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Tissue Inhibitor of Metalloproteinases-2 Inhibits the 4-Aminophenylmercuric Acetate-Induced Activation and Autodegradation of the Free Promatrix Metalloproteinase-2

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Matrix metalloproteinase-2 (MMP-2; 72-kDa gelatinase; 72-kDa type IV collagenase; gelatinase A) plays an important role in normal physiological processes and in many pathologic processes such as arthritis and metastasis of cancer. Tissue inhibitor of metalloproteinases-2 (TIMP-2) binds to proMMP-2 or mature MMP-2 at a 1:1 ratio and inhibits the catalytic activity of MMP-2. We demonstrated that the baculovirus/insect cell system does not have TIMP-2 activity. The human proMMP-2 free of TIMP-2 was expressed in the expression system and purified by one-step affinity chromatography using gelatin-Sepharose. The free proMMP-2 was autoactivated to the mature MMP-2 and autodegraded into smaller molecular weight forms in the absence of external activator. The activation and autodegradation of the proMMP-2 was much more rapid in the presence of 4-aminophenylmercuric acetate (APMA). Addition of TIMP-2 inhibits both APMA-induced activation and autodegradation of the free proMMP-2. However, an increasing concentration of TIMP-2 more readily inhibited activation of the free proMMP-2 than autodegradation. These results demonstrate that TIMP-2 plays roles in inhibition of both activation and autodegradation of the free proMMP-2 in addition to inhibition of the catalytic activity of MMP-2.

Keywords: 4-Aminophenylmercuric acetate (APMA), Baculovirus, Promatrix metalloproteinase (ProMMP)-2, Tissue inhibitor of metalloproteinases (TIMP)-2.

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Introduction

Matrix metalloproteinases (MMPs) are an important group of zinc enzymes responsible for degradation of the extracellular matrix components. Matrix metalloproteinase-2 (MMP-2; 72-kDa gelatinase; 72-kDa type IV collagenase; gelatinase A) can degrade various components of the extracellular matrix including type IV collagen, which is a major component of basement membranes, as well as collagen types V, VII, and X, fibronectin, elastin, and gelatin (Woessner, 1991). MMP-2 is involved in normal processes such as embryonic development and wound healing, and in pathological processes such as metastasis of cancer and arthritis (Stetler-Stevenson, 1990; Liotta *et al.*, 1991; Woessner, 1991; Cawston, 1995).

MMP-2, as with all known MMPs, is secreted as a zymogen and is activated by removal of the amino-terminal propeptide. ProMMP-2, which is a zymogen of MMP-2, was isolated from the culture media of various cancer cell lines, but was complexed with TIMP-2 (Goldberg *et al.*, 1989; Lee *et al.*, 1995). TIMP-2 is a 22-kDa protein that binds to mature MMP-2 as well as to proMMP-2 at 1:1 stoichiometric ratio (De Clerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989a). Because binding of TIMP-2 to MMP-2 inhibits the catalytic activity of MMP-2, it is known that the MMP-2 activity is controlled in part by the level of TIMP-2 in the extracellular matrix or culture medium (Stetler-Stevenson *et al.*, 1989b).

Because proMMP-2 is purified as a complexed form with TIMP-2 or as a mixture of the free proMMP-2 and the complexed form, properties of proMMP-2 free of TIMP-2 and roles of TIMP-2 on free proMMP-2 have been poorly investigated. It was shown that free proMMP-2 purified from the culture media of the human rheumatoid synovial cells is rapidly activated and autodegraded by 4-aminophenylmercuric acetate (APMA) (Okada *et al.*, 1990) and

