Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation

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Abstract Pro-MMP2 activation is a two-step process resulting in (1) an intermediate 64 kDa form generated by the MT1-MMP activity, and (2) a mature 62 kDa form. Addition of plasminogen to HT1080 cells cultured under various conditions, or to their membrane preparation, induced a complete conversion of the intermediate MMP-2 form to the mature one, and processing of pro-MMP-9. The pro-MMP-2 activation was inhibited by plasmin inhibitors and anti-uPA antibody. These results provide evidence for involvement of the PA/plasmin system in the second step of MMP-2 activation.

Key words: MMP-2; MMP-9; uPA/plasmin system; Activation

1. Introduction

MMP-2 (gelatinase A, collagenase type IV) is a member of the matrixin enzyme family (MMP) [1]. By its ability to degrade type IV collagen, MMP-2 is believed to play a key role in the basement membrane remodeling occurring during normal (embryonic development, tissue repair, angiogenesis) and pathologic (metastasis) processes [2–4]. The pro-enzyme (72 kDa, pro-MMP-2) acquires its full proteolytic activity after an activation step, which essentially involves removal of an 80-amino-acid residue N-terminus (the prodomain), resulting in a lower molecular mass form (62 kDa) [5].

Increasing evidence is accumulating that, unlike the other MMPs, pro-MMP-2 is activated via a membrane-associated mechanism. We have previously shown that gelatinase A binds to the cell surface of mammary carcinoma cells [6]. A membrane type MMP (MT1-MMP) has been identified and associated with pro-MMP-2 activation [7]. Three additional membrane proteins (MT2-MMP, MT3-MMP and MT4-MMP) have been reported and are probably involved in the processing of progelatinase A [8–10]. The pro-MMP-2 activation is believed to involve two consecutive proteolytic cleavages. The first one, associated with the MT1-MMP activity, processes the pro-MMP-2 (72 kDa) to an intermediate form (64 kDa) with amino-terminus Leu^{67} [11,12]. The second one generates the mature active form of MMP-2 and is supposed to result from an autoproteolytic cleavage [13].

The involvement of plasminogen activator (PA)/plasmin system in the activation of pro-MMP-2 has been a subject of controversial interpretation. Keski-Oja et al. reported that uPA is able to activate pro-MMP-2 [14]. Leupeptin and its synthetic analogues inhibited pro-MMP-2 activation and the in vitro migration of fibrosarcoma cells [15]. On the contrary, Lim et al. demonstrated that, in HT1080 cell line, MMP-2 and PA/plasmin system are differentially regulated and pro-MMP-2 activation is independent of serine proteases [16].

In an attempt to better understand the possible role of the PA/plasmin system in pro-MMP-2 activation, we used HT1080 cells which express constitutively PA, MMP-2 and MMP-9 [17,18]. Our results demonstrate that, although PA/plasmin system cannot initiate pro-MMP-2 processing, it converts totally the intermediate 64 kDa form of MMP-2 to a mature, proteolytically active, 62 kDa form. Therefore, these results suggest a cooperation between plasmin and MT1-MMP in the activation process of pro-MMP-2.

2. Materials and methods

2.1. Cell culture

The human fibrosarcoma HT1080 cells and human breast adenocarcinoma MCF7 cells were grown to 80% confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, glutamine (292 mg/ml), sodium bicarbonate (2.1 g/l), ascorbic acid (50 μg/ml) and penicillin–streptomycin (100 U/ml). Cells were seeded on plastic or type I collagen (20 ng/well)-coated 24-well plates. Treatments with TPA (10 ng/ml), concanavalin A (ConA, 50 μg/ml), plasminogen (2–30 μg/ml), aprotinin (5 μg/ml) or TIMP-2 (100 nM) were done under serum-free conditions. All chemicals were purchased from Sigma (St. Louis, MO) and Gibco (Gaithersburg, MD). Human plasminogen was purified by affinity chromatography on lysine–Sepharose [19]. Recombinant human MMP-2 produced from transfected CHO cells was purified as previously described [20]. TIMP-2 was produced by transformed Chinese hamster ovary cells (CHO) using dihydrofolate reductase-methotrexate selection and amplification system. TIMP-2 was purified from CHO-conditioned medium on Hitrap $Cu2^{+}$-chelating affinity column and Hitrap Q-column (Pharmacia Biotech. Inc.) according to the procedure described by DeClerck et al. [21].

2.2. Cell membranes preparations

Crude plasma membrane preparations were obtained as previously described [22]. Essentially, cells were lysed with ice-cold buffer consisting of 20 mM Tris-HCl, pH 7.6, 0.02% (w/v) Brij-35 and passed 10 times through a narrow-gauge needle. The whole-cell lysate was centrifuged at $10\,000 \times g$ for 1 h at 4°C. The supernatant was retained and centrifuged at $100\,000 \times g$ for 1 h at 4°C. The pellet was suspended in buffer A, consisting of 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% (w/v) Brij-35.

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Abbreviations: *p*-APMA, *p*-aminophenylmercuric acetate; ECM, extracellular matrix; MMP, matrix metalloproteinase; PA, plasminogen activator(s); TIMP, tissue inhibitor of metalloproteinase; MT-MMP, membrane type matrix metalloproteinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; uPA, urokinase plasminogen activator; ε-ACA, ε-amino caproic acid.

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Protein concentrations were determined by using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to 2 mg/ml.

Aliquots of membrane preparation were incubated with recombinant MMP-2 at 37°C for 16 h. The reaction was stopped by adding non-reducing sample buffer for polyacrylamide gel electrophoresis. Following centrifugation (12 000 × *g*, 15 min), samples were taken for gelatin zymography.

2.3. Gelatin zymography

Media conditioned by cells (200 000 cells/well) were collected and clarified by centrifugation. Aliquots of conditioned media were standardized for cell DNA content [23], mixed with electrophoresis sample buffer without reducing agent and applied without boiling to 10% acrylamide gels containing gelatin 1 mg/ml. Gels were run at 20 mA, washed in 2% Triton X-100 for 1 h and incubated at 37°C for 16 h in activation buffer containing 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3. In some experiments, the chelating agent EDTA (5 mM) or ε-amino caproic acid (εACA, 100 mM) were added to the activation buffer to prove the metalloproteinase or serine proteinase nature of the gelatinolytic activities, respectively. After staining with Coomassie brilliant blue R-250, the gelatinolytic activities were detected as clear bands against the blue background. Molecular weight standard proteins (Bio-Rad Laboratories, Hercules, CA) were run simultaneously. The degree of digestion was estimated by laser scanning densitometry using a model GS-700 Imaging Densitometer (Bio-Rad, Richmond, CA) equipped with molecular Analyst software.

2.4. Enzyme assay

MMP-2 activity was assayed using heat-denatured, ^{3}H-labelled type I collagen as a substrate. The enzyme was first activated by incubation with membrane preparation of HT1080 cells for 24 h in the presence or absence of plasminogen. Thereafter, the radiolabelled substrate was added. As a positive control of the enzymatic reaction, we used 1 mM 4-amino-phenylmercuric acetate (APMA)-activated MMP-2. As a negative control, 10 mM EDTA was added to the incubation mixture.

2.5. Northern blot analysis

Confluent HT1080 human fibrosarcoma cell line was cultured for 24 h in serum-free DMEM. After removing conditioned media, cells were washed extensively with PBS and RNAs were extracted as previously described [24]. From each experimental condition, 15 μg of RNA were separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred to a nylon filter (Hybond-N, Amersham) for Northern blot hybridization. Filters were allowed to dry and RNAs were fixed under UV light for 5 min. Prehybridization and hybridization were performed according to Maniatis et al. [25].

