chymotrypsin, plasmin, plasma kallikrein, thrombin, cathepsin G, neutrophil elastase and thermolysin) were tested for their ability to activate proMMP-9. Of them, trypsin was the most effective activator of proMMP-9. Only partial activation (10–30 %) was observed with plasmin, cathepsin G and chymotrypsin. The active forms generated by trypsin were identified as 80 kDa, 74 kDa and 66 kDa by their abilities to bind to α_2-macroglobulin. In the presence of an equimolar amount of TIMP, proMMP-9 was also converted into the same molecular-mass species by trypsin, but they were not proteolytically active. This suggests activated MMP-9 is inhibited by TIMP. Activated MMP-9 digested gelatin, type-V collagen, reduced carboxymethylated transferrin and, to a lesser extent, type-IV collagen and laminin A chain. The specific activity against gelatin was estimated to be 15000 units/mg (1 unit = 1 μg of gelatin degraded/min at 37 °C) by titration with α_2-macroglobulin. Comparative studies on digestion of gelatin and collagen types IV and V by MMP-9 and MMP-2 indicated that both enzymes degrade these substrates into similar fragments. However, the susceptibilities of laminin, fibronectin and reduced carboxymethylated transferrin to these two MMPs were sufficiently different to indicate differences in substrate specificities between these two closely related proteinases.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of related metalloendopeptidases that are secreted in zymogens from connective-tissue cells, inflammatory phagocytes and a number of transformed cells [see Matrisian (1990) and Woessner (1991) for review]. On activation, these enzymes are capable of digesting various components of extracellular matrix. Thus MMPs are considered to play important roles in morphogenesis, tissue remodelling, reproduction and control of cell behaviour under physiological conditions. In excess, they may participate in accelerated pathological destruction of extracellular matrices associated with various connective-tissue diseases and cancer-cell invasion and metastasis. Eight distinct members of the MMP family have been identified by cDNA cloning, and seven have been characterized for their ability to digest extracellular-matrix macromolecules (see Woessner, 1991). Among them, two enzymes can be sub-grouped as so-called 'gelatinases/type IV collagenases', as they readily digest denatured collagens (gelatins) and, to a certain extent, native type-IV collagen. The two MMPs have molecular masses of 72 kDa and 92 kDa in their respective zymogen forms, and their primary structures are closely related (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). Studies by Wilhelm *et al.* (1989) and Murphy *et al.* (1989*a*) indicate that the substrate specificities of the two gelatinases/type-IV collagenases against protein substrates are indistinguishable. Many connective-tissue cells in culture synthesize the 72 kDa enzyme (Murphy *et al.*, 1985, 1989*b*; Collier *et al.*, 1988; Okada *et al.*, 1990). The 92 kDa enzyme was originally recognized as a gelatinolytic enzyme in polymorphonuclear leucocytes (Sopata & Dancemicz, 1974) and purified from human (Hibbs *et al.*, 1985) and pig (Murphy *et al.*, 1989*a*) neutrophils. Macrophages secrete a metalloproteinase that digests gelatins and type-V collagen (Mainardi *et al.*, 1984). These enzymes have now been shown by immunochemical criteria to be identical with the 92 kDa form of gelatinase/type-IV collagenase from various transformed cells (Hibbs *et al.*, 1987; Murphy *et al.*, 1989*b*; Wilhelm *et al.*, 1989; Moll *et al.*, 1990). According to the numerical distinction of the members of the MMP family, the 72 kDa and the 92 kDa zymogens are designated proMMP-2 and proMMP-9 (Nagase *et al.*, 1992).

Human proMMP-9 has been purified from the culture medium of SV40-transformed fetal lung fibroblasts (Wilhelm *et al.*, 1989), phorbol 12-myristate 13-acetate (PMA)-treated monocytic leukaemia cell lines U937 and HL60 (Wilhelm *et al.*, 1989; Davis & Martin, 1990) and PMA-treated fibrosarcoma HT1080 cells (Moll *et al.*, 1990). ProMMP-9 purified from these sources was also shown to be non-covalently complexed with a 29 kDa tissue inhibitor of metalloproteinases (TIMP) (Wilhelm *et al.*,

Abbreviations used: α_2M, α_2-macroglobulin; APMA, 4-aminophenylmercuric acetate; BCA, bicinchoninic acid; Cm-Tf, reduced carboxymethylated transferrin; DMEM, Dulbecco's modified Eagle's medium; Dip-F, di-isopropyl phosphorofluoridate; EHS, Engelbreth–Holmes–Schwarm; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitors of metalloproteinases of 29 kDa.

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1989; Moll *et al.*, 1990). The proMMP-9–TIMP complexes are shown to be tight, so that they cannot be dissociated unless they are treated with denaturants such as SDS in 0.1% trifluoroacetic acid (Wilhelm *et al.*, 1989). This observation indicates that not only the activation studies of human proMMP-9 can be complicated but also the action of MMP-9 on the protein substrates may be underestimated by the presence of TIMP complexed with proMMP-9.

In this paper we have developed a method to isolate proMMP-9 from U937 cells and HT1080 cells free of TIMP without denaturing the zymogen. This allowed us to study the activation process of proMMP-9 by an organomercurial compound, 4-aminophenylmercuric acetate (APMA), and proteinases in the absence and presence of TIMP. Active forms of MMP-9 were identified by their ability to bind to α_2-macroglobulin (α_2M). The specificities of MMP-2 and MMP-9 were compared by their abilities to digest various protein substrates. Our present study demonstrates that the subsite requirements of the two homologous enzymes are different, and that the activation process of proMMP-9 is interfered with by the presence of TIMP.

EXPERIMENTAL

Materials

APMA, Brij 35, chymotrypsin, concanavalin A–Sepharose, di-isopropyl phosphorofluoridate (Dip-F), methyl α-D-mannoside, plasminogen (human), PMA, thrombin (human), transferrin (human), trypsin (bovine), urokinase, type-V collagen (human) and alkaline phosphatase-conjugated donkey anti-(sheep IgG) IgG were from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM), antibiotics, fetal-calf serum and lactalbumin hydrolysate were from Gibco. YM-10 membrane and Green A Dyematrex gel were from Amicon Corp. DEAE-cellulose (DE-52) was from Whatman. Sephacryl S-200 was from Pharmacia. Type-IV collagen and laminin isolated from mouse Engelbreth–Holmes–Schwarm (EHS) tumour were from Collaborative Research Inc. Bicinchoninic acid (BCA) protein assay reagents were from Pierce Chemicals Co. Human fibronectin was purified from plasma by the method of Ruoslahti & Engvall (1978). Human plasma kallikrein was purified as described by Nagase & Barrett (1981). ProMMP-2 was purified from the culture medium of human rheumatoid synovial fibroblasts as described by Okada *et al.* (1990). Human neutrophil elastase, cathepsin G and α_2M were gifts from Dr. G. Salvesen and Dr. J. J. Enghild at Duke University Medical Center (Durham, NC, U.S.A.). The human monocytic leukaemia cell line U937 and the human fibrosarcoma cell line HT1080 were obtained from the American Type Culture Collection.

Cell cultures

U937 and HT1080 cell lines were cultured in DMEM containing 10% fetal-cal serum. To stimulate the production of proMMP-9 from these lines, the cells were washed with phosphate-buffered saline (1.2 mM-KH_2PO_4/8.1 mM-Na_2HPO_4/138 mM-NaCl/2.7 mM-KCl/0.9 mM-$CaCl_2$/0.5 mM-$MgCl_2$) three times and treated with PMA (50 ng/ml) in serum-free DMEM supplemented with 0.2% lactalbumin hydrolysate. After 2–3 days, the conditioned medium was harvested and stored at −20 °C after removal of the cells by centrifugation (400 *g*, 5 min).

Enzyme assays

Gelatinolytic activity was measured by using heat-denatured [^{14}C]acetylated type-I collagen (guinea pig) by the method of Harris & Krane (1972). One unit of gelatinolytic activity degraded 1 μg of gelatin/min at 37 °C. Activity against reduced [^{14}C]-carboxymethylated transferrin ([^{14}C]Cm-Tf) was measured as described previously (Okada *et al.*, 1986). One unit of [^{14}C]Cm-Tf-degrading activity generated 1 μg of substrate fragments soluble in 3% trichloroacetic acid in 1 min at 37 °C. All the enzyme assays were carried out in TNC buffer [50 mM-Tris/HCl buffer (pH 7.5)/0.15 M-NaCl/10 mM-Ca^{2+}/0.02% NaN_3/0.05% Brij 35].

Purification of proMMP-9

ProMMP-9 was purified from the culture media of the U937 cells and the HT1080 cells stimulated with PMA. All purification procedures were carried out at 4 °C in TNC buffer unless otherwise stated. The crude culture medium was first concentrated approx. 20-fold with an Amicon Diaflo apparatus fitted with a YM-10 membrane, and applied to a column (1.5 cm × 22 cm) of DEAE-cellulose equilibrated with 50 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/10 mM-Ca^{2+}/0.02% NaN_3 to remove glycosaminoglycans and other anionic compounds. The unbound fraction was then passed through an anti-(human MMP-3) immunoadsorbent column (75 ml), and applied to a column (1.5 cm × 14 cm) of Green A Dyematrex gel equilibrated with TNC buffer without Brij 35. ProMMP-9 was eluted with 0.3 M-NaCl and 0.5 M-NaCl. The fractions eluted with a 0.3 M-NaCl were free from TIMP (see Fig. 1), but contained proMMP-2. They were then concentrated to 45 ml with a YM-10 membrane and applied to a column (2.5 cm × 8 cm) of gelatin–Sepharose equilibrated with 50 mM-Tris/HCl (pH 7.5)/0.6 M-NaCl. ProMMP-9 and proMMP-2 were eluted with the same buffer containing 1 M-NaCl and 5% (v/v) dimethyl sulphoxide, dialysed against TNC buffer and passed through an anti-(human proMMP-1) immunoadsorbent column to remove a trace amount of proMMP-1. The pool containing proMMP-9 and proMMP-2 was applied to a concanavalin A–Sepharose column equilibrated with TNC buffer to separate the proMMP-9 (glycosylated) from proMMP-2 (unglycosylated). The bound proMMP-9 was eluted from the column with TNC buffer containing 0.6 M-NaCl and 1 M-methyl α-D-mannoside, and further purified by gel-permeation chromatography on a Sephacryl S-200 column (1.5 cm × 120 cm) in TNC buffer containing 0.4 M-NaCl.

Purification of TIMP

TIMP was purified from the culture medium of HT1080 cells treated with PMA. The initial purification steps up to Green A Dyematrex gel chromatography are the same as those for proMMP-9. The fractions eluted from Green A Dyematrex gel with 0.5 M-NaCl were concentrated with a YM-10 membrane and applied to a column (2.5 cm × 8 cm) of gelatin–Sepharose equilibrated with 50 mM-Tris/HCl (pH 7.5)/0.4 M-NaCl. The unbound fractions containing free TIMP were concentrated and subjected to gel-permeation chromatography on a Sephacryl S-200 column (1.5 cm × 120 cm) equilibrated with TNC buffer containing 0.4 M-NaCl. The final product was homogeneous on SDS/PAGE. The concentration of TIMP was determined by titration with the known amount of MMP-3 (stromelysin) which binds to TIMP in 1:1 stoichiometry (Okada *et al.*, 1988*b*).

Electrophoresis and zymography

SDS/PAGE was performed by the method of Bury (1981). After electrophoresis, the proteins were stained with either $AgNO_3$ (Wray *et al.*, 1981) or Coomassie Brilliant Blue R-250. The molecular-mass standards were phosphorylase *b* (94 kDa), transferrin (77 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soya-bean trypsin inhibitor (21 kDa) and lysozyme (14 kDa).

Zymography was conducted with SDS/polyacrylamide gels

containing gelatin (0.8 mg/ml). The samples to be tested were mixed with the SDS/PAGE sample buffer without a reducing agent and subjected to electrophoretic analysis without boiling. Enzyme activity was detected as described by Hibbs *et al.* (1985).

Protein determination

The concentrations of proteins were determined by the BCA protein assay by the method of Smith *et al.* (1985), with crystalline BSA as standard.

Digestion of extracellular matrix macromolecules

ProMMP-2 and proMMP-9 were maximally activated with 1 mM-APMA at 37 °C and incubated with various protein substrates in TNC buffer at the indicated temperature. The reactions were stopped with 20 mM-EDTA and products analysed by SDS/PAGE under reducing conditions.

RESULTS

Purification of proMMP-9

Typical results of purification of proMMP-9 from the culture medium of U937 cells are shown in Table 1. Similar purification results were obtained with the culture medium of HT1080 cells.

