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Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2

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ABSTRACT **Simian virus 40 (SV40)-transformed human lung fibroblasts secrete both 72-kDa type IV collagenase and a closely related 92-kDa type IV collagenase that was not detected in the parental cell line. The 92-kDa type IV procollagenase purified from these cells exists in a noncovalent complex with the tissue inhibitor of metalloproteases, TIMP. Here we report that the 72-kDa type IV procollagenase purified from *HRAS*-transformed human bronchial epithelial cells, SV40-transformed lung fibroblasts, and normal skin fibroblasts exists in a stable but noncovalent stoichiometric complex with a 24-kDa inhibitor referred to here as "TIMP-2." TIMP-2 is closely related to TIMP, as demonstrated by comparison of the partial amino acid sequence of this protein to that of TIMP, although it does not cross-react with TIMP-specific antibody. The TIMP-2 inhibitor interacts with the 72-kDa type IV collagenase in preference to the 92-kDa type IV collagenase that forms a complex exclusively with TIMP. The 72-kDa type IV collagenase–TIMP-2 complex can be activated with organomercurials to yield a catalytically competent enzyme. Activation occurs concomitantly with autoproteolytic cleavage of the amino terminus of the protein and does not require dissociation of the complex. Both activity and activation of the complex can be completely inhibited by further addition of stoichiometric quantities of purified TIMP-2 or recombinant TIMP.**

Tissue inhibitor of metalloproteases (TIMP) is a ubiquitous (1) glycoprotein (M_r 30,000) that originally was purified from serum-free medium conditioned by normal human skin fibroblasts (2). Inhibition of interstitial collagenase is stoichiometric, with a 1:1 molar ratio of inhibitor to enzyme being required for complete inhibition of enzyme activity. Interstitial procollagenase can neither interact with TIMP nor bind to collagen; both require extracellular activation of the collagenase zymogen (3, 4). Active enzyme binds with nearly equal affinity to both monomeric collagen and aggregated fibrils. The K_i of enzyme–inhibitor binding was determined to be less than 10^{-9} M (4).

The gene encoding TIMP has been cloned as a collagenase inhibitor (5, 6) and as a factor with erythroid-potentiating activity (7) that stimulates colony formation by relatively mature erythroid precursors (CFU-E). The *TIMP* gene has been localized to the X chromosome (8, 9). The secreted protein consists of 184 amino acid residues and contains six disulfide bonds and two glycosylation sites containing N-linked oligosaccharides. The sequence of the inhibitor has no substantial homology to previously sequenced protease inhibitors with the exception of the metalloprotease inhibitor isolated from bovine scapular cartilage (10) and from normal murine fibroblasts (11, 12). The inhibitor extracted from cartilage has a molecular mass of 27,400 with an NH_2-terminal sequence of 45 residues that shows 65% homology to TIMP.

Recent observations suggest that expression of TIMP may be involved in suppression of tumorigenicity of immortal murine 3T3 cells (13). Mouse 3T3 cell lines constitutively synthesizing an RNA complementary to the messenger RNA encoding TIMP were shown to secrete a reduced amount of TIMP into the culture medium. Unlike the parental cells, these cells were invasive in a human amnion invasion assay and were tumorigenic and metastatic in athymic mice. In addition, in an *in vitro* amnion invasion assay system, TIMP inhibited the invasion of B16-F10 murine melanoma cells through the human amniotic membrane (14), while intraperitoneal injection of recombinant TIMP into mice showed a significant inhibition of metastatic lung colonization by these cells. The anticolonization effect of recombinant TIMP was found to be due to its action on invasion rather than on tumor growth.

In this report we describe a human metalloprotease inhibitor, TIMP-2, closely related to TIMP, that forms an exclusive complex with the 72-kDa type IV collagenase.

MATERIALS AND METHODS

Cell Culture. Simian virus 40 (SV40)-transformed fetal lung fibroblasts, the parental line IMR-90, and the monocytic leukemia strain U937 were cultured as described (15). U937 cells (2×10^6 cells per ml) were treated with phorbol 12-myristate 13-acetate (50 ng/ml; PMA) in Dulbecco's modified Eagle's/F-12 medium containing 0.1% fetal bovine serum for 24–48 hr.

Enzyme Purification. The 92-kDa (15) and 72-kDa (16) type IV collagenases were purified from conditioned medium of SV40-transformed fibroblasts or PMA-differentiated U937 cells by using a three-step procedure. Serum-free medium was adjusted to 0.01 M Tris·HCl (pH 7.5) and applied to a 2.5 × 10 cm column of reactive red agarose (Sigma) equilibrated in 0.02 M Tris·HCl (pH 7.5) containing 0.005 M $CaCl_2$ (Tris/$CaCl_2$ buffer) and 0.15 M NaCl. The enzyme was eluted from the column with a 0.15–2.0 M NaCl linear gradient in Tris/$CaCl_2$ buffer, and fractions were assayed by gelatin zymography (17).

Fractions containing gelatinolytic activity were pooled, adjusted to contain 0.5 M NaCl and 0.01% Brij-35, and chromatographed on a 1.0 × 10 cm column of gelatin-Sepharose (Sigma) as described (16, 18) except that the enzyme was eluted by using a 0–10% dimethyl sulfoxide gradient, which permitted resolution of two enzymes. Note that free inhibitor does not bind to this affinity column. Gelatin-Sepharose-purified enzyme was dialyzed into 0.005 M Tris·HCl (pH 7.5) containing 0.0001 M $CaCl_2$ and 0.005% Brij-35 and was stored at −80°C. An optional step includes gel-filtration chromatography on an Ultrogel AcA-44 column

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Abbreviations: TIMP, tissue inhibitor of metalloproteases; APMA, *p*-aminophenylmercuric acetate; SV40, simian virus 40; PMA, phorbol 12-myristate 13-acetate.

(LKB) equilibrated in Tris/$CaCl_2$ buffer containing 1.0 M NaCl and 0.01% Brij-35.

Purification of TIMP-2 and Sequence Analysis. The purified 72-kDa type IV collagenase–TIMP-2 complex was subjected to gel filtration in 0.02 M Tris·HCl, pH 7.5/0.05 M NaCl/0.1% $NaDodSO_4$ to separate the proenzyme and TIMP complex. The fractions containing TIMP-2 were pooled and dialyzed against 0.005 M Tris·HCl, pH 7.5/0.0001 M $CaCl_2$/0.005% Triton X-100 (LB buffer).

The gelatin-Sepharose-purified enzyme–inhibitor complex was subjected to reverse-phase HPLC with an Applied Biosystems 130-Å microbore separation system with a 2.1 × 30 mm column equilibrated in 0.07% trifluoroacetic acid. The column was developed with a linear gradient of 0–70% acetonitrile in 0.07% trifluoroacetic acid. HPLC-purified TIMP-2 was identified by silver staining of the $NaDodSO_4$/PAGE, lyophilized, and subjected to S-pyridylethylation (Applied Biosystems User Bulletin no. 28, 470A/477A-120A). The TIMP-2 peak was collected, reconstituted in 50 mM ammonium bicarbonate, and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Worthington). The peptides were separated by microbore reverse-phase HPLC as described above. Several of the resolved peptides and the untreated TIMP-2 were subjected to amino acid sequence determination with an Applied Biosystems 470A gas-phase sequencer.

Enzyme Assays. Samples of purified proenzyme were activated by the organomercurial compound, *p*-aminophenylmercuric acetate (APMA) as described (16). The activated enzyme was preincubated with inhibitor as indicated for 5 min at 25°C, and the samples were divided into equal aliquots. Enzyme activity, with [^{14}C]gelatin (40,000 cpm/mg) as the substrate, was determined as described (16, 19). The other aliquot was subjected to $NaDodSO_4$/PAGE analysis and laser densitometry. Alternatively, inhibitor was added prior to the addition of APMA, and activation was then initiated as described above.

RESULTS

The 24-kDa Inhibitor TIMP-2 Preferentially Interacts with 72-kDa Type IV Collagenase to Form a Stable Stoichiometric Complex. Human bronchial epithelial cells secrete a 72-kDa type IV collagenase in response to transformation with *HRAS* (16). This metalloprotease is also secreted in large quantities by other human transformed cell lines as well as normal human skin fibroblasts. A 92-kDa enzyme is secreted by several transformed cell lines of fibroblast origin and other tumor cells (15); this enzyme is normally expressed by human macrophages and epidermal keratinocytes in preference to the 72-kDa enzyme (15). In addition, SV40-transformed human lung fibroblasts secrete both the 72-kDa type IV collagenase and the 92-kDa type IV collagenase, which is not detectable in the parental cell line, IMR-90 (15).

We have recently demonstrated (15) that the 92-kDa type IV procollagenase exists in a noncovalent complex with TIMP, which can be activated by APMA, yielding an enzyme with a substrate specificity similar to that of the 72-kDa type IV collagenase. The complex has been purified from phorbol ester-differentiated U937 monocytic leukemia cells, the human fibrosarcoma cell strain HT-1080, and SV40-transformed human lung fibroblasts. The results (Fig. 1*A*) show that the 92-kDa type IV collagenase purified from conditioned medium of SV40-transformed cells contains stoichiometric amounts of a 30-kDa TIMP, while the 72-kDa type IV collagenase, purified from the same starting material, contains a 24-kDa protein. This protein was not detected in any preparations of affinity-purified 92-kDa enzyme. Conversely, TIMP was not detected in preparations of the 72-kDa enzyme, purified by the same procedure. This observation shows that, although the same cells secrete all four compo-

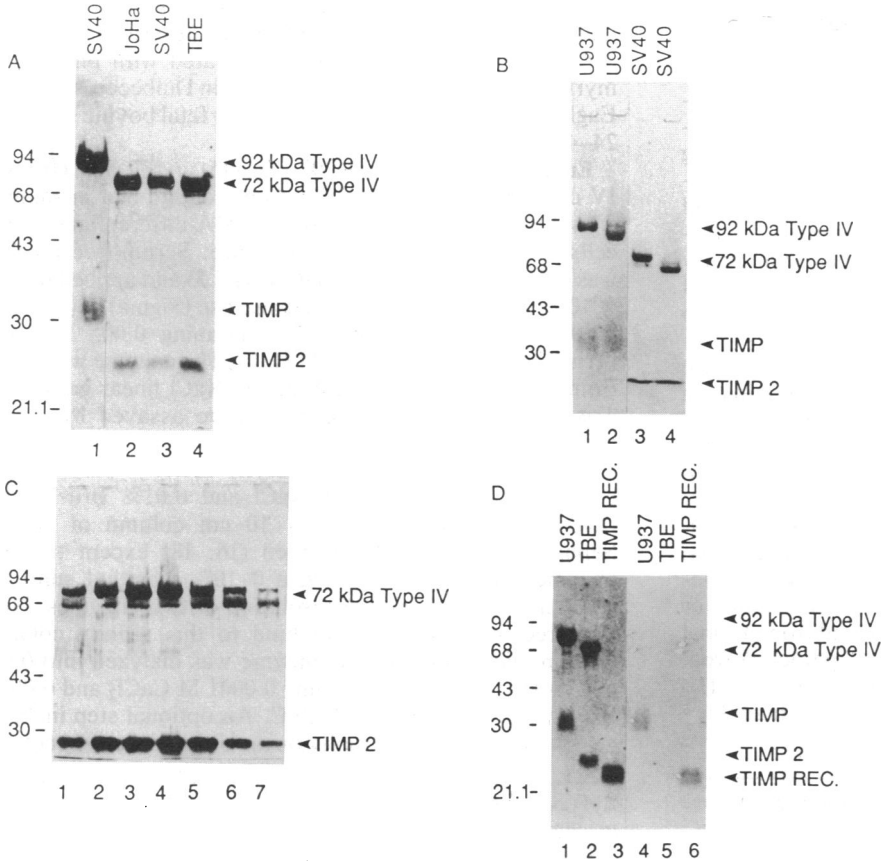

FIG. 1. Analysis of the type IV collagenase–inhibitor complex. (*A*) Samples (1 μg) of purified type IV collagenase–inhibitor complex from the indicated cell source were subjected to $NaDodSO_4$/PAGE on a 13% gel. (*B*) The enzyme–inhibitor complex was subjected to $NaDodSO_4$/PAGE as in *A* before (lanes 1 and 3) and after (lanes 2 and 4) activation with APMA. (*C*) An APMA-activated 72-kDa–type IV collagenase complex was size-fractionated on an Ultrogel AcA-44 gel filtration column, and fractions were analyzed on silver-stained 13% $NaDodSO_4$/PAGE. (*D*) Purified complex (lanes 1, 2, 4, and 5) and purified recombinant TIMP (lanes 3 and 6) were stained (lanes 1–3) or subjected to immunoblot analysis with anti-TIMP antibody (lanes 4–6). Note that the molecular mass of the recombinant TIMP is lower because of the absence of glycosylation.

nents involved in the formation of the two enzyme–inhibitor complexes, TIMP associates exclusively with the 92-kDa type IV procollagenase, and the 24-kDa protein is found complexed with the 72-kDa enzyme. The gelatin affinity column does not bind free TIMP or 24-kDa protein (data not shown).

The 24-kDa protein found in preparations of the type IV collagenase did not react with TIMP-specific antibody (Fig. 1*D*), but both recombinant TIMP and the TIMP associated with the 92-kDa enzyme did react with anti-TIMP antibody. Gel filtration chromatography of the gelatin-Sepharose-purified 72-kDa enzyme in 1.0 M NaCl (data not shown) failed to resolve the putative complex between these two proteins. However, gel filtration in the presence of 0.1% $NaDodSO_4$ did separate the proenzyme from the complexed protein. These observations indicate that the 72-kDa purified proenzyme and the 24-kDa protein exist in a stable noncovalent complex.

The results in Fig. 2 show the dissociation of the complex after acidification of the sample with trifluoroacetic acid and

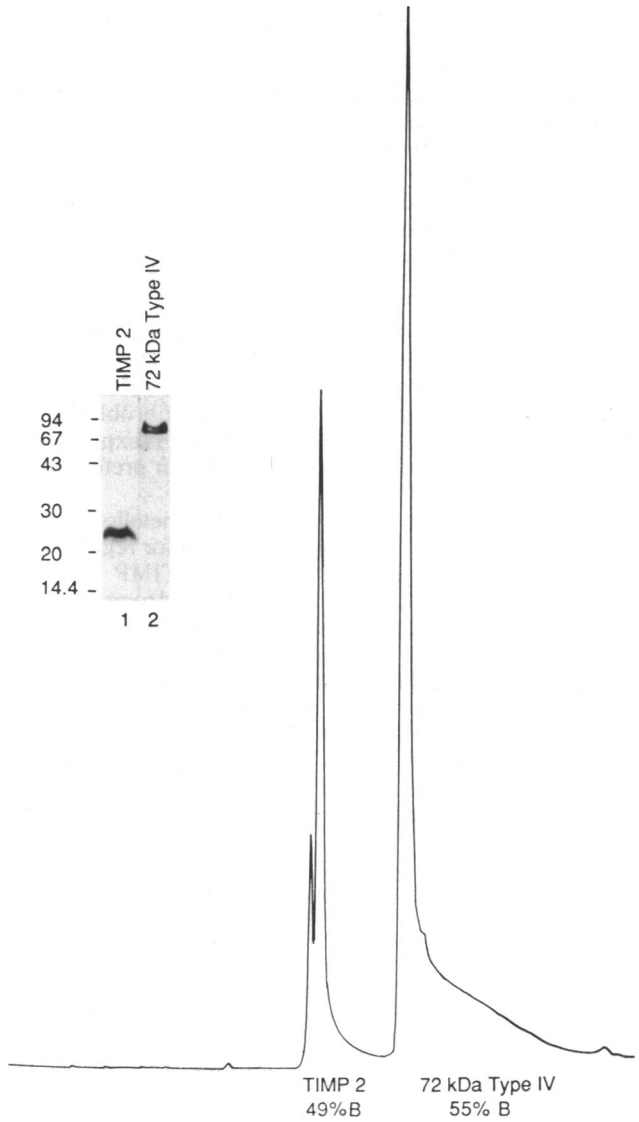

FIG. 2. Reverse-phase HPLC of the 72-kDa type IV procollagenase–TIMP-2 complex. A purified preparation of the 72-kDa type IV collagenase–TIMP-2 complex from TBE cells was chromatographed on a 130-Å microbore separation system as described. (*Inset*) Each peak was collected, lyophilized, and analyzed on silver-stained $NaDodSO_4$/PAGE.

HPLC chromatography. This procedure permits purification of the 24-kDa protein free of the enzyme as shown in Fig. 2. The procedure does not permit the isolation of the free enzyme because it is inactivated in the course of chromatography.

To obtain a partial amino acid sequence of the 24-kDa protein, the first peak (Fig. 2) was subjected to digestion with trypsin and further chromatographed as in Fig. 2. The resulting peptides were subjected to amino acid sequence analysis. Undigested protein was used to determine the sequence of the amino terminus. The determined peptide sequences are presented in Fig. 3. The homology between the sequences of these peptides and TIMP clearly shows the close relationship of the two proteins. Based on this observation we refer to the 24-kDa protein found in complex with the 72-kDa type IV collagenase as TIMP-2.

APMA-Induced Activation and Enzymatic Activity of the TIMP-2-Type IV Collagenase Complex Can Be Inhibited by Further Addition of Stoichiometric Amounts of TIMP-2. Treatment of the 72-kDa type IV collagenase with organomercurials results in the proteolytic processing of the amino-terminal domain with a loss of 6 kDa in molecular mass and conversion of the proenzyme into a catalytically competent form (16, 20). The formation of a complex between TIMP-2 and the 72-kDa proenzyme did not prevent its activation by treatment with the organomercurial APMA (Fig. 1*B*). Similarly, autoactivation of the TIMP–92-kDa type IV collagenase complex initiated by APMA resulted in a loss of 8 kDa as determined by $NaDodSO_4$/PAGE (Fig. 1*B*; ref. 15), corresponding to the cleavage of 73 amino acid residues from the amino-terminal domain (15). Activation did not affect dissociation of the enzyme–inhibitor complex, since gel filtration chromatography of the activated enzyme failed to resolve the enzyme from TIMP-2 (Fig. 1*C*). The specific activity of the APMA-activated enzyme–TIMP-2 complex against [^{14}C]-gelatin was between 900 and 1200 units/mg of enzyme protein.

What is the activity of the complex compared to the activity of the free enzyme? The answer to this question is not clear, since enzyme preparations from available sources contain TIMP-2 and the conditions inducing dissociation of the complex cause inactivation of the enzyme.

Does TIMP-2 inhibit the activity of the 72-kDa type IV collagenase? Since the amount of TIMP-2 in gelatin-affinity-purified preparations of the enzyme is approximately stoichiometric, the activity of the complex might be explained by the presence of a minor fraction of the free enzyme. If so, one would predict that addition of small quantities of purified TIMP-2 will cause complete inhibition of enzyme activity. To investigate this possibility we have obtained a purified preparation of TIMP-2. Purified 72-kDa type IV collagenase–TIMP-2 complex was dissociated and subjected to gel filtration chromatography on Ultrogel ACA-

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TIMP2 NT : SPVHPQQAFCNADVVIRAKAVS
TIMP     : V P   T    S L   F   G

TIMP2 TP1: ITLCDFIVPWDTLSTTQK
TIMP     :   T S VA  NS  LA R

TIMP2 TP2: CPMIPCYISSPDECL
TIMP     :  LS   KLQ GTH
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FIG. 3. Amino acid sequence of peptides derived from human inhibitor TIMP-2. Tryptic peptides (TP1 and TP2) were fractionated and sequenced as described. Purified 72-kDa type IV collagenase–TIMP-2 complex was electroblotted onto a polyvinylidene difluoride (PVDF, Millipore) membrane and a protein band corresponding to TIMP-2 was subjected to amino-terminal sequence analysis (NT). In each pair of lines, the TIMP-2 amino acid sequence is the upper line, and the human TIMP sequence is the lower line, in which blank spaces indicate identical residues.

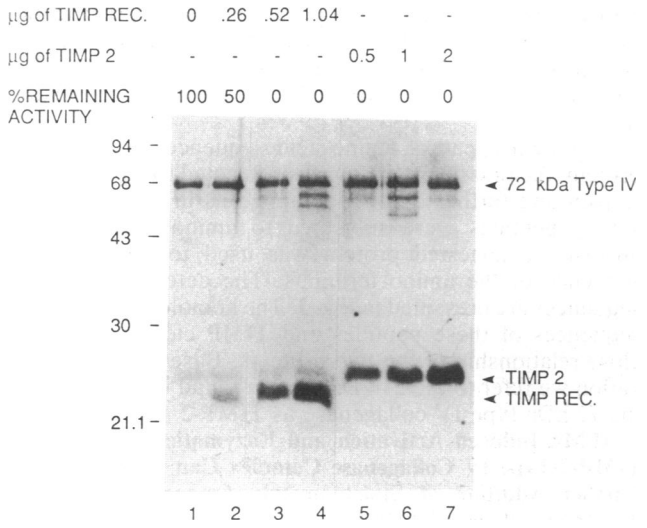

FIG. 4. Activation of the 72-kDa type IV procollagenase–TIMP-2 complex and inhibition of its activity with TIMP and TIMP-2. One microgram of the enzyme–inhibitor complex purified from TBE cells was activated with 1 mM APMA as described (lane 1) and briefly incubated with the indicated amounts of recombinant (REC.) TIMP (lanes 2–4) or purified TIMP-2 (lanes 5–7). The reaction mix was separated into two aliquots and electrophoresed on a 13% $NaDodSO_4$ gel or assayed for gelatinolytic activity against C_{14} gelatin.

44 in the presence of 0.1% $NaDodSO_4$. The fractions containing free TIMP-2 were pooled after analysis on silver-stained $NaDodSO_4$/PAGE. The protein was then reconstituted by using equilibrium dialysis against LB buffer containing 0.005% Triton X-100. This preparation was used to study the effect of addition of TIMP-2 on the type IV collagenase–TIMP complