Expression of human recombinant 72 kDa gelatinase and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme

Rafael FRIDMAN,*§ Robert E. BIRD,* Matti HOYHTYA,* Marc OELKUOT,* Dan KOMAREK,* Chi-Ming LIANG,* Michael L. BERMAN,* Lance A. LIOTTA,† William G. STETLER-STEVENSON‡ and Thomas R. FUERST†

* Molecular Oncology Inc., 19 Firstfield Road, † MedImmune Inc., 35 West Watkins Mill Road, Gaithersburg, MD 20878,

and ‡ Laboratory of Pathology, NCI, NIH, Bethesda, MD 20892, U.S.A.

The human 72 kDa gelatinase/type IV collagenase is a metalloproteinase that is thought to play a role in metastasis and angiogenesis. The 72 kDa progelatinase can be isolated from conditioned media as a complex with the tissue inhibitor of metalloproteinase-2 (TIMP-2). To investigate 72 kDa gelatinase–TIMP-2 interactions and to compare the activity of the complex versus that of the free enzyme, we have expressed and purified human 72 kDa progelatinase and TIMP-2 as single proteins in a recombinant vaccinia virus mammalian cell expression system. The recombinant 72 kDa progelatinase was able to bind TIMP-2, and it digested gelatin and collagen type IV after activation by *p*-aminophenylmercuric acid (APMA). The specific activity of the recombinant free enzyme was 20-fold

higher than the activity of an APMA-treated stoichiometric complex of recombinant 72 kDa progelatinase and TIMP-2. Also, TIMP-2 caused an 86 % inhibition of activity when added to the activated enzyme at a 1:1 molar ratio. Activation of the free recombinant 72 kDa progelatinase yielded the 62 kDa species and two fragments of 46 and 35 kDa that cross-reacted with monoclonal antibodies to the 72 kDa proenzyme. TIMP-2 inhibited the conversion of the recombinant proenzyme to the 62 kDa species and the appearance of the 45 and 35 kDa bands. These results suggest that TIMP-2 is not only a potent inhibitor of the activated enzyme but also prevents the generation of low-molecular-mass species and full enzymic activity from the zymogen.

INTRODUCTION

The 72 kDa gelatinase/type IV collagenase is a neutral metalloproteinase capable of degrading a variety of extracellular matrix (ECM) components, including collagen types IV, V, VII and X, gelatin, fibronectin [1,2] and elastin [3]. Because of its ability to cleave the triple-helical domain of collagen type IV, it has been suggested that the 72 kDa gelatinase plays a role in the turnover of basement membranes during development, wound healing, metastasis and angiogenesis [4,5]. Previous studies have shown that the latent form of the 72 kDa gelatinase isolated from conditioned media is secreted in a non-covalent stoichiometric complex with the tissue inhibitor of metalloproteinase-2 (TIMP-2; molecular mass 21 799 Da) [6,7]. Although biologically active TIMP-2 can be purified from the complex by reverse-phase h.p.l.c., this procedure inactivates the enzyme [6,7]. TIMP-2 has been shown to inhibit the proteolytic activity of the activated complex [6,7], angiogenesis [8] and tumour cell invasion *in vitro* [9,10]. The properties of the 72 kDa gelatinase free of TIMP-2 have recently been studied using enzyme purified from acid-treated complex [11] and native enzyme isolated from human skin [12] and from cultured human rheumatoid synovial cells [13,14]. These studies have shown [11,13,14] a higher specific activity of the free enzyme than that previously seen with the native complex, although the interactions with TIMP-2 were not investigated.

Previous studies have shown that treatment of the native progelatinase–TIMP-2 complex with organomercurials yields enzyme with the first 80 amino acids removed and having enzymic activity [6,7,11]. The observation that stoichiometric amounts of TIMP-2 inhibited the activated complex led to the

hypothesis that an inhibitor/enzyme ratio of 2:1 was required to achieve complete inhibition of activity [5,6]. However, Goldberg and co-workers suggested the possibility that the activity of the activated complex was due to the presence of free enzyme [6]. Since the 72 kDa gelatinase/TIMP-2 ratio in cell supernatants may be affected by culture conditions and/or by the presence of growth factors, as reported for transforming growth factor- β (TGF- β) [11,15], the stoichiometry of the complex may vary. Thus the precise relationship between TIMP-2 and the 72 kDa enzyme could not be readily determined. In an attempt to define the role of TIMP-2 in the complex and its effects on free 72 kDa gelatinase, both latent and activated, we have expressed human 72 kDa progelatinase and TIMP-2 in a recombinant vaccinia virus mammalian cell expression system [12] and isolated biologically active proteins. Here we show that free 72 kDa gelatinase is more active than either an activated stoichiometric complex of recombinant 72 kDa gelatinase and TIMP-2 or a native gelatinase–TIMP-2 complex. We also show that TIMP-2 is a potent inhibitor of autoproteolytic activation and of enzymic activity.

EXPERIMENTAL

Construction of plasmid vectors and recombinant vaccinia viruses

The construction of the recombinant plasmid pTF7EMC-1 containing the bacteriophage T7 promoter–terminator cassette positioned between vaccinia virus thymidine kinase sequences for homologous recombination has been described previously [16,17]. To clone either the human 72 kDa gelatinase or the human TIMP-2 [18] cDNAs containing the respective original signal sequences into pTF7EMC-1, PCR was used to prepare

Abbreviations used: APMA, *p*-aminophenylmercuric acetate; ECM, extracellular matrix; mAb, monoclonal antibody; (r)TIMP-2, (recombinant) tissue inhibitor of metalloproteinase-2; TGF- β , transforming growth factor- β .

§ To whom all correspondence and reprints requests should be addressed, at: Department of Pathology, Wayne State University, School of Medicine, 540 E. Canfield, Detroit, MI 48201, U.S.A.

fragments containing appropriate restriction enzyme sites at the 5' and 3' termini. The 72 kDa gelatinase (5', *Afl*III; 3', *Hpa*I) and the TIMP-2 (5', *Nco*I; 3', *Bgl*II) cDNA fragments were cloned into the *Nco*I and *Bam*HI sites of pTF7EMC-1 and the resulting plasmids, pT7GEL and pT7TIMP-2, were isolated. For cloning purposes, the 5' end PCR primer for the 72 kDa gelatinase added a serine codon immediately after the initiator ATG. The PCR inserts were sequenced by using the dideoxy method [19] with recombinant T7 DNA polymerase and the Sequenase Version 2.0 sequencing kit (United States Biochemicals, Cleveland, OH, U.S.A.), as described by the manufacturer. Only one amino acid mutation (valine to alanine) was found at position 28 in the signal sequence of the collagenase. Both the serine after the first methionine and the mutation had no apparent effect on synthesis and secretion of the recombinant enzyme. The nucleotide sequence of the TIMP-2 PCR fragment did not indicate any amino acid substitutions. Recombinant vaccinia viruses containing either 72 kDa gelatinase (vT7GEL) or TIMP-2 (vT7TIMP-2) cDNAs were obtained by homologous recombination as previously described [20]. Recombinant vaccinia virus (vTF7-3) containing the bacteriophage T7 RNA polymerase gene has been also described [20].

Metabolic labelling

Transient expression and metabolic labelling of the 72 kDa gelatinase and TIMP-2 with [³⁵S]methionine (Amersham) were achieved by transfecting the respective expression plasmids into normal monkey kidney BSC-1 cells (ATCC-CCL 26) that had been previously infected with vTF7-3 virus as described [20]. At 5–6 h after infection, the medium was removed and replaced with Dulbecco's modified Eagle's medium lacking methionine (Gibco BRL) and supplemented with 1% dialysed fetal bovine serum and 100 µCi/ml [³⁵S]methionine. The cells were then incubated for 18 h at 37 °C. After labelling, the medium was collected, clarified by centrifugation in an Eppendorf Microfuge (13000 g, 15 min, 4 °C) and frozen at –70 °C until used.