It has been reported that proMMP-9 secreted from U937 cells of HT1080 cells is tightly complexed with TIMP secreted from the same cells (Wilhelm *et al.*, 1989; Moll *et al.*, 1990). The proMMP-9–TIMP complexes have been shown to be not readily dissociable. Indeed, when the crude culture medium of U937 cells after the DE-52 step was applied directly to the gelatin–Sepharose column, proMMP-9 was co-purified with TIMP (Fig. 1*a*). However, Green A Dyematrex gel chromatography proved to be a good step for isolation of proMMP-9 free from TIMP; the proMMP-9 fraction eluted with 0.3 M-NaCl from the column was virtually free from TIMP of molecular mass 29 kDa (Fig. 1*a*, lane 2). On the other hand, proMMP-9 was co-eluted with TIMP in the 0.5 M-NaCl fraction, presumably as a complex, since subsequent purification of this fraction did not change the ratio of the two components. Thus the 0.3 M-NaCl fraction (39% recovery of proMMP-9) was subjected to further purification using gelatin–Sepharose. Since U937 cells and HT1080 cells synthesize proMMP-2 as well as proMMP-9 and both zymogens are adsorbed by gelatin–Sepharose, the major contaminant at this stage is proMMP-2. Separation of proMMP-2 and proMMP-9 was accomplished by using concanavalin A–Sepharose. The final product of proMMP-9 was free from TIMP, and the molecular mass was estimated to be 92 kDa with reduction and

Table 1. Purification of proMMP-9 from U937 cells

See the text for detailed purification procedures. proMMP-9 activity was monitored after activation with 1 mM-APMA for 36 h at 37 °C

Step	Total proteins (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude medium	3096	4390	1.4	1	100
DEAE-cellulose (DE-52)	600	4290	7.1	5	98
Anti-(MMP-3) immunoadsorption	533	4250	8.0	5.7	97
Green A Dyematrex (0.3 M-NaCl fraction)	5.3	1630	308	220	39
Gelatin–Sepharose	1.2	1360	1130	806	31
Anti-(proMMP-1) immunoadsorption	1.0	1140	1140	814	26
Concanavalin A–Sepharose	1.8	965	536*	382*	22
Sephacryl S-200	0.2	762	3760	2690	19

* Decrease in specific activity and purification is due to the contamination of concanavalin A.

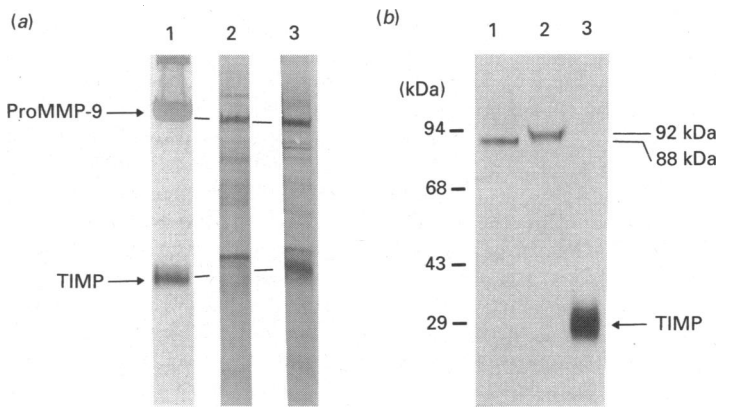

Fig. 1. SDS/PAGE analysis of proMMP-9 preparation

(*a*) proMMP-9 at different purification stages was analysed under reducing conditions. Lane 1, proMMP-9 eluted from a gelatin–Sepharose column after the DE-52 step; lanes 2 and 3, fractions eluted from a Green A Dyematrex gel column with 0.3 M-NaCl and 0.5 M-NaCl respectively. (*b*) SDS/PAGE analysis of purified proMMP-9 and TIMP. Lanes 1 and 2, proMMP-9 (0.1 μg) run without and with reduction respectively. Lane 3, TIMP (0.3 μg) purified from the culture medium of HT1080 cells. Proteins were stained with silver. Protein standards were phosphorylase *a* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

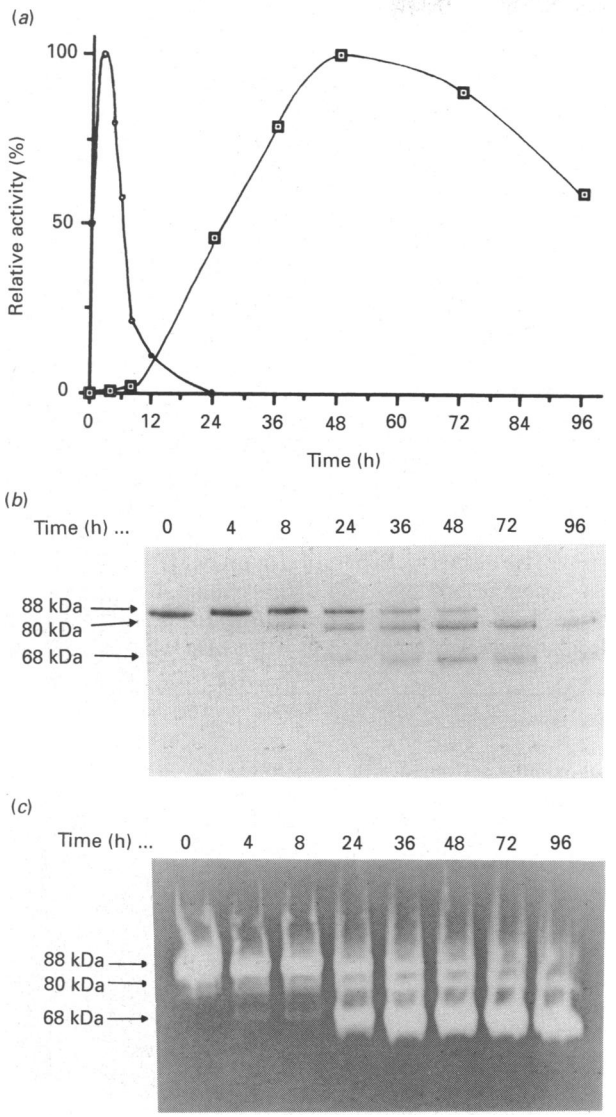

Fig. 2. Activation of proMMP-9 by APMA

ProMMP-9 (28 μg/ml) was incubated with 1 mM-APMA at 37 °C for the period indicated. Then the samples were used (*a*) to measure gelatinolytic activity (⊡), (*b*) for SDS/PAGE without reduction and (*c*) for zymographic analyses. The time course of activation of proMMP-2 (○) by 1 mM-APMA at 37 °C is shown in (*a*) for comparison.

88 kDa without reduction on SDS/PAGE (Fig. 1*b*). TIMP-free proMMP-9 was used for zymogen-activation studies and characterization of the enzyme activity.

Activation of proMMP-9

No gelatinolytic activity was detected with the purified proMMP-9. Many of the zymogens of MMPs can be activated by treating with mercurial compounds or proteinases. Therefore we first examined a time-course of proMMP-9 activation by 1 mM-APMA. Unlike the case of proMMP-2, activation of proMMP-9 by APMA was very slow: the maximal specific activity, 3800 units/mg, was attained only after a 48 h incubation at 37 °C, whereas proMMP-2 was fully activated within 45 min (Fig. 2*a*). The correlation between the activation and the changes in molecular mass of proMMP-9 on APMA treatment was examined by SDS/PAGE and zymography under non-reducing conditions (Figs. 2*b* and 2*c*). ProMMP-9 of molecular mass 88 kDa was first converted into the 80 kDa form and then the

Table 2. Changes in molecular mass on activation of proMMP-9 by APMA or trypsin

Molecular-mass estimations under non-reducing conditions are based on both SDS/PAGE and zymography, and those under reducing condition are solely based on SDS/PAGE. The presence (+) or absence (−) of proteolytic activity was assessed by the reactivity with α_2M in zymographic analyses under non-reducing conditions. [ProMMP-9+TIMP] is a 1:1 molar complex of proMMP-9 and TIMP.

	Molecular mass (kDa)	
Treatment	Non-reduced	Reduced
ProMMP-9	88 (−)	92
ProMMP-9+APMA	80 (−)	84 and 82
	68 (+)	70
ProMMP-9+trypsin	80 (+)	82
	74 (minor) (+)	
	66 (minor) (+)	
	56 and 53 (−)	68
		45
[ProMMP-9+TIMP]+APMA	80 (−)	84 and 82
[ProMMP-9+TIMP]+trypsin	80 (−)	82
	74 minor (−)	
	66 minor (−)	
	56 and 53 (−)	68
		45

68 kDa form. Under reducing conditions the corresponding molecular masses were slightly higher (see Table 2). Zymographic analyses at each time point exhibited gelatinolytic activity in both 80 kDa and 68 kDa species as well as the 88 kDa zymogen, but the major activity was associated with the 68 kDa species. Furthermore, the appearance of the 68 kDa species correlated with the enzyme activity detected in a conventional assay using [^{14}C]gelatin in solution, suggesting that this may be the active species of MMP-9 generated by APMA treatment. This was confirmed by its ability to bind to α_2M (see below).

Next, we examined eight endopeptidases for their abilities to activate proMMP-9. These included trypsin, chymotrypsin, plasmin, plasma kallikrein, thrombin, cathepsin G, neutrophil elastase and thermolysin (1–10 μg/ml for various incubation times). Of these, trypsin activated proMMP-9 most effectively. Treatment with chymotrypsin or cathepsin G at a concentration of 10 μg/ml resulted in only partial (22–30%) activation which required 24–48 h incubation at 37 °C. The maximal activation observed with plasmin (10 μg/ml) was only 10% of the full activity after incubation for 48 h at 37 °C. The effects of other enzymes on proMMP-9 activation were negligible. We further investigated the activation process of proMMP-9 by trypsin. A time-course analysis of proMMP-9 activation with 10 μg of trypsin/ml showed that the maximal activity of 3800 units/mg was attained after incubation at 37 °C for 0.5–1 h (Fig. 3*a*). SDS/PAGE analyses showed that the 88 kDa zymogen was converted into 80 kDa, 56 kDa and 53 kDa forms (Fig. 3*b*). Zymograms at each time point indicated that gelatinolytic activity was associated with the 80 kDa, 74 kDa and 66 kDa species (Fig. 3*c*). The protein bands corresponding to the latter two species were not visible in Fig. 3(*b*), but they could be detected by prolonged silver staining (results not shown). The proteins of molecular mass 56 kDa and 53 kDa did not exhibit any gelatinolytic activity.

Zymographic analyses suggested that several active species with different molecular masses were generated after activation of proMMP-9 by APMA or trypsin. However, it is difficult to assess whether or not all of these forms indeed have enzymic activity, since even the 88 kDa zymogen exhibits gelatinolysis

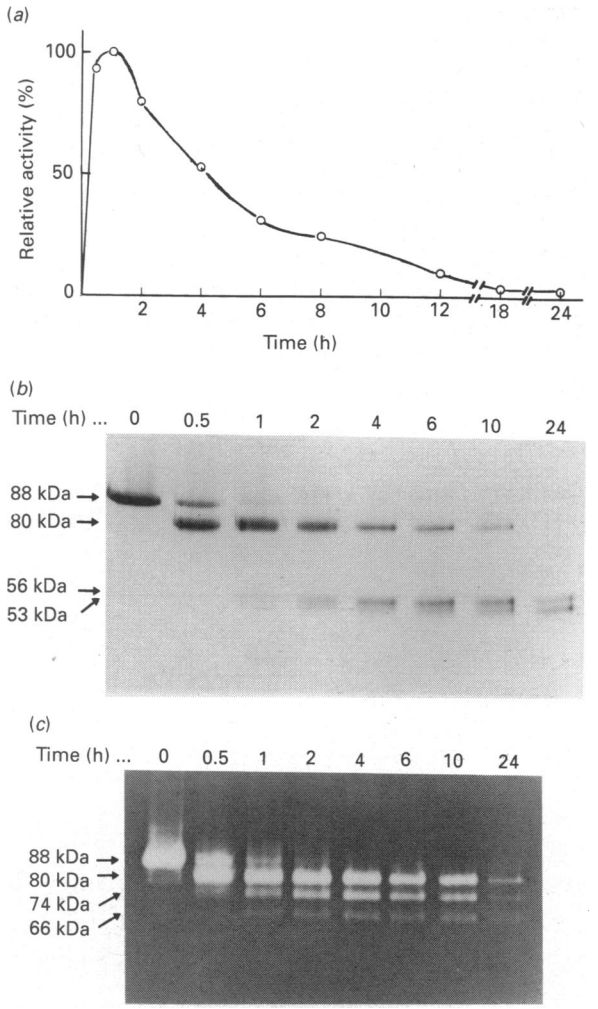

Fig. 3. Activation of proMMP-9 by trypsin

ProMMP-9 (28 μg/ml) was incubated with trypsin (10 μl/ml) at 37 °C for the period indicated and the reaction terminated with 2.5 mM-Dip-F. Samples were then used (*a*) to measure gelatinolytic activity, (*b*) for SDS/PAGE without reduction and (*c*) for zymographic analyses.