has a higher specific activity than the complex formed with TIMP-2 (Kolkenbrock *et al.*, 1991). In addition, it was suggested that TIMP-2 may prevent generation of the low molecular weight (MW) species and full enzymatic activity from the free proMMP-2, which was expressed using a vaccinia virus/HeLa cell system and purified by single affinity chromatography (Friedman *et al.*, 1993). However, it is not clear whether the free proMMP-2 purified from the culture media of the mammalian cells was completely free of TIMP-2. To obtain proMMP-2 completely free of TIMP-2, the proMMP-2/TIMP-2 complex was separated by reverse-phase high performance liquid chromatography in the presence of trifluoroacetic acid, but proMMP-2 was autoactivated during neutralization and degraded into smaller MW forms (Howard *et al.*, 1991; Kleiner *et al.*, 1993). In order to study properties of the free proMMP-2 and characterize additional roles of TIMP-2 on proMMP-2 other than on inhibition of catalytic activity of the mature MMP-2, we have used a baculovirus/*Sf*9 insect cell system as a convenient source of proMMP-2 free of TIMP-2. Here, we demonstrate that the baculovirus/insect cell system does not have the TIMP-2 activity and is thus a valuable system to express proMMP-2 in a condition free of TIMP-2. In addition, we show that the TIMP-2-free proMMP-2 expressed in the baculovirus/insect cell system is readily autoactivated and autodegraded, but addition of TIMP-2 to the proMMP-2 efficiently inhibits activation and autodegradation of proMMP-2.

Materials and Methods

Cell culture *Spodoptera frugiperda Sf*9 cells were cultured in TNM-FH insect medium (Sigma, St. Louis, USA) containing 100 unit/0.1 mg penicillin/streptomycin (Sigma, St. Louis, USA) and 0.25 μg/ml fungizone (GIBCO BRL, Gaithersburg, USA) in the presence of 10% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, USA) at 27°C (Jeang *et al.*, 1987). Human fibrosarcoma HT-1080 cells were cultured in DMEM medium (GIBCO BRL, Gaithersburg, USA) containing 100 unit/0.1 mg penicillin/streptomycin and 2 mM glutamine in the presence of 10% fetal bovine serum at 37°C (Rasheed *et al.*, 1974). When culture media were recovered for protein purification, serum-free media were used.

Baculoviral transfer vector containing the human proMMP-2 cDNA The full-length proMMP-2 cDNA cloned in pBluescript (pBS-Gel) (Stratagene, La Jolla, USA) was a generous gift from Dr. G. I. Goldberg (Collier *et al.*, 1988). The 3.1-kb *Eco*RI-*Xba*I fragment of proMMP-2 cDNA was subcloned into the unique *Eco*RI and *Xba*I sites of a baculoviral transfer vector, pBacPAK9 (Clontech, Palo Alto, USA).

Recombinant baculovirus containing the human proMMP-2 cDNA Procedures for transfection, plaque isolation, and baculovirus multiplication were followed according to the BacPAK Baculovirus Expression System manual (Clontech, Palo Alto, USA). The resultant recombinant vector (pBacPAK9-GEL) was cotransfected with a linearized parental viral DNA (BacPAK6) into *Sf*9 cells and the extracellular viruses in the medium were recovered at 4 d after the transfection. To isolate recombinant virus plaques, the viruses were infected into *Sf*9 cells, overlaid with the complete medium containing 120 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and 0.5% (w/v) agarose. The *Sf*9 cells were stained with 50 μg/ml neutral red at 5 d after infection in order to visualize recombinant white plaques and parental blue plaques. The recombinant viruses eluted from the individual white plaques were infected *Sf*9 cells and the extracellular viruses were harvested at 4 d after the infection (Koh *et al.*, 1998).

Screening of the recombinant viruses containing the MMP-2 cDNA was performed by polymerase chain reaction (PCR) using the baculoviral DNAs as templates and MMP-2 cDNA-specific oligonucleotides as a primer pair; 5′-CCATCGAGACCATGCG GAAG-3′ and 5′-CCCAAGGTCCATAGCTCATC-3′. PCR conditions were 93°C for 120 s during predenaturation, 35 cycles of 93°C for 40 s in denaturation, 51°C for 90 s in annealing, and 72°C for 120 s in extension, followed by 72°C for 300 s (Sambrook *et al.*, 1989). The pBacPAK9-GEL plasmid DNA was used as a positive control.

Expression of the human proMMP-2 in *Sf*9 cells For expression of the recombinant human proMMP-2, *Sf*9 cells were plated at a density of 2×10^6 cells/ml in the complete medium and were washed twice with the serum-free medium. The *Sf*9 cells were infected with the recombinant baculovirus stock at a multiplicity of infection (m.o.i.) of 1.6. After 1 h, the serum-free medium was added to the cells. The culture medium was recovered at 4 d after infection or after the described incubation period. Mock-infection was done with the serum-free medium without virus inoculum.