Immunoprecipitations and Western blots

Samples (25–50 µl) of [³⁵S]methionine-labelled supernatants were diluted to a final volume of 0.25 ml with immunoprecipitation buffer (Tris-buffered saline, pH 7.5, containing 1% Brij-35, 1 mM phenylmethanesulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 0.01% sodium azide) and incubated with either a rabbit polyclonal antibody raised against native 72 kDa–TIMP-2 complex or monoclonal antibodies (mAbs) to either the 72 kDa gelatinase or TIMP-2 obtained in our laboratory. Antigen–antibody complexes were precipitated with Protein G coupled to Sepharose beads (Pharmacia LKB). The immunoprecipitates were diluted in sample buffer (2×) containing β-mercaptoethanol, boiled (3 min) and applied to an 8–16% acrylamide SDS/PAGE mini-gel (Novex, Encinitas, CA, U.S.A.). Labelled proteins were visualized by fluorography.

Immunodetection of antigens blotted on to nitrocellulose membranes was achieved using mAbs to either the 72 kDa gelatinase or TIMP-2. Antigen–antibody complexes were detected using the immunoperoxidase ABC Kit (Vector Laboratories) as described by the manufacturer.

Purification of recombinant proteins

To isolate recombinant 72 kDa progelatinase and TIMP-2 proteins, suspension cultures of HeLa cells grown in MEM

spinner medium (Quality Biological, Inc., Gaithersburg, MD, U.S.A.) supplemented with 5% horse serum were co-infected with a mixture of either vTF7-3 and vT7GEL or vTF7-3 and vT7TIMP-2 recombinant vaccinia viruses as previously described [23]. After infection, the cells were cultured for 2 days in Opti-MEM I (Gibco BRL) medium. The media were then collected by centrifugation and the supernatant was processed for protein purification. The recombinant 72 kDa progelatinase was purified by affinity chromatography on a gelatin–Sepharose (Sigma) column equilibrated with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl₂ and 0.02% Brij-35 [1]. The proenzyme was eluted with 7% dimethyl sulphoxide diluted in the same buffer and the fractions containing enzyme protein, identified by absorbance at 280 nm, were pooled and immediately dialysed against equilibration buffer at 4 °C. Samples of purified progelatinase were stored at –70 °C. Recombinant TIMP-2 was purified by affinity chromatography using an anti-TIMP-2 monoclonal antibody coupled to CMBr-activated Sepharose beads (Pharmacia LKB). Bound TIMP-2 was eluted with 0.5 M ammonium acetate at pH 3.0. The fractions containing protein were pooled and dialysed against 20 mM sodium acetate, pH 6.0, containing 0.5 mM NaCl. The dialysed protein solution was applied to a CM-Sepharose fast-flow column (Pharmacia LKB) and the TIMP-2 protein was eluted with a linear gradient from 1 to 400 mM NaCl in 40 mM sodium acetate, pH 6.0. The fractions containing TIMP-2 were pooled and stored at –70 °C. Native TIMP-2 and 72 kDa gelatinase–TIMP-2 complex were isolated from conditioned media of A2058 human melanoma cells as described [1,2,7].

Zymograms and enzyme assays

Zymograms were performed using SDS/10% PAGE pre-cast mini-gels containing 1% gelatin (Novex) as previously described [15]. For gelatinase assays, purified enzyme samples in 50 mM Tris/HCl, pH 7.5, containing 5 mM CaCl₂, 200 mM NaCl and 0.02% Brij-35 were activated at 37 °C with a final concentration of 1 mM *p*-aminophenylmercuric acetate (APMA) from a 10× stock solution in 50 mM NaOH. Control enzyme received an equal amount of NaOH without APMA. In some experiments the recombinant 72 kDa progelatinase was incubated with rTIMP-2 for 10 min at 22 °C before or after activation by APMA. Gelatinase activity was determined using heat-denatured rat [³H]collagen type I (18000 c.p.m./µg) (du Pont) as a substrate. The reaction mixture (55 µl) was incubated at 37 °C for 20 min, and the reaction was terminated by the addition of an EDTA/BSA solution to a final concentration of 3.5 mM EDTA/0.03% BSA. Undigested [³H]gelatin was then precipitated with 0.06% tannic acid/1% trichloroacetic acid (final concentrations) and the radioactivity of the supernatant was measured by scintillation spectroscopy.

Determination of protein concentration

The protein concentrations of homogeneous solutions of the two proteins were estimated spectrophotometrically from A_{280} data using absorption coefficients estimated from the tyrosine and tryptophan contents of the proteins as described previously [21]. For the 72 kDa progelatinase, $\epsilon = 137040 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was calculated by multiplying the number of tyrosine residues (30) by $1413 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and adding this product to the product of the number of tryptophan residues (15) multiplied by $6310 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The same calculation was made for TIMP-2, which contains seven tyrosine and four tryptophan residues; the resulting ϵ value was $35131 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Based on molecular

masses of 70952 Da for the 72 kDa gelatinase and 21799 Da for TIMP-2, the calculated absorption coefficients are $A^{0.1\%} = 1.93 \text{ ml/mg}$ and $A^{0.1\%} = 1.61 \text{ ml/mg}$ for the two proteins respectively.

RESULTS

Purification and biochemical properties of recombinant 72 kDa gelatinase

Suspension cultures of HeLa cells co-infected with each of the recombinant vaccinia viruses and with vTF7-3 were used to produce recombinant proteins for purification. Figure 1 shows a silver-stained SDS/PAGE gradient gel loaded with 10 μg of

Figure 1 Silver-stained SDS/PAGE gel of affinity-purified recombinant 72 kDa progelatinase and TIMP-2

Lane 1, ^{14}C -labelled molecular mass markers; lane 2, recombinant 72 kDa gelatinase; lane 3, rTIMP-2. Each lane contains 10 μg of purified protein under reducing conditions.

Figure 2 Zymogram of recombinant 72 kDa gelatinase

Crude media from infected HeLa cells (lane 1, 1 μl) containing recombinant 72 kDa progelatinase and affinity-purified recombinant 72 kDa progelatinase before (lane 4, 5 ng) and after (lane 5, 5 ng) activation by APMA (15 min, 37 $^{\circ}\text{C}$) were electrophoresed on gelatin-containing gels (zymograms) as described in the Experimental section. Lane 2, flow through after affinity purification; lane 3, molecular mass standards.

Figure 3 Gelatinase activity of recombinant 72 kDa gelatinase as a function of time

Purified recombinant 72 kDa progelatinase (5 ng/reaction) was activated with 1 mM APMA at 37 $^{\circ}\text{C}$ for various periods of time and incubated with [^3H]gelatin (18000 c.p.m./ μg) for 20 min at 37 $^{\circ}\text{C}$. The solubilized products were measured in a liquid scintillation spectrometer as described in the Experimental section. Inset: the samples (20 ng/lane) were electrophoresed in gelatin-containing gels. Upper arrow, latent 72 kDa form; middle arrow, activated 62 kDa form; lower arrow, 45 kDa species. Similar results were obtained in four independent experiments.

either recombinant 72 kDa progelatinase (lane 2) or rTIMP-2 (lane 3).

The purified recombinant 72 kDa progelatinase was tested for its ability to digest gelatin by zymography before and after exposure to APMA (30 min, 37 $^{\circ}\text{C}$). As shown in Figure 2 (lanes 1 and 4), a gelatinolytic band of 72 kDa was observed with the latent form of the recombinant proenzyme. After activation with APMA, the purified proenzyme yielded a major active species of 62 kDa and a minor species of 45 kDa (Figure 2, lane 5) with gelatinase activity. Assays using soluble [^3H]gelatin showed that the activated recombinant enzyme had a specific activity of 330 μg of digested gelatin/h per μg of enzyme.

Activation studies (Figure 3) showed that maximal activity against [^3H]gelatin was achieved after 15 min of exposure to APMA, after which time the enzymic activity decreased. Zymograms showed (Figure 3, inset) that the 45 kDa species could be observed after 5 min of exposure to APMA.

Inhibition by rTIMP-2

To test the effect of TIMP-2 on the activity of the recombinant 72 kDa gelatinase, equal amounts of activated enzyme were mixed with increasing concentrations of either recombinant or native TIMP-2, and the activity of the mixture was tested in the gelatinase assay. As shown in Figure 4, an 86% inhibition of enzyme activity was observed at a 1:1 enzyme/inhibitor molar ratio (arrow). The activity of the rTIMP-2 was indistinguishable from that observed with native TIMP-2.

The effect of APMA on the activation of the recombinant 72 kDa proenzyme in the presence or absence of stoichiometric amounts of rTIMP-2 was analysed in gelatinase assays, zymograms and Western blots. Table 1 shows that exposure

Figure 4 Effect of TIMP-2 on the activity of recombinant 72 kDa gelatinase

Purified recombinant 72 kDa progelatinase (20 ng/reaction) was activated with APMA (30 min, 37 °C) and incubated (5 min, 22 °C) with increasing amounts of either recombinant TIMP-2 (○) or native TIMP-2 (●). At the end of the incubation period, the gelatinase activity was measured as described in the Experimental section. The arrow indicates an enzyme/inhibitor molar ratio of 1:1.

Table 1 [³H]Gelatin degradation by activated and latent recombinant 72 kDa gelatinase

Purified free recombinant 72 kDa progelatinase or a stoichiometric recombinant 72 kDa progelatinase-rTIMP-2 complex was treated or not with APMA (30 min, 37 °C) and then incubated (20 ng of enzyme/reaction) with approx. 1 µg of [³H]gelatin (18 000 c.p.m./µg) for 20 min at 37 °C. Solubilized products were counted for radioactivity in a liquid scintillation spectrometer.

Enzyme	³ H-labelled products (c.p.m.)
71 kDa gelatinase	
— APMA	1929
+ APMA	16 524
72 kDa gelatinase-rTIMP-2 complex	
— APMA	1325
+ APMA	2104
Blank	1730

(30 min, 37 °C) of the free recombinant 72 kDa progelatinase to APMA generates an enzyme with high enzymic activity. In contrast, little activity was observed in a recombinant 72 kDa progelatinase-rTIMP-2 stoichiometric complex treated with APMA. The calculated specific activity of the recombinant complex was 16.6 µg of digested gelatin/h per µg of enzyme. We also compared the gelatinase activity of a native 72 kDa progelatinase-TIMP-2 complex isolated from the conditioned medium of A2058 cells, and found that 60 ng of native complex activated with APMA (1 h, 37 °C) solubilized 1013 c.p.m. (corrected for blanks) of [³H]gelatin, whereas 20 ng of the recombinant free enzyme solubilized 14 501 c.p.m. under the same conditions. Thus treatment of a stoichiometric 72 kDa progelatinase-TIMP-2 complex (native or recombinant) with APMA generates an enzyme with very low proteinase activity when compared with recombinant free enzyme.