A cDNA probe for the MT1-MMP was designed by RT-PCR on JAR cell poly A-RNA as template (American Type Collection no. HTB144) as previously described [26]. The probe was labelled (average of 10^9 cpm/μg DNA) with ^{32}P-deoxyribonucleotides by random priming DNA labelling method according to the manufacturer (Boehringer-Mannheim). Filters were washed, dried and exposed to X-ray films. Uptake of radioactivity was determined by scanning the autoradiograms using an Ultroscan XL Laser Densitometer, LKB (Pharmacia, Uppsala, Sweden). The relative amount of RNA loaded on the gel was estimated from ethidium bromide staining.

3. Results

3.1. Effect of plasminogen on gelatinases activation by HT1080 cells cultured on plastic

When human fibrosarcoma HT1080 cells were cultured on plastic for 48 h, they secreted pro-MMP-9 (92 kDa), pro-MMP-2 (72 kDa) and its intermediate form (64 kDa) (Fig. 1A, lane 1). We investigated the effect of increasing concentrations of plasminogen on pro-MMP-9 and pro-MMP-2 activation. After 48 h of incubation in the presence of plasminogen (5–30 μg/ml), the gradual appearance of activated MMP-9, in a concentration-dependent manner, was clearly observed (Fig. 1A, lanes 3–6). Addition of plasminogen resulted also in the conversion of the intermediate MMP-2 (64 kDa) into active MMP (62 kDa). The mature MMP-2 form (62 kDa) was already identified in samples treated with low concentration of plasminogen (2 μg/ml) and its level was not affected by higher concentration of this serine protease. The metalloproteinase nature of these gelatinolytic bands was assessed by their inhibition by 10 mM EDTA (data not shown). The conversion of inactive plasminogen to active plasmin by HT1080 cells was verified by gelatin zymography performed, in the absence or in the presence of ε-ACA, on medium conditioned by HT1080 cells treated with plasminogen for 24 h (data not shown).

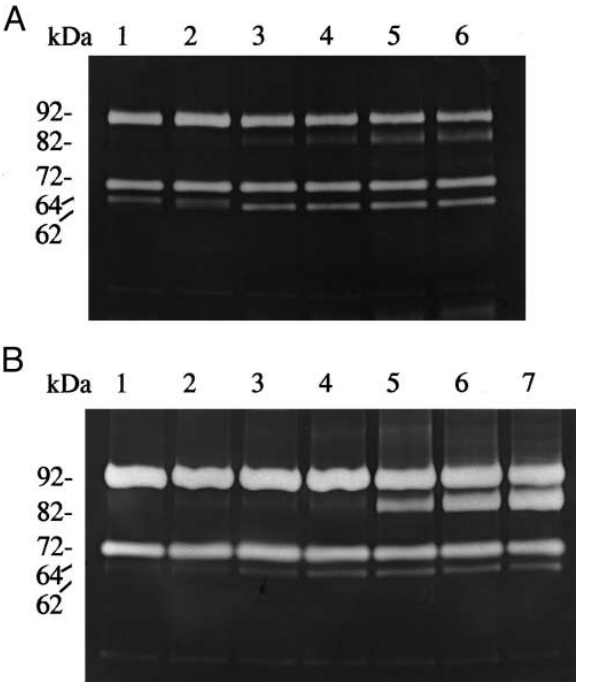

Fig. 1. Zymographic analysis of medium conditioned by HT1080 cells cultured on plastic and treated with increasing concentrations of plasminogen (A) or plasmin (B). A: Cells were cultured in the absence (lane 1) or presence of plasminogen: 2 μg/ml (lane 2), 5 μg/ml (lane 3), 10 μg/ml (lane 4), 20 μg/ml (lane 5) and 30 μg/ml (lane 6). B: Cells were cultured in the absence (lane 1) or presence of plasmin: 0.5 μg/ml (lane 2), 1 μg/ml (lane 3), 2 μg/ml (lane 4), 4 μg/ml (lane 5), 8 μg/ml (lane 6), 12 μg/ml (lane 7).

In order to confirm that the observed plasminogen effects on gelatinases activation could be ascribed to plasmin generated by cells, we treated HT1080 cells with increasing concentrations of plasmin (0.5–12 μg/ml). Under these conditions, the activation of pro-MMP-9 and the conversion of the intermediate MMP-2 form into the mature (62 kDa) species were similarly observed (Fig. 1B).

To rule out the possibility that active MMP-2 generated by plasmin was involved in pro-MMP-9 processing, cells were treated with different concentrations of plasminogen (5–30 μg) and TIMP-2 (100 nM). Although, the level of activated MMP-9 was increased in the presence of plasminogen, it was not affected by the addition of TIMP-2 (Fig. 2). On the contrary, TIMP-2 inhibited the spontaneous formation of intermediate MMP-2 form (64 kDa) and consequently that of the mature MMP-2 (Fig. 2, lanes 3,5,7, and 9). These results confirm the involvement of MMP in the first step of pro-

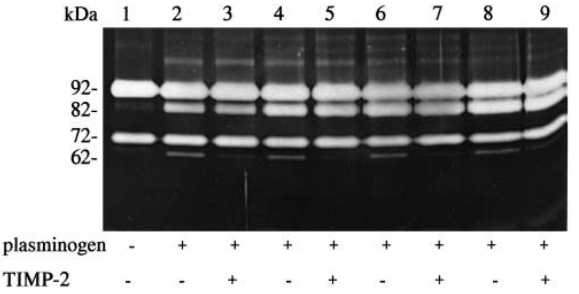

Fig. 2. Effect of TIMP-2 (100 nM) on the activation of pro-MMP-9 and pro-MMP-2 produced by HT1080 cells cultured on plastic. Cells were treated according to the above indications. Concentrations of plasminogen were: 5 μg/ml (lanes 2 and 3); 10 μg/ml (lanes 4 and 5); 20 μg/ml (lanes 6 and 7); 30 μg/ml (lanes 8 and 9).

MMP-2 activation. This MMP has previously been identified as the MT1-MMP [7].

3.2. *Effect of plasminogen on gelatinases activation by HT1080 cells treated with ConA, TPA or seeded on type I collagen layer*

We next focused our interest on the conversion of the intermediate MMP-2 form to the active MMP-2 by plasmin. HT1080 cells were always treated with plasminogen at a concentration (2 μg/ml) which did not affect MMP-9 activation but which influenced the second step of pro-MMP-2 activation. The cells were stimulated with ConA, TPA or seeded on type I collagen layer, which are known to enhance gelatinase A processing and expression of its membrane activator, the MT1-MMP. Under these conditions, we confirmed an increased expression of MT1-MMP mRNA by Northern blot analysis (Fig. 3).

When HT1080 cells were cultured on plastic in the presence of ConA or TPA, the appearance of the intermediate and mature forms of MMP-2 in conditioned medium was observed (Fig. 4, lanes 3 and 7, respectively). The addition of plasminogen to these stimulated cells provoked total conversion of the intermediate MMP-2 form to the mature one (Fig. 4, lanes 4 and 8, respectively). This process was inhibited by aprotinin (Fig. 6, lanes 5 and 9, respectively), indicating that the PA/plasmin system was implicated in the proteolytic cleavage of MMP-2 intermediate. Interestingly, TIMP-2 inhibited

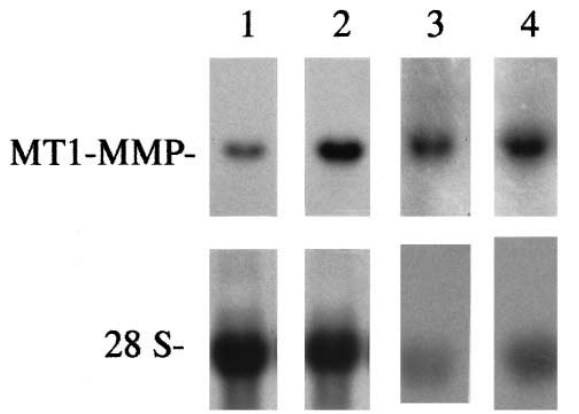

Fig. 3. Northern blot analysis of HT1080 cells. Cells were cultured on plastic (lane 1) and treated with TPA (lane 2), or ConA (lane 3) or cultured on type I collagen layer (lane 4).

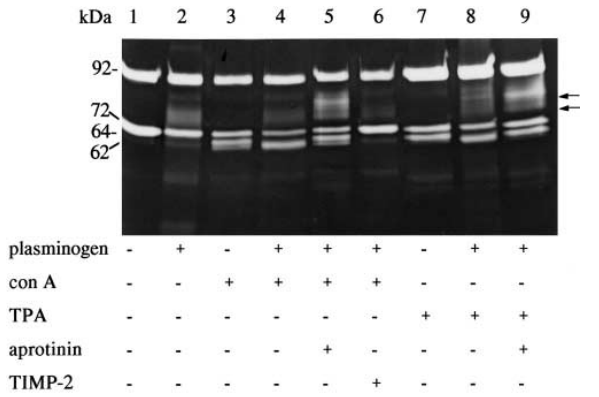

Fig. 4. Zymographic analysis of medium conditioned by HT1080 cells stimulated with ConA or TPA, treated or not treated with plasminogen (2 μg/ml) for 24 h and in the presence of aprotinin or TIMP-2 according to the above indications. The arrows on the right indicate the proteolytic activity associated with plasmin.

the induction of the MMP-2 intermediate form (64 kDa) by ConA (Fig. 4, lane 6) or TPA (data not shown). In the presence of TIMP-2, plasminogen had no effect on the zymogen (Fig. 4, lane 6) and was thus unable to initiate the pro-MMP-2 processing.

We next cultured HT1080 cells on a type I collagen layer. Under these culture conditions, the medium conditioned by fibrosarcoma cells contained gelatinolytic activities corresponding to pro-MMP-9, pro-MMP-2, and the two activated forms of the latter (64 and 62 kDa) (Fig. 5, lane 1). The addition of plasminogen induced the conversion of the intermediate form to the mature one (Fig. 5, lane 2). The effect of ConA or TPA on MMP-2 activation was additive to that of type I collagen (Fig. 5, lanes 3 and 7). Addition of plasminogen induced total processing of the intermediate form (Fig. 5, lanes 4 and 8). Aprotinin inhibited the conversion of the intermediate species to the mature MMP-2 form (Fig. 5, lanes 5 and 9), indicating again that this process was dependent on the serine protease activity. In the presence of TIMP-2, the 72 kDa zymogen was the only MMP-2 form detected, thus confirming that the initial cleavage was metalloproteinase-dependent (Fig. 5, lane 6).

3.3. *Effect of plasminogen on gelatinases activation by membranes of HT1080 cells*

The involvement of plasminogen in MMP-2 processing was further analysed by using membrane preparations of HT1080 cells incubated with recombinant pro-MMP-2. The membrane preparation was able to process the pro-MMP-2 to its intermediate and mature forms (Fig. 6, lane 2). This suggested that the activator (MT1-MMP) was proteolytically functional and generated the intermediate form. The activation ratio (62 kDa form/72 kDa+64 kDa+62 kDa forms) was estimated by scanning densitometry. In the presence of plasminogen, the intermediate MMP-2 form was completely processed to the mature one (Fig. 6, lane 3) and gave rise to an activation ratio of 0.239. Aprotinin, added to the incubation mixture, containing the membrane preparation, pro-MMP-2 and plasminogen, inhibited the conversion of the intermediate MMP-2 to the mature form (activation ratio = 0.044), while a mAb against uPA considerably slowed down this reaction (activation ratio = 0.074) (Fig. 6, lanes 4 and 5, respectively). These results

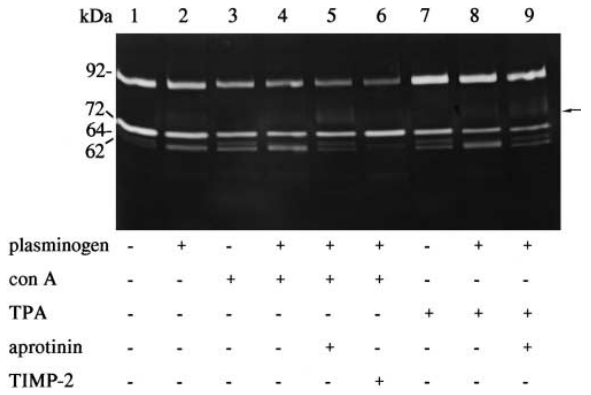

Fig. 5. Zymographic analysis of medium conditioned by HT1080 cells cultured on type I collagen layer. Cells were stimulated with ConA or TPA, treated with plasminogen (2 μg/ml), aprotinin or TIMP-2 for 24 h according to the above indications. The arrow on the right indicates the proteolytic activity associated with plasmin.

showed that the PA/plasmin system was involved in the processing of the intermediate MMP-2 form.

Additional confirmation for the implication of PA/plasmin system only in the second step of pro-MMP-2 activation was obtained with membrane preparation from MCF7 mammary carcinoma cells. This cell line is characterized by a functional PA/plasmin system [28] but it does not express MT1-MMP mRNA. In the presence or absence of plasminogen, the membrane preparation did not activate the pro-MMP-2 (Fig. 7, lanes 2 and 3).

The pro-MMP-9 secreted in the medium conditioned by HT1080 cells was activated in the presence of plasminogen, by membranes of both MCF7 cells (Fig. 7, lane 4) and HT1080 cells (data not shown), which is consistent with the presence of a functional PA/plasmin system in both membranes preparations.

In order to determine if the mature MMP-2 generated by PA/plasmin system is proteolytically active, a quantitative enzymatic assay was carried out with ^{3}H-labelled gelatin substrate (Fig. 8. The percentage of substrate degradation was similar when we used pro-MMP-2 activated by APMA ($18 \pm 5\%$), membrane-activated pro-MMP-2 in the absence ($22 \pm 5\%$) or in the presence ($21 \pm 3\%$) of plasminogen.

4. Discussion

The human fibrosarcoma HT1080 cell line represents a suitable model for studying the cooperation between MMPs and PA/plasmin system in the activation processes of gelatinases (MMP-9 and MMP-2). Fibrosarcoma HT1080 cells are able to activate exogenously added plasminogen to plasmin, as a result of the membrane receptor bound uPA activity [17]. These cells also secrete both pro-MMP-9 and pro-MMP-2. We studied the role of PA/plasmin system in the activation of both pro-MMP-9 and pro-MMP-2.

HT1080 cell monolayers treated with plasminogen or plasmin activated pro-MMP-9 in a concentration-dependent manner. Studies with purified enzymes have shown the ability of plasmin to activate pro-MMP-9 [29]. Fibroblast monolayers treated with plasminogen activated exogenous pro-MMP-9 [30]. Our results confirm that plasmin can activate the endogenously secreted MMP-9. The activation mechanism of MMP-9 is not elucidated, but it is considered to be a membrane-independent process. This is supported by our finding that membrane preparations of MCF7 cells or HT1080 cells were unable to activate pro-MMP-9 present in medium conditioned by HT1080 cells. Under these conditions, addition of plasmin or plasminogen is required in order to observe the activation of pro-MMP-9. Pro-MMP-9 has been shown to be activated by MMP-2 in vitro [31], and this process was inhibited by TIMP-2. We demonstrate that TIMP-2 fails to affect the activation of pro-MMP-9 in the presence of plasminogen, suggesting that the mature MMP-2 is unlikely involved in pro-MMP-9 processing.

Membrane-pro-MMP-2 activation is thought to be a two-step process: the first one involving MT1-MMP activity generates the intermediate form (64 kDa), and the second one, due to an intermolecular autocatalytic reaction, results in the formation of mature MMP-2 form. MT1-MMP is produced by HT1080 cells and its level of expression can be modulated by TPA, ConA and type I collagen. The cooperation between MT1-MMP and plasma membrane generated plasmin in MMP-2 activation has not yet been clearly documented. However, it has been shown that isolated extracellular matrices of human fibroblasts treated with uPA caused processing of matrix-bound 72 kDa gelatinase to 64 and 62 kDa [14]. Leupeptin and a synthetic analogue suppressed in vitro invasion of HT1080 cell line and MMP-2 activation [15]. These data are in support of the implication of the serine protease system in pro-MMP-2 processing.

We provide evidence that although the PA/plasmin system cannot initiate pro-MMP-2 activation, it may be involved in the second step of this reaction. This is supported by the following observations. (1) The treatment of HT1080 cells with plasminogen converted the intermediate MMP-2 form to the mature one. (2) A similar effect was observed by treatment with plasmin. (3) The plasminogen treatment of cells stimulated with ConA, TPA or type I collagen led to the total conversion of the intermediate MMP-2 form to the mature one; this process was inhibited by aprotinin and a monoclonal antibody (mAb) raised against uPA.

ConA, TPA and type I collagen induced an enhancement of

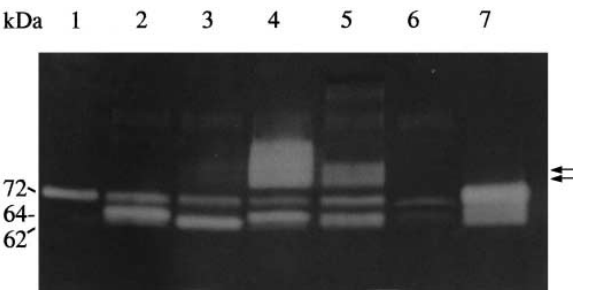

Fig. 6. Zymographic analysis of recombinant pro-MMP-2 incubated with membranes of HT1080 cells. Lane 1: pro-MMP-2; lane 2: pro-MMP-2 incubated with membrane preparation; lane 3: pro-MMP-2 incubated with membrane preparation and plasminogen; lane 4: pro-MMP-2 incubated with membrane preparation, plasminogen and aprotinin; lane 5: pro-MMP-2 incubated with membrane preparation, plasminogen and a mAb against uPA; lane 6: membrane preparation; lane 7: pro-MMP-2, activated with APMA, used as a marker of the molecular mass of the intermediate and mature MMP-2 forms. The arrows on the right indicate the proteolytic activity associated with plasmin.

pro-MMP-2 activation, resulting in the appearance of the intermediate, and mature forms of MMP-2 produced by different cell types [11,27,32,33]. The HT1080 cells express constitutively MT1-MMP and thus process spontaneously the pro-MMP-2 to the intermediate form. In accordance with a previous work [13], we show that TIMP-2 inhibits the first step of pro-MMP-2 activation and that addition of plasminogen in the presence of TIMP-2 has no effect on zymogen processing. These results indicate that the serine protease system cannot initiate pro-MMP-2 activation. The fact that relatively low concentration of plasminogen induces total processing of the intermediate MMP-2 form suggests that this interaction takes place on the cell membrane, where MT1-MMP and the receptor bound uPA colocalize. Therefore, we used membrane preparations of HT1080 which were able to induce the first step of pro-MMP-2 activation. In the presence of plasminogen, the intermediate MMP-2 form was completely converted to a mature one. This reaction was inhibited by aprotinin, and a blocking mAb raised against uPA, indicating that the uPA/plasmin system is involved in this reaction.

Confirmation of the role of plasmin in the second step of pro-MMP-2 activation was obtained by using membrane preparations of MCF7 human breast carcinoma cells. These cells do not express MT1-MMP mRNA [34] and their membranes were unable to activate the exogenously added pro-MMP-2 to the intermediate form. Even in the presence of plasminogen, the activation of pro-MMP-2 was not initiated. However, the exogenously added pro-MMP-9 was activated, thus suggesting that the membrane preparation generated plasmin.

The mature MMP-2 form, generated by HT1080 membrane preparation in the presence or absence of plasminogen, hydrolysed the ^{3}H-labelled substrate to the same extent, as did the APMA activated pro-MMP-2. This indicates that plasmin processed the intermediate form to a proteolytically active final species. However, we cannot exclude a stepwise activation mechanism, i.e. after the initial cleavage by MT1-MMP, plasmin removes part of the propeptide domain by specific cleavage which is completed by autoproteolysis, thereby generating the mature MMP-2 form.

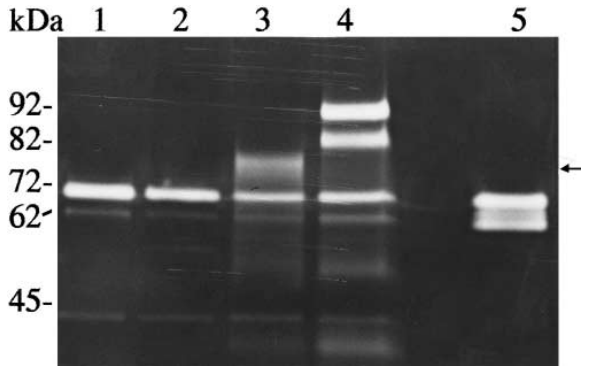

Fig. 7. Zymographic analysis of pro-MMP-2 incubated with membranes of MCF7 cells. Lane 1: recombinant pro-MMP-2; lane 2: membrane preparation incubated with recombinant pro-MMP-2; lane 3: membrane preparation incubated with recombinant pro-MMP-2 and plasminogen; lane 4: membrane preparation incubated with plasminogen and HT1080 conditioned medium containing MMP-9 and pro-MMP-2; lane 5: pro-MMP-2 activated with APMA, used as a marker for the molecular mass of the intermediate and mature MMP-2 forms. The arrows on the right indicate the proteolytic activity associated with plasmin.

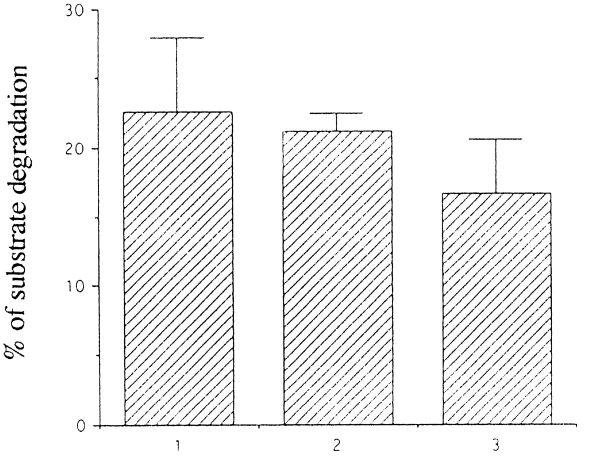

Fig. 8. ^{3}H-labelled gelatin substrate hydrolysis by membrane activated pro-MMP-2. (1) pro-MMP-2 activated by HT1080 membranes; (2) pro-MMP-2 activated by HT1080 membranes in the presence of plasminogen (2 μg/ml); (3) pro-MMP-2 activated with APMA.

Altogether our data provide the first evidence for a cooperation between a membrane MMP and the PA/plasmin system in the activation of pro-MMP-2. Although procollagenase-3 (MMP-13) was activated in vitro by MT1-MMP, the addition of plasminogen to ConA stimulated fibroblast monolayers had no effect on the processing rate [35]. Thus, in this cell system a physiological role for activation of MMP-13 by plasmin was excluded. Therefore, the implication of uPA/plasmin system in the activation of MMP-2 represents a new activation cascade and may be of great importance in vivo.

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