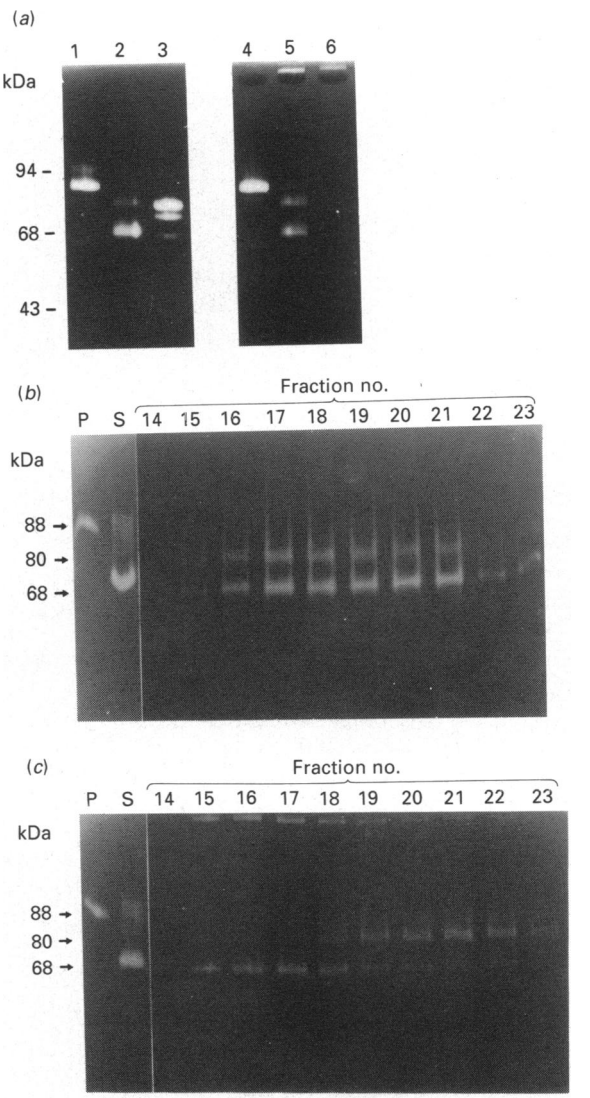

Fig. 4. Identification of active species of MMP-9 by complex-formation with α_2M

(*a*) proMMP-9 (lanes 1 and 4), APMA-activated MMP-9 (lanes 2 and 5) and trypsin-activated MMP-9 (lanes 3 and 6) were subjected to zymography before (lanes 1–3) and after (lanes 4–6) complexing with a 4 M excess of α_2M. Active MMP-9 was complexed with α_2M, and the zones of gelatin lysis were observed at the top of the gel together with α_2M dimers. (*b*) and (*c*), specific binding of the APMA-generated 68 kDa species to α_2M. APMA-activated proMMP-9 containing 80 kDa and 68 kDa species was subjected to gel-permeation chromatography on a column (0.5 cm × 20 cm) of Sephacryl S-300 before (*b*) and after (*c*) complexation with a 4 M excess of α_2M. Fractions (200 μl) were collected, and a portion of each was subjected to zymographic analysis. The shift of the 68 kDa species to the earlier fractions together with α_2M as demonstrated in (*c*) indicates that only the 68 kDa species is proteolytically active. P, ProMMP-9; S, the APMA-activated sample before gel-permeation chromatography.

due to autoactivation induced by molecular perturbation in SDS during electrophoresis. To identify the active molecules, we used the property of α_2M of binding only to active endopeptidases (Barrett & Starkey, 1973). α_2M bound stoichiometrically to activated MMP-9 within 1 h at 37 °C and inhibited its gelatinolytic activity (results not shown). This establishes that all active species complex with α_2M, and that the MMP-9 proteins which fail to bind to α_2M do not possess endopeptidase activity. Indeed, proMMP-9 did not bind to α_2M even after incubation with a 4 M excess of α_2M at 37 °C for 1 h, which was demonstrated by the failure to shift the gelatin-lysis zone at 88 kDa to the top of the gel on zymography (Fig. 4*a*). When APMA-activated proMMP-9 was incubated with α_2M, only the 68 kDa species bound to α_2M. As shown in Fig. 4(*a*), most of the gelatinolytic activity at 68 kDa was shifted to the top of the gel where α_2M–proteinase complexes run on SDS/PAGE under non-reducing conditions (Fig. 4*a*, lane 5). The small amount of activity remaining at 68 kDa was probably due to partial dissociation of the enzyme from the complex (see below). The failure of the 80 kDa species to bind to α_2M indicates that it does not possess endopeptidase activity unless activated by the SDS treatment in zymography. The specific complex-formation of the 68 kDa MMP-9, but not the 80 kDa species, with α_2M was also demonstrated by the separation of the α_2M–68 kDa MMP-9 complex from the 80 kDa species by gel filtration (Figs. 4*b* and 4*c*). Some gelatinolytic activity detected at 68 kDa in the α_2M fraction (earlier fractions in Fig. 4*c*) is derived from partial dissociation from the α_2M–enzyme complexes due to SDS treatment. Using the same technique, we concluded that the 80 kDa, 74 kDa and 66 kDa species generated by trypsin are all active (Fig. 4*a*, lanes 3 and 6).

Titration of active MMP-9 with α_2M also allowed us to make

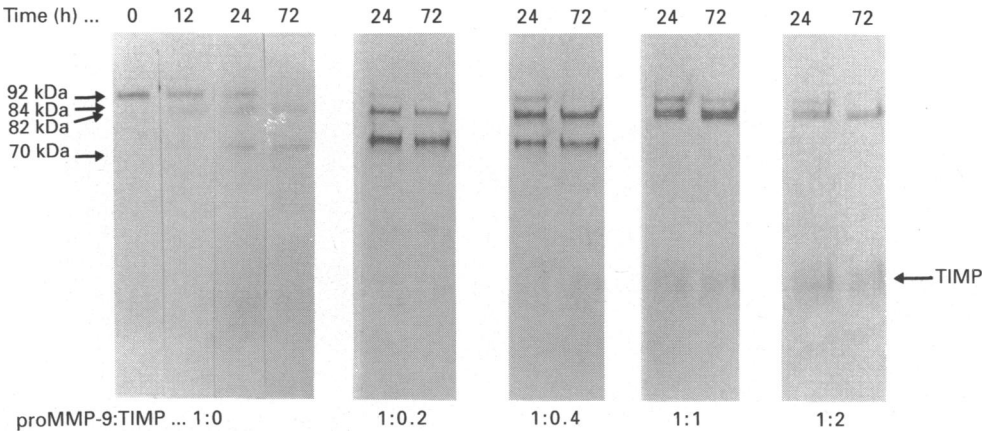

Fig. 5. Effect of TIMP on proMMP-9 processing by APMA

ProMMP-9 was mixed with TIMP at various molar ratios as indicated and subjected to activation by APMA for 24 h or 72 h at 37 °C. APMA activation was terminated with 20 mM-EDTA, and the samples were analysed by SDS/PAGE (7.5% acrylamide) under reducing conditions. The amount of TIMP was determined by titration with MMP-3.

Table 3. Effect of TIMP on proMMP-9 activation by APMA and trypsin

ProMMP-9 was mixed with TIMP at the various molar ratios, and the mixture was activated by treatment with 1 mM-APMA for 36 h at 37 °C or with 10 μg of trypsin/ml for 1 h at 37 °C followed by inactivation with 2.5 mM-Dip-F. MMP-9 activity was examined by using [^{14}C]gelatin as substrate by the method of Harris & Krane (1972).

TIMP/proMMP-9 (mol/mol)	MMP-9 activity (%)	
	APMA activation	Trypsin activation
0	100	100
0.1	78	80
0.2	58	61
0.4	26	48
0.6	19	30
0.8	3	4
1.0	0	0
1.5	0	0

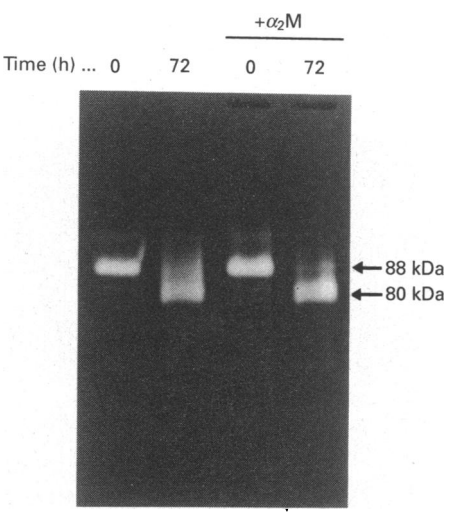

Fig. 6. Lack of proteolytic activity of the 80 kDa species generated by APMA in the presence of TIMP

ProMMP-9 was mixed with an equimolar amount of TIMP and the mixture was activated with 1 mM-APMA for 72 h at 37 °C. Portions of the APMA-activated samples were then subjected to zymographic analyses before or after reacting with α_2M for 1 h at 37 °C. The sample without activation with APMA (time 0) shows the 88 kDa zymogen.

an accurate assessment of the specific activity of MMP-9. Assuming that α_2M forms a complex with MMP-9 in 1:1 molar stoichiometry, the specific activity of MMP-9 against gelatin was estimated to be 15000 units/mg, approx. 3.3 times higher than that estimated on the basis of the amount of protein determined by the BCA assay using BSA as a standard.

Effect of TIMP on proMMP-9 activation

ProMMP-9 purified by gelatin affinity chromatography, without using Green A Dyematrex gel, contained a considerable amount of TIMP (Fig. 1*a*, lane 1). When this material was treated with APMA, proMMP-9 was converted into the 80 kDa species, but only a small amount of active form (68 kDa) was generated (results not shown). This led us to consider the effect of TIMP in the proMMP-9 activation process. To investigate this further the TIMP-free proMMP-9 preparation was mixed with TIMP at various molar ratios and the processing of proMMP-9 by APMA examined by SDS/PAGE under reducing conditions. As shown in Fig. 5, conversion of proMMP-9 (92 kDa) into the 70 kDa (68 kDa without reduction; see Table 2 for molecular-mass conversion) active species was prevented by the presence of TIMP in a dose-dependent manner, and a complete block was observed at a 1:1 molar ratio even after 72 h incubation at 37 °C. Measurement of the gelatinolytic activity of these samples indicated that generation of the 70 kDa species correlated with the expression of the enzyme activity (Table 3). Although the 84 kDa and 82 kDa species (80 kDa without reduction) exhibited gelatinolytic activity on zymography (Fig. 6), the generation of these species did not correlate with gelatinolytic activity in the solution assay. Lack of proteolytic activity in these species was further confirmed by their failure to bind to α_2M (Fig. 6).

The ability of trypsin to activate proMMP-9 in the presence of TIMP was also examined (Table 3). As in the case of APMA, the expression of gelatinolytic activity was completely blocked by a stoichiometric amount of TIMP during trypsin activation.

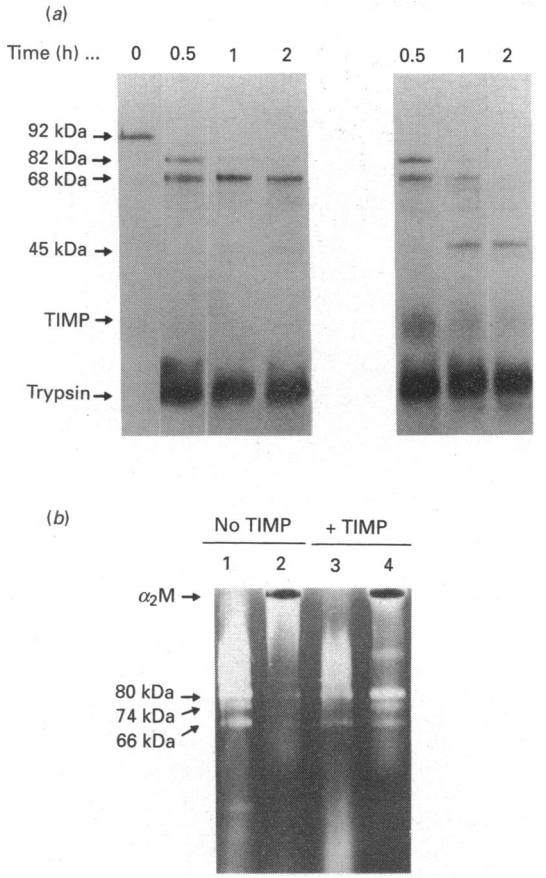

Fig. 7. Processing of proMMP-9 by trypsin in the presence of TIMP

(*a*) proMMP-9 was incubated with 10 μg of trypsin/ml at 37 °C for the indicated period in the absence (left panel) and presence (right panel) of an equimolar amount of TIMP. After inactivation of trypsin with 2.5 mM-Dip-F, samples were analysed by SDS/PAGE under reducing conditions. Proteins were stained with silver. (*b*) ProMMP-9 was activated with 10 μg of trypsin/ml at 37 °C for 30 min in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of an equimolar amount of TIMP. After inactivation of trypsin with 3 mM-Dip-F, the samples were allowed to react with (lanes 2 and 4) or without (lanes 1 and 3) α_2M for 1 h at 37 °C and subjected to zymography. Note that the trypsin-activated MMP-9 was complexed with α_2M in the absence of TIMP, but not in the presence of TIMP.

Table 4. Substrate specificity of MMP-9 and MMP-2 at 32 °C

Substrate	Activity (μg of substrate digested/min per mg of protein)	
	MMP-9	MMP-2
Gelatin (type I)*	15000	12000
Cm-Tf*	89	226
Type-IV collagen	16	4
Type-V collagen	2500	600
Laminin	–†	–†
Fibronectin	< 2	50

* Measured at 37 °C.
† Activity detected but not quantified.

SDS/PAGE analysis of the proMMP-9 and TIMP mixture after trypsin treatment indicated that proMMP-9 was converted into 82 kDa and 68 kDa species, as in the case of proMMP-9 alone (Fig. 7*a*). However, a faster degradation of the 68 kDa species to 45 kDa was evident in the presence of TIMP (Fig. 7*a*). This suggests that the binding of TIMP to proMMP-9 and/or the processed MMP-9 induces conformational changes in the MMP-9 molecule. The α_2M-binding studies also indicated that the MMP-9 generated by trypsin in the presence of TIMP was not proteolytically active (Fig. 7*b*).

Properties of MMP-9

The activity of MMP-9 was inhibited by typical inhibitors of MMPs, including TIMP, 1,10-phenanthroline, EDTA and dithiothreitol. We have reported that glycine was inhibitory to MMP-2 (Okada *et al.*, 1990), but a significant inhibitory effect of glycine was not observed with MMP-9.

The ability of MMP-9 to digest various components of the extracellular matrix was examined and compared with that of MMP-2 (Table 4). MMP-2 and MMP-9 digested type-I gelatin to a similar extent. SDS/PAGE analysis of the products showed that the major cleavage sites on type-I gelatin by the two enzymes were similar (Fig. 8). Digestion products of type-IV collagen and type-V collagen generated by the two enzymes were also indistinguishable (Fig. 8). However, the specific activities of MMP-

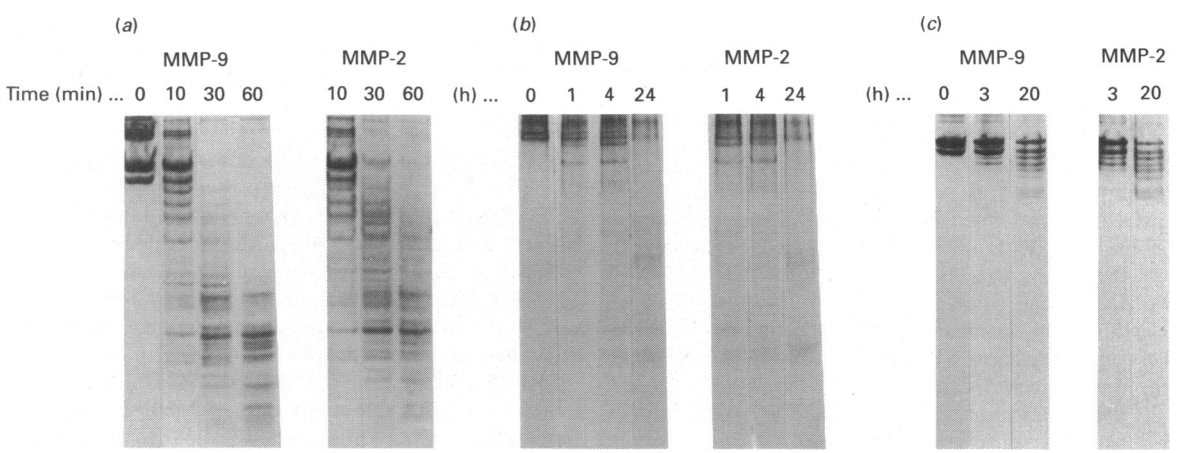

Fig. 8. Digestion of (*a*) type-I gelatin and (*b*) types-IV and (*c*) -V collagens by MMP-9 and MMP-2

Guinea-pig gelatin type I (15 μg), type-IV collagen from EHS tumour (18 μg) and human type-V collagen (20 μg) were digested by equivalent units of MMP-2 or MMP-9 at 37 °C for gelatin and at 32 °C for collagen types IV and V for the indicated period. The reaction was stopped with 25 mM-EDTA and products were analysed by SDS/PAGE.

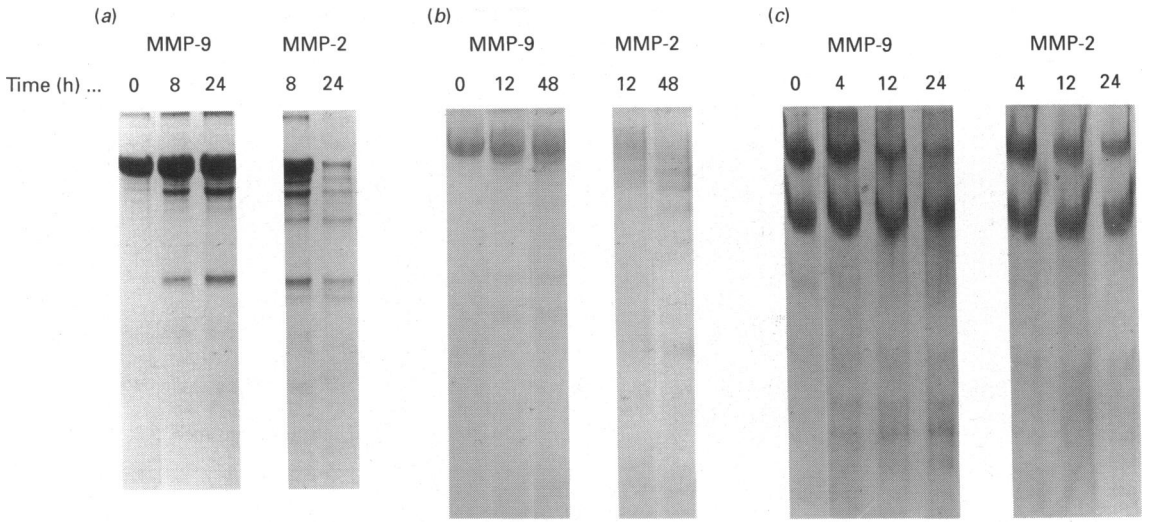

Fig. 9. Differential actions of MMP-9 and MMP-2 on (*a*) Cm-Tf, (*b*) fibronectin and (*c*) laminin

Cm-Tf (30 μg), fibronectin (30 μg) and laminin (30 μg) were incubated with 20 units of MMP-9 or MMP-2 at 32 °C for the period indicated. The reaction was terminated with 25 mM-EDTA, and the products were analysed by SDS/PAGE.

9 towards these macromolecules are about 4-fold higher than those of MMP-2. It is also notable that both enzymes digested type-V collagen more readily than type-IV collagen. These results suggest that MMP-9 and MMP-2 attack these extracellular macromolecules at least at similar loci, if not at identical bonds.

The actions of MMP-9 and MMP-2 on Cm-Tf were very different: 1 mg of MMP-9 digested only 89 μg of Cm-Tf in 1 min at 37 °C. This was about 40% of the activity of MMP-2. SDS/PAGE analyses of the Cm-Tf digestion products showed that only two major fragments of molecular mass 64 kDa and 32 kDa were generated from the 77 kDa Cm-Tf by MMP-9, whereas fragments of 71 kDa, 64 kDa, 53 kDa, 32 kDa, 28 kDa, 24 kDa and 16 kDa were produced by MMP-2 (Fig. 9). The susceptibilities of fibronectin and laminin to MMP-9 and MMP-2 were also different: MMP-2 had significant activity toward fibronectin, but the activity of MMP-9 on this substrate was negligible; and MMP-9 digested primarily the A chain of laminin, whereas both A and B chains increased their electrophoretic mobility after MMP-2 digestion. These results indicate that the subsite requirements are different for these two closely related MMPs.

DISCUSSION

ProMMP-9 has been purified from human (Hibbs *et al.*, 1985) and pig neutrophils (Murphy *et al.*, 1989*a*), and from the culture medium of several transformed human cells (Wilhelm *et al.*, 1989; Davis & Martin, 1990; Moll *et al.*, 1990) and rat mammary carcinoma cells (Lyons *et al.*, 1991). ProMMP-9 isolated from the transformed cells was shown to be non-covalently complexed with TIMP. Thus characterization of MMP-9 from these cells has been limited, since the effects of TIMP on proMMP-9 activation and its action on substrates are not known. In this study we purified proMMP-9 free from TIMP as far as we could detect on SDS/PAGE. The key step was chromatography on Green A Dyematrex gel. This allowed us to investigate the activation process of proMMP-9 by APMA and trypsin and the enzymic properties of MMP-9.

Like other zymogens of MMPs, proMMP-9 was activated by APMA, but this process was very slow. The two proMMP-9 preparations from U937 cells and HT1080 cells showed a very similar activation time course. This can be contrasted with pig neutrophil proMMP-9, which was fully activated within 5 min by 0.5 mM-APMA at 37 °C (Murphy *et al.*, 1989*a*). On APMA treatment, human proMMP-9 (88 kDa) was first converted into 80 kDa and then 68 kDa species. Similar changes in molecular mass have been reported for human and pig proMMP-9 on organomercurial treatment (Murphy *et al.*, 1989*a*; Wilhelm *et al.*, 1989; Davis & Martin, 1990; Moll *et al.*, 1990), but the active forms have not been identified. The zymographic method is often used for easier detection of molecular-mass changes, but this method does not allow identification of the proteolytically active form, since even the 88 kDa zymogen exhibits apparent gelatinolytic activity. We have identified the catalytically active form of MMP-9 by using α_2M, which binds only to proteolytically active forms. This method allowed us to determine that only the 68 kDa form is proteolytically active after APMA treatment. Interestingly, in the presence of an equimolar amount of TIMP, proMMP-9 was converted only into the 80 kDa species (84 kDa and 82 kDa under reducing conditions). These forms also exhibited apparent gelatinolytic activity in gelatin zymograms, but no enzymic activity was detected in a conventional gelatinase assay in solution (Table 3), and it failed to bind to α_2M. This suggests that either the 80 kDa form is an inactive intermediate, or it is bound to TIMP. When the proMMP-9–TIMP complex was treated with APMA and subjected to gel-permeation chromatography, MMP-9 and TIMP were eluted together (results not shown), suggesting that the latter is the likely case. This is further supported by the increased generation of the 80 kDa intermediate species and decreased formation of the 68 kDa active MMP-9 in the presence of increasing amounts of TIMP during APMA activation (Fig. 5). It may also be suggested that the binding of TIMP to the intermediate is tight and that TIMP does not transfer from the intermediate to the active MMP-9. This is supported by the observation that the amount of the 80 kDa intermediate did not change even after a longer activation period with APMA (72 h) in an excess of proMMP-9. This tight binding of TIMP to the MMP-9 intermediate can be contrasted with the interaction of MMP-3 (stromelysin) intermediates and TIMP reported by Ward *et al.* (1991). These authors have shown that TIMP has the ability to bind to MMP-3 intermediates during APMA activation, but when the molar ratio of proMMP-3 and TIMP is 2:1, all MMP-3 intermediates are eventually converted into fully active 46–50 kDa MMP-3, by

the transfer of TIMP from the TIMP–intermediate complex to active MMP-3 (Ward *et al.*, 1991). Dissociation of the 80 kDa MMP-9 intermediate and TIMP complex might have occurred during SDS/PAGE, which thus exhibits the apparent gelatinolytic activity on zymography (Fig. 6). It was also notable that there was a small amount of activity detected around 80 kDa on zymograms even after prolonged incubation of proMMP-9 with APMA (Fig. 2). This suggests that our proMMP-9 preparation might have contained a trace amount of TIMP which could not be detected by SDS/PAGE.

Of eight endopeptidases tested, trypsin activated proMMP-9 most effectively. Plasmin, which recognizes arginine and lysine residues which participate in proMMP-1 (tissue procollagenase) (Suzuki *et al.*, 1990) and proMMP-3 (prostromelysin) (Okada *et al.*, 1988*a*; Nagase *et al.*, 1990) activation processes, was not a good activator of proMMP-9. Three different active species (80 kDa, 74 kDa, 66 kDa) were identified after trypsin treatment by the combination of zymography and the α_2M-binding assay. Treatment of proMMP-9 with trypsin in the presence of an equimolar amount of TIMP did not produce any proteolytic activity. The zymographic analyses indicated that proMMP-9 was converted into the similar 80 kDa, 74 kDa and 66 kDa forms, but they failed to bind to α_2M. This also suggests that MMP-9 is complexed with TIMP after activation. It is also evident that the enzyme–inhibitor complexes were degraded after longer treatment with trypsin.

TIMP binds to proMMP-9 non-covalently. Thus it may be postulated that the binding of TIMP to this zymogen is through interaction at sites other than the reactive centre of the two molecules, since proMMP-9 exhibits autolytic activity even in the form of the proMMP-9–TIMP complex (Wilhelm *et al.*, 1989). Activation of the proMMP-9–TIMP complex resulting in a MMP-9–TIMP complex indicates the temporal release of TIMP from proMMP-9 and its reassociation with activated MMP-9. The transfer of TIMP from the proMMP-9–TIMP complex to the active MMP-2 has been reported by Ward *et al.* (1991), and it has been suggested that the binding of TIMP and proMMP-9 is weaker than the complex-formation of TIMP and active MMPs. Our attempt to form the proMMP-9–TIMP complex by mixing the purified components also indicated that only about 30% of TIMP binds to proMMP-9 even with a 2 M excess of proMMP-9 (Y. Ogata & H. Nagase, unpublished work). Nonetheless, the level of TIMP expression in transformed cells seems to be important for governing the expression of MMP-9 activity. Treatment of HT1080 cells with PMA has been shown to increase the steady-state concentration of TIMP mRNA approx. 2-fold (Stetler-Stevenson *et al.*, 1990).

Two gelatinolytic enzymes, MMP-2 and MMP-9, are thought to be involved in digesting similar extracellular-matrix macromolecules such as gelatins and collagen types IV and V, and the two MMPs are considered to have indistinguishable substrate specificities for these macromolecules (Wilhelm *et al.*, 1989; Murphy *et al.*, 1989*a*). Both enzymes have been shown to digest elastin and cartilage proteoglycans at a reasonable rate (Senior *et al.*, 1991; Murphy *et al.*, 1991). In the present report we have further examined the substrate specificity of MMP-2 and MMP-9, using several protein substrates. Our results indicate that, although the actions of the two enzymes against gelatin, collagen types IV and V are indeed similar, their actions on fibronectin and laminin are clearly distinguishable. Furthermore, their actions on Cm-Tf were more strikingly different. The results indicate that MMP-9 has far more strict subsite requirements than MMP-2. Indeed, fibronectin is susceptible to MMP-2 and degraded into several fragments, but it is not readily digested by MMP-9. Further analyses of the cleavage sites of Cm-Tf by MMP-2 and MMP-9 may allow us to design specific substrates and/or inhibitors which may be useful for distinguishing between the activities of these two closely related MMPs.

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