Figure 5 Zymogram and Immunoblot of latent and activated free recombinant 72 kDa gelatinase and recombinant 72 kDa gelatinase-TIMP-2 complex

Samples obtained after 30 min of incubation with or without APMA, as described in Figure 4, were analysed by either zymography (a) or immunoblots (b) as described in the Experimental section. Lanes 1, free recombinant 72 kDa progelatinase; lanes 2, APMA-treated free recombinant 72 kDa gelatinase; lanes 3, latent complex; lanes 4, APMA-treated complex. Proteins in the immunoblots were electrophoresed under reducing conditions.

The zymogram in Figure 5(a) shows that treatment of the free recombinant 72 kDa progelatinase with APMA (30 min, 37 °C) yielded the 62 kDa form and an additional active species of 45 kDa (Figure 5a, lane 2). In the presence of a stoichiometric amount of TIMP-2, the conversion of the proenzyme to the 62 kDa and 45 kDa forms was partially inhibited (lane 4). Little conversion to the low-molecular-mass forms was observed in either the free recombinant 72 kDa progelatinase (lane 1) or the recombinant complex (lane 3) without APMA addition. Western blots of the same samples (Figure 5b) developed with a mixture of two different mAbs to the 72 kDa enzyme and with an anti-TIMP-2 mAb, showed a complete conversion of the free recombinant 72 kDa proenzyme to the 62 kDa, 45 kDa and 35 kDa forms after exposure to APMA (lane 2). In contrast, the APMA-treated recombinant complex showed only the 72 kDa species and some 62 kDa form (lane 4). Thus TIMP-2 prevents the generation of small active species from the 72 kDa progelatinase.

DISCUSSION

Active recombinant human 72 kDa progelatinase and TIMP-2 have been expressed in mammalian cells using a recombinant vaccinia virus expression system known for its ability to produce eukaryotic proteins in the proper configuration [20,22,23]. Both recombinant 72 kDa progelatinase and TIMP-2 were secreted into the medium, purified and found to have similar activities to the native proteins. Since vaccinia virus causes a shut-off of host cell protein synthesis, formation of complex between the recombinant proteins and endogenous gelatinase and TIMP-2 was thus minimized. This facilitated the isolation of recombinant 72 kDa progelatinase free of TIMP-2, and TIMP-2 free of 72 kDa gelatinase.

The recombinant 72 kDa gelatinase digested gelatin and native collagen type IV after exposure to organomercurial compounds, and was equally inhibited by stoichiometric amounts of either recombinant or native TIMP-2, with a consistent 1:1 molar inhibition of the 72 kDa gelatinase by TIMP-2 as reported previously [7]. The recombinant 72 kDa progelatinase formed a complex with rTIMP-2 that showed an extremely low proteinase activity after exposure to organomercurial. The specific activity of the recombinant complex was 16.6 µg of digested gelatin/h

per μg of enzyme, while that of the activated recombinant 72 kDa gelatinase was 330 μg of digested gelatin/h per μg of enzyme. This is in agreement with a previous study showing a higher activity of free 72 kDa gelatinase than of the native 72 kDa gelatinase-TIMP-2 complex [14]. Furthermore, a recombinant 92 kDa gelatinase free of TIMP produced in bacteria was shown to be more active than activated native 92 kDa gelatinase-TIMP complex [3]. In a recent study, the specific activity reported for the native complex was 1.5 μg of digested gelatin/h per μg of enzyme; however, after acid treatment of the complex to remove TIMP-2, the self-activated gelatinase showed a specific activity of 45 μg of digested gelatin/h per μg of enzyme [11]. This activity is approximately 7-fold lower than that of our vaccinia recombinant 72 kDa enzyme, probably due to the loss of activity after the acid treatment of the native enzyme [11].

We have found that stoichiometric amounts of rTIMP-2 inhibited the conversion of the recombinant 72 kDa progelatinase to the low-molecular-mass forms and caused an almost complete inhibition of enzymic activity. In contrast, organomercurial activation of the recombinant 72 kDa progelatinase free of TIMP-2 generated a potent enzyme and yielded the previously reported 62 kDa form [1,2]. A fraction of the free enzyme also generated two additional fragments of 45 and 35 kDa which could not be detected in the purified zymogen. The 45 kDa protein showed gelatinolytic activity in zymograms. The generation of low-molecular-mass forms with enzymic activity from the zymogen has been reported with other metalloproteinases [24–26] and with the 72 kDa gelatinase [11,27]. Overall and co-workers reported the presence of an active 43 kDa species derived from 72 kDa gelatinase in the medium of fibroblast cells stimulated with concanavalin A [27]. Likewise, a 42.5 kDa fragment with high specific activity and an inactive 37 kDa fragment were observed with autoactivated 72 kDa progelatinase free of TIMP-2 [11]. We have recently expressed and purified two recombinant C-terminally truncated forms of the 72 kDa progelatinase with a specific activity similar to that of the full-length enzyme, but with a reduced sensitivity to TIMP-2 inhibition [28], further suggesting the existence of smaller fragments of the 72 kDa gelatinase with enzymic activity.

The data presented here and previous studies with the native complex [7,11,13,14] demonstrate that the 72 kDa enzyme associated with TIMP-2 has very little proteinase activity. Since the ratio between the enzyme and the inhibitor in the complex isolated from conditioned media may vary depending on the culture conditions [15], and since significant differences in the specific activity of the native complex have been reported by various laboratories [7,11], it is possible that the low activity observed with the native 72 kDa gelatinase-TIMP-2 complex may be due to the presence of small amounts of free enzyme [6]. However, Kleiner et al. have shown that APMA activation of cross-linked native 72 kDa progelatinase-TIMP-2 complex results in enzymic activity [29]. The specific activity of free recombinant 72 kDa enzyme seen here and that of free native enzyme shown by others [13,14] would indicate that only a small fraction of free enzyme would be sufficient to generate the low activity of the complex. If this is the case, then the role of TIMP-2 bound to the zymogen may be to neutralize the enzyme by maintaining it in a latent inactive state. Howard et al. [11] proposed a stabilizing role for TIMP-2 against autoactivation of acid-treated free progelatinase. However, we did not observe a significant autoactivation of the free recombinant 72 kDa progelatinase, which required organomercurial activation to yield the low-molecular-mass forms and to become enzymically competent. This is in agreement with previous studies showing that

purified preparations of 72 kDa progelatinase free of TIMP-2 contain enzyme in the latent form [12–14].

The observation that both free native and recombinant 72 kDa gelatinases [11,13,14] are significantly more active than the activated complex suggests that degradation of the ECM *in vivo* may be more efficiently accomplished by generating a 72 kDa gelatinase free of TIMP-2. Although the physiological mechanisms are still unknown, this could be achieved by inactivating TIMP-2 from the complex, by an enhanced production of free enzyme and/or by a down-regulation of TIMP-2 expression, similar to that reported with TIMP [30,31]. The measurement of the relative levels of TIMP-2 and the 72 kDa gelatinase and the identification of activated species in both normal and pathological situations will help to define the role of the 72 kDa gelatinase in a more precise manner.

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REFERENCES

- Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A. and Goldberg, G. I. (1988) *J. Biol. Chem.* **263**, 6579–6587
- Stetler-Stevenson, W. G., Kruttsch, H. C., Wachter, M. P., Margulies, I. M. K. and Liotta, L. A. (1989) *J. Biol. Chem.* **264**, 1353–1356
- Senior, R. M., Griffin, G. L., Fliszar, C. J., Shapiro, S. D., Goldberg, G. I. and Welgus, H. G. (1991) *J. Biol. Chem.* **266**, 7870–7875
- Ura, H., Bonfil, R. D., Reich, R., Reddel, R., Pfeifer, A., Harris, C. C. and Klein, A. J. P. (1989) *Cancer Res.* **49**, 4615–4621
- Liotta, L. A., Steeg, P. S. and Stetler-Stevenson, W. G. (1991) *Cell* **64**, 327–336
- Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S. and He, C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8207–8211
- Stetler-Stevenson, W. G., Kruttsch, H. C. and Liotta, L. A. (1989) *J. Biol. Chem.* **265**, 17374–17378
- Takigawa, M., Nishida, Y., Suzuki, F., Kishi, J., Yamashita, K. and Hayakawa, T. (1991) *Biochem. Biophys. Res. Commun.* **171**, 1264–1271
- DeClerck, Y. A., Yean, T. D., Chan, D., Shimada, H. and Langley, K. E. (1991) *Cancer Res.* **51**, 2151–2157
- Albini, A., Melchiori, A., Santi, L., Liotta, L. A., Brown, P. D. and Stetler-Stevenson, W. G. (1991) *J. Natl. Cancer Inst.* **83**, 775–779
- Howard, E. W., Bullen, E. C. and Banda, M. J. (1991) *J. Biol. Chem.* **266**, 13064–13069
- Seltzer, J. L., Akers, K. T., Weingarten, H., Grant, G. A., McCourt, D. W. and Eisen, A. Z. (1990) *J. Biol. Chem.* **265**, 20409–20413
- Okada, Y., Morodomi, T., Enghild, J. J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G. and Nagase, H. (1990) *Eur. J. Biochem.* **194**, 721–730
- Kolkenbrock, H., Orgel, D., Hecker-Kia, A., Noack, W. and Ulbrich, N. (1991) *Eur. J. Biochem.* **198**, 775–781
- Brown, P. T., Levy, A. T., Margulies, I. M. K., Liotta, L. A. and Stetler-Stevenson, W. G. (1990) *Cancer Res.* **50**, 6184–6191
- Elroy-Stein, O., Fuerst, T. M. and Moss, B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6126–6130
- Mackett, M., Smith, G. L. and Moss, B. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7415–7419
- Stetler-Stevenson, W. G., Brown, P. D., Onisto, M., Levy, A. T. and Liotta, L. A. (1990) *J. Biol. Chem.* **265**, 13933–13938
- Sanger, F., Niklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Fuerst, T. M., Niles, E. G., Studier, W. F. and Moss, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8122–8126
- Pantoliano, M. W., Bird, R. E., Johnson, S., Asel, E. D., Dodd, S. W., Wood, J. F. and Hardman, K. D. (1991) *Biochemistry* **30**, 10117–10125
- Mizukami, T., Fuerst, T. M., Berger, E. A. and Moss, B. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9273–9277
- Fuerst, T. M., Earl, P. L. and Moss, B. (1987) *Mol. Cell. Biol.* **7**, 2538–2544
- Matrisian, L. M. (1990) *Trends Genet.* **6**, 121–125
- Grant, G. A., Eisen, A. Z., Marmer, B. L., Roswit, W. T. and Goldberg, G. I. (1987) *J. Biol. Chem.* **262**, 5885–5889
- Nagase, H., Enghild, J. J., Suzuki, K. and Salvesen, G. (1990) *Biochemistry* **29**, 5783–5789

- 27 Overall, C. M. and Sodek, J. (1990) *J. Biol. Chem.* **265**, 21141–21151
- 28 Fridman, R., Fuerst, R. F., Bird, R. E., Hoythya, M., Oelkelt, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L. and Stetler-Stevenson, W. G. (1992) *J. Biol. Chem.* **267**, 15398–15405
- 29 Kleiner, D. E., Krutzsch, H. C., Unsworth, E. J. and Stetler-Stevenson, W. G. (1992) *Biochemistry* **31**, 1665–1672
- 30 Khokha, R., Waterhouse, P., Yagel, S., Lala, P. K., Overall, C. M., Norton, G. and Denhardt, D. T. (1989) *Science* **243**, 947–950
- 31 Ponton, A., Coulombe, B. and Skup, D. (1991) *Cancer Res.* **51**, 2138–2143

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