Gelatin zymography Gelatinolytic activities of MMP-2 were analyzed by gelatin zymography according to the method of Miyazaki *et al.* (1990). Under nonreducing conditions, samples in SDS sample buffer [2% (w/v) SDS, 60 mM Tris·HCl (pH 6.8), 0.05% bromophenol blue, and 5% (v/v) glycerol] were loaded into 10% SDS-polyacrylamide gels containing 2 mg/ml gelatin without denaturation. After electrophoresis, samples separated on the gels were renatured by gentle shaking in 2.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), and 0.1 M NaCl at room temperature for 1 h with two changes. The gel was incubated in 50 mM Tris·HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 37°C for 18 h. Then, it was stained with 0.25% Coomassie brilliant blue, 45% (v/v) ethanol, and 10% (v/v) acetic acid for 30 min followed by destaining with 50% methanol and 10% acetic acid and then 0.5% methanol, 0.4% glycerol, and 0.7% acetic acid.

Purification of the recombinant proMMP-2 All purification steps were done at 4°C. To purify the recombinant proMMP-2 expressed in *Sf*9 cells, the medium sample recovered at 4 d after the baculovirus infection with the recombinant was loaded on a gelatin-Sepharose (Sigma, St. Louis, USA) column. The column was equilibrated with a column buffer [50 mM Tris·HCl (pH 7.5), 500 mM NaCl, 1 mM $CaCl_2$, and 0.05% Brij-35] prior to loading and washed with the column buffer after loading (Collier *et al.*, 1988; Lee *et al.*, 1995). The proMMP-2 was eluted with 7%

dimethyl sulfoxide in the column buffer. The fractions containing proMMP-2 were detected by gelatin zymography and the pooled active fractions were dialyzed against the column buffer. The purified proMMP-2 was stored at −70°C in small aliquots.

To compare the recombinant proMMP-2 with the proMMP-2 from a human source, proMMP-2 was partially purified from HT-1080 cells (Moon *et al.*, 1996). The medium sample was recovered from the HT-1080 culture incubated with the serum-free medium for 24 h and the protein fraction was precipitated by addition of ammonium sulfate to 80% (Lee *et al.*, 1995). The column chromatography was followed by the methods described above.

Labeled gelatin degradation assay The MMP-2 activity was measured by [^{3}H]-labeled gelatin degradation assay using heat-denatured rat [^{3}H] type I collagen (Cat. # NET660, Dupont NEN, Boston, USA) as a substrate (Liotta *et al.*, 1980). ProMMP-2 (5 ng) in 160 μl assay buffer [50 mM Tris·HCl (pH 7.5), 10 mM $CaCl_2$, 200 mM NaCl, 0.02% NaN_3, and 0.05% Brij-35] was incubated in the presence of the recombinant TIMP-2 (Cat. # PF021, Calbiochem, La Jolla, USA) or the tested sample at 37°C for 30 min and activated in the presence of a final concentration of 1 mM APMA at 37°C for 1 h. Sixty ng of denatured [^{3}H] type I collagen and 5 μg of denatured cold type I collagen (Sigma, St. Louis, USA) in the assay buffer were added to the reaction mixture (final volume of 200 μl) and incubated at 37°C for 1 h. The reaction was stopped by the addition of 50 mM EDTA at final concentrations. Undigested substrates were precipitated with 0.03% tannic acid, 0.6% trichloroacetic acid, and 40 μg gelatin at final concentrations. After precipitation on ice for 20 min, the reactions were spun at 13,000 rpm for 10 min and the radioactivity in the supernatant was measured by liquid scintillation counting.

Analysis of APMA-induced activation and autodegradation of proMMP-2 ProMMP-2 (1.85 μg/ml) in the assay buffer with 0.1 mg/ml BSA was treated with a final concentration of 1 mM APMA for various time intervals to examine the process of APMA-induced activation and autodegradation of proMMP-2. To examine the inhibitory effects of TIMP-2 on activation and autodegradation of proMMP-2, various concentrations of the recombinant TIMP-2 were added to proMMP-2 at the same time of APMA treatment and incubated at 37°C for 30 min for inhibition of the activation process, and for 12 h for inhibition of the autodegradation process. Processed products from proMMP-2 were detected by gelatin zymography.

Results and Discussion

Baculovirus/insect cell system expresses neither gelatinolytic MMPs nor TIMP-2 Immortalized mammalian cells usually express several MMPs which are closely related to each other in many properties, as well as express TIMPs which are natural inhibitors of MMPs binding with high affinity (De Clerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989a). Thus, purification of a specific MMP from mammalian cells requires laborious steps, such as partial denaturation to dissociate tightly bound TIMPs from MMPs and multiple column chromatography to separate MMPs with similar characteristics and TIMPs (Howard *et al.*, 1991; Lee *et al.*, 1995). In an attempt to express and purify human proMMP-2 in the absence of expression of TIMP-2 and other gelatinolytic MMPs, we have employed a baculovirus/insect cell system.

To examine whether the baculovirus/*Sf*9 insect cell system is free of gelatinolytic MMPs and TIMP-2, relative gelatinolytic activity was measured in the presence of various culture media of *Sf*9 cells (Table 1). The gelatinolytic activity was slightly decreased when the supernatant of day-4 culture medium of *Sf*9 cells was added to the reaction, and slightly increased when the supernatant of day-4 culture medium of *Sf*9 cells infected with a parental baculovirus, BacPAK6 (Clontech, Palo Alto, USA), was added. Although these differences were statistically invaluable ($p>0.05$ in t-test), it is possible that the slight increase of gelatinolytic activity in the latter was due to the nonspecific action of intracellular proteases released upon lysis of the *Sf*9 cells. In addition, we could detect no gelatinolytic bands by gelatin zymography and no TIMP bands by reverse gelatin zymography in these samples (data not shown). We have thus concluded that the baculovirus/insect cell system expresses neither gelatinolytic MMPs nor TIMP-2.

Isolation of the recombinant proMMP-2 baculovirus To express the recombinant human proMMP-2 in the baculovirus/insect cell system, the full-length proMMP-2 cDNA was subcloned into a transfer vector, pBacPAK9. The resultant plasmid (pBacPAK9-GEL) was cotransfected with the linearized parental viral BacPAK6 DNA into *Sf*9 cells via lipofection. The transfer vector has an essential gene but the linearized parental viral DNA partially deletes the essential gene. Thus, the recombinant virus generated by homologous recombination can only release their progenies into the culture medium (Kitts and Possee, 1993). The supernatant was recovered at 4 d post-infection

Table 1. Relative gelatinolytic activity in the presence of various culture media of *Sf*9 cells.

Sample	Relative MMP-2 activity[a] (%)
*Sf*9 serum-free medium	100.0
Day-4 culture medium of *Sf*9 cells	95.0 ± 8.0
Day-4 culture medium of *Sf*9 cells infected with parental virus[b]	109.1 ± 8.4
TIMP-2 (15 ng)	3.0 ± 2.1

[a]Relative gelatinolytic activity was measured by [^{3}H]-labeled gelatin degradation assay as described in the Materials and Methods section. The results are expressed as mean ± standard deviation from three independent experiments of duplicate reactions.

[b]*Sf*9 cells were infected with the parental virus at m.o.i. of 1.8 as described in the Materials and Methods section.

and subjected to plaque assay. White recombinant plaques were isolated and their DNAs were subjected to PCR screening using a primer pair specific to the human proMMP-2 cDNA. A recombinant virus containing the human proMMP-2 cDNA was named as the 72Gel baculovirus.

Expression and purification of the human proMMP-2

For detection of the recombinant proMMP-2 expression, *Sf*9 cells were infected with the 72Gel virus at 1.6 m.o.i. and the supernatants were analyzed by gelatin zymography (Fig. 1). The supernatant at 2 d post-infection started to show a 68-kDa gelatinolytic band, which co-migrated with the proMMP-2 derived from the human fibrosarcoma HT-1080 cells. The 68-kDa recombinant proMMP-2 accumulated in the supernatant until 6 d post-infection. However, smaller MW forms autodegraded from the proMMP-2 started to be detected in the supernatant at 6 d post-infection and upon infection for longer periods.

For purification of the human proMMP-2, the 72Gel viruses were infected into *Sf*9 cells at 1.6 m.o.i. and the supernatant was recovered at 4 d post-infection to avoid inclusion of the autodegraded smaller MW forms. The free proMMP-2 was easily purified by one-step affinity chromatography using gelatin-Sepharose. Such easy purification was possible since our expression system was free of TIMP-2, other gelatinolytic MMPs, and autodegraded fragments of proMMP-2. The final yield of the human proMMP-2 was 35–50 μg per 100 ml serum-free medium.

The recombinant proMMP-2 purified from the infected *Sf*9 cells was compared with the proMMP-2 partially purified from HT-1080 cells by SDS-PAGE and gelatin zymography analyses. The recombinant proMMP-2 showed the same mobility as the human proMMP-2 purified from HT-1080 cells in both analyses (Fig. 2). These results demonstrate that post-translational modifications of proMMP-2 in *Sf*9 cells would be very similar to those in human cells.

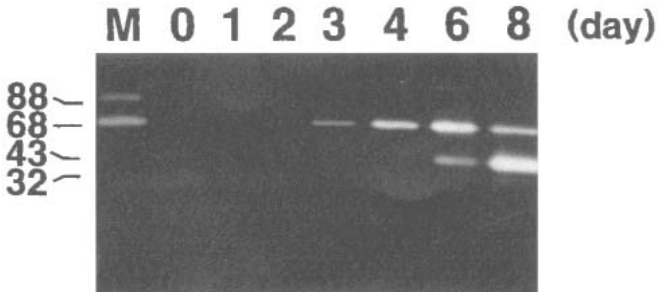

Fig. 1. Detection of the recombinant proMMP-2 by gelatin zymography. Culture media of *Sf*9 cells infected with the 72Gel baculovirus were recovered after given incubation periods and analyzed by gelatin zymography. Lanes 0, 1, 2, 3, 4, 6, and 8; incubation periods (day) after infection. Lane M, the culture medium of HT-1080 cells, known to secrete 68-kDa proMMP-2 and 88-kDa pro-MMP9, as size marker. The molecular weights (kDa) of the detected bands are indicated on the left.

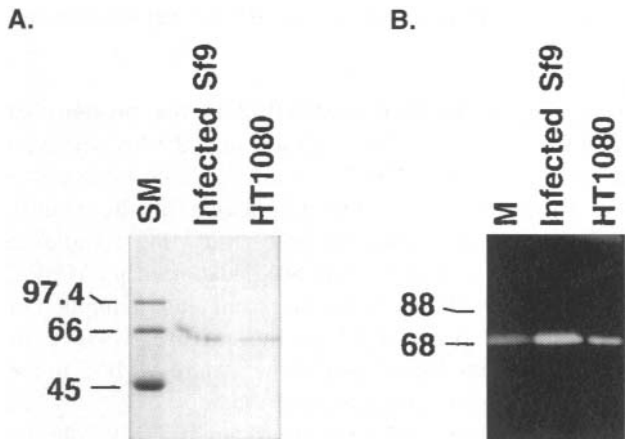

Fig. 2. Analysis of the purified proMMP-2. The recombinant proMMP-2 purified by single chromatography using gelatin-Sepharose was subjected to 10% SDS-PAGE in non-reducing conditions (A) and to gelatin zymography (B). Lane Infected *Sf*9, the recombinant proMMP-2 purified from the culture medium of *Sf*9 cells infected with the 72Gel virus; Lane HT-1080, proMMP-2 partial-purified from the culture medium of HT-1080 cells. Lane SM, molecular weight size marker; Lane M, the culture medium of HT-1080 cells.

During the expression of the recombinant proMMP-2 in the baculovirus/insect cell system, we found that the free proMMP-2 was autodegraded into smaller MW forms including the 43 kDa, 38 kDa, and 32 kDa gelatinolytic fragments. Similar autodegradation without external activators was also detected during the purification process of proMMP-2 from the proMMP-2/TIMP-2 complex (Howard *et al.*, 1991) and in the purified recombinant proMMP-2 expressed in the vaccinia virus/HeLa cell system (Fridman *et al.*, 1993). In addition, Bergmann *et al.* (1995) reported the expression of free proMMP-2 in a baculovirus/insect cell system while our study was in progress, and the purified free proMMP-2 was auto-activated and autodegraded. In contrast, the proMMP-2 partially purified from HT-1080 cells, most of which was complexed with TIMP-2, was more resistant to autodegradation than the recombinant free proMMP-2 (data not shown; see below).

A 64-kDa intermediate form and the 62-kDa mature MMP-2 were found in the activation process of the free proMMP-2 in the presence of external activators such as APMA (see below; Stetler-Stevenson *et al.*, 1989b) and MT1-MMP (Sato *et al.*, 1994). Interestingly, the intermediate form and the mature MMP-2 were not observed at detectable levels during the autodegradation process in the absence of external activators, as shown in Fig. 1 and reported by others (Bergmann *et al.*, 1995). However, it is thought that at least a trace amount of the mature MMP-2 is required for triggering the auto-degradation. Therefore, these results suggest that auto-activation of the free proMMP-2 to the mature MMP-2 is a rate-limiting step and that autodegradation from the mature MMP-2 into

smaller MW forms without external activators is a fast step.

Processing of the free proMMP-2 in the presence of APMA Our results have shown that the recombinant proMMP-2 free of TIMP-2 was autodegraded into smaller MW forms without detectable levels of the mature MMP-2, and much faster than the proMMP-2 complexed with TIMP-2. Inhibition of autodegradation of proMMP-2 by TIMP-2 would thus be resulted from either inhibition of activation of proMMP-2 to the mature MMP-2 or inhibition of autodegradation of the mature MMP-2 to the smaller MW forms itself, or from both.

Prior to analysis of the inhibition step by TIMP-2 during autodegradation of the free proMMP-2, we examined the kinetics of activation and autodegradation of the free proMMP-2 in the presence of APMA. The proMMP-2 was incubated for various periods of time in the presence of APMA and the reaction products were analyzed by gelatin zymography (Fig. 3). Eighty percent of the proMMP-2 was converted into the intermediate form (64 kDa) and the mature MMP-2 (62 kDa) at 30 min after incubation with APMA. The mature MMP-2 was detected at highest levels after 1 h incubation but kept decreasing upon further incubation due to autodegradation of the mature MMP-2 into the smaller gelatinolytic fragments (43 kDa, 38 kDa, and 32 kDa) and smaller non-gelatinolytic fragments (data not shown). To study the role of TIMP-2 on processing of proMMP-2, the activation process was examined after 30 min incubation and the autodegradation process after 12 h incubation in the presence of APMA.

Inhibition of activation and autodegradation of the free proMMP-2 by TIMP-2 To examine the effect of TIMP-2 on the activation of the free proMMP-2, activation of proMMP-2 was induced by APMA for 30 min in the absence or presence of various amounts of TIMP-2. Levels of proMMP-2 and its processed forms were analyzed by gelatin zymography (Fig. 4). In the absence of TIMP-2, most of the free proMMP-2 was processed into the 64 kDa intermediate form and the mature 62 kDa MMP-2. When the free proMMP-2 was incubated with TIMP-2 at a 1:1 proMMP-2/TIMP-2 molar ratio, small amounts of the mature MMP-2 were observed in the gelatin zymography. Increased amounts of TIMP-2 slowly decrease the processing of proMMP-2 into the intermediate form. This result demonstrates that TIMP-2 inhibits the activation processes both from proMMP-2 to an intermediate form and from the intermediate form to the mature MMP-2, although the latter process is inhibited more effectively than the former under our experimental conditions.

The effect of TIMP-2 on autodegradation of the free proMMP-2 was also examined by measuring levels of various MMP-2 products incubated for 12 h in the presence of APMA and various concentration of TIMP-2 (Fig. 5). In the absence of TIMP-2, only small amounts of the mature MMP-2 and small MW gelatinolytic fragments were detected in the gelatin zymogram due to autodegradation of the proMMP-2 into the non-gelatinolytic fragments. The presence of TIMP-2 resulted in accumulation of the mature MMP-2 and smaller MW fragments at a 1:1 proMMP-2/TIMP-2 molar ratio and a greater accumulation of them at a 1:2 proMMP-2/TIMP-2 molar ratio. Complete inhibition of autodegradation from the mature MMP-2 to the smaller MW forms was observed at a 1:5 proMMP-2/TIMP-2 molar ratio.

It has been reported that activation of proMMP-2 by APMA is triggered by the molecular perturbation of proMMP-2 and is followed by dissociation of the Zn^{2+}-Cys^{73} interaction (Itoh *et al.*, 1995). The resultant activated proMMP-2 (proMMP-2*), in which the pro-domain is not processed, is partially active and is

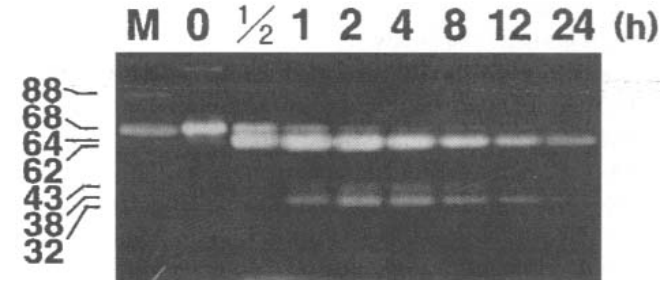

Fig. 3. Processing of the recombinant proMMP-2 in the presence of APMA. The recombinant proMMP-2 (1.85 μg/ml at a final concentration) was incubated with a final concentration of 1 mM APMA for various time intervals. The reaction products were analyzed by gelatin zymography. Lanes 0, 1/2, 1, 2, 4, 8, 12, and 24; incubation periods (h) in the presence of APMA. Lane M, the culture medium of HT-1080.

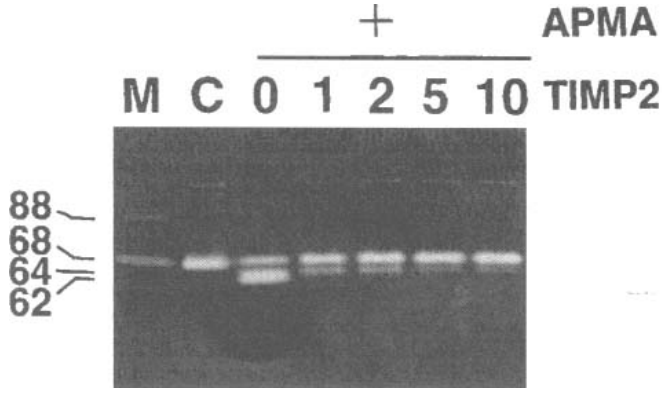

Fig. 4. Inhibition of activation of proMMP-2 by TIMP-2. The recombinant proMMP-2 (1.85 μg/ml at a final concentration) with various amounts of TIMP-2 were incubated for 30 min in the presence of APMA to allow activation of the proMMP-2. The reaction products were analyzed by gelatin zymography. Lanes 0, 1, 2, 5, and 10, the absence or presence of TIMP-2 to achieve the proMMP-2/TIMP-2 molar ratios of 1:0, 1:1, 1:2, 1:5, and 1:10, respectively, in the presence of 1 mM APMA (+). Lane C, the recombinant proMMP-2 as a control; lane M, the culture medium of HT-1080.

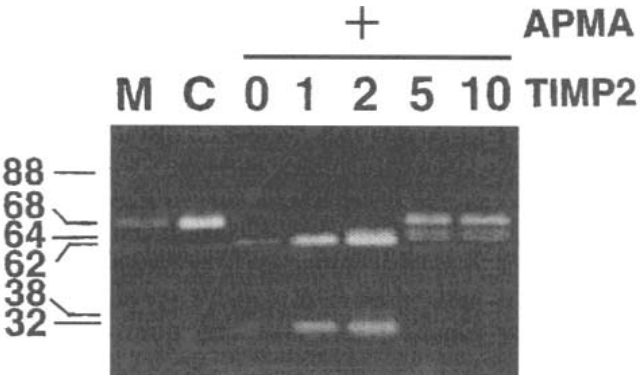

Fig. 5. Inhibition of autodegradation of proMMP-2 by TIMP-2. The recombinant proMMP-2 (1.85 μg/ml at a final concentration) with various amounts of TIMP-2 were incubated for 12 h in the presence of APMA to allow autodegradation of the proMMP-2. The reaction products were analyzed by gelatin zymography. Lanes, same as those in Fig. 4.

processed to the fully active mature MMP-2 (62 kDa) via the intermediate form (64 kDa) by serial cleavage of the pro-domain. It is known that TIMP-2 binds to at least one binding site of the C-terminal domain of proMMP-2 but not to the catalytic domain. However, TIMP-2 binds more tightly to the mature MMP-2 through the catalytic domain and the C-terminal domain than to proMMP-2 (Howard and Banda, 1991; Fridman *et al.*, 1992; Murphy *et al.*, 1992). Because proMMP-2* and the intermediate form generated during APMA treatment expose their catalytic domains, TIMP-2 would bind to proMMP-2* and the intermediate form as efficiently as the mature MMP-2 and then form the inactive enzyme/inhibitor complexes. Our results that TIMP-2 inhibits the APMA-induced activation processes of proMMP-2 (Fig. 3) are thus explained by a decrease of the active MMP-2 species due to the formation of the inactive enzyme/inhibitor complexes.

Although TIMP-2 inhibits the activation processes both from proMMP-2 to the intermediate form and from the intermediate form to the mature MMP-2, inhibition of the former process has shown much slower kinetics than that of the latter process as a function of TIMP-2. Two possible mechanisms can explain this finding. First, TIMP-2 has an additional binding site on the C-terminal side of the prodomain of the proMMP-2* and the N-terminal side of the pro-domain of proMMP-2* shields the binding site. In this case, TIMP-2 would bind to the intermediate form at higher affinity than to proMMP-2* and then block the processing from the intermediate form to the mature MMP-2 more efficiently than that from proMMP-2* to the intermediate form. A second possibility is that the various MMP-2 species/TIMP-2 complexes may still have residual catalytic activity. Kolkenbrock *et al.* (1991) reported that the formation of the proMMP-2/TIMP-2 complex does not completely prevent its activation by APMA and that the activated complex exhibits gelatinolytic activity equivalent to approximately 10% of the mature MMP-2. This residual activity of the complex may process a portion of the proMMP-2* to the intermediate form but it may not be enough to process further to the mature MMP-2.

The APMA-induced autodegradation of the free proMMP-2 (Fig. 5) was examined after 12 h incubation in the presence of APMA, whereas the APMA-induced activation was examined after 30 min incubation (Fig. 4). From a comparison of these results, we could find the fate of free proMMP-2 in the presence of various concentrations of TIMP-2 as a function of incubation time. As shown in Fig. 5, the presence of TIMP-2 drastically retarded APMA-induced autodegradation of proMMP-2. However, the partially processed products of free proMMP-2 during 30 min incubation (Fig. 4) were further processed into the mature MMP-2 and the smaller MW fragments at 1:1-2 proMMP-2/TIMP-2 molar ratios and into the mature MMP-2 at 1:3-5 molar ratios, during 12 h incubation. These results strongly support the possibility that the various MMP-2 species/TIMP-2 complexes would still have residual catalytic activity in the presence of APMA. The residual catalytic activity of MMP-2 in the complex would activate and eventually autodegrade each other, even at a very slow rate.

While inhibition of the proMMP-2 activation by TIMP-2 was gradually increased by the increasing amounts of TIMP-2 (Fig. 4), inhibition of autodegradation was sharply increased, especially between 1:2 and 1:5 proMMP-2/TIMP-2 molar ratios. It reflects the fact that the extent of autodegradation is not a simple function of the amount of active MMP-2 species. Thus, it is likely that TIMP-2 plays a role in the stabilization of MMP-2. As mentioned above, TIMP-2 binds at least two sites of the activated MMP-2; the catalytic domain and the C-terminal domain. It is suggested that the binding of TIMP-2 to the C-terminal domain of MMP-2 may contribute to the stabilization of MMP-2 (Howard and Banda, 1991; Howard *et al.*, 1991). This supports our hypothesis that TIMP-2 plays a role in the stabilization of MMP-2.

It was previously shown that TIMP-2 inhibits the catalytic activity of the mature MMP-2 by its binding to the catalytic domain. Here, we have demonstrated that TIMP-2 has additional roles both in inhibition of the proMMP-2 activation and in inhibition of the autodegradation of MMP-2. We used APMA as an external activator to expedite the activation and autodegradation of proMMP-2. Efficient activators of proMMP-2 such as MT1-MMP are also present *in vivo*. We thus expect that additional roles of TIMP-2 that inhibits both activation and autodegradation of proMMP-2 would be true *in vivo* as well as *in vitro*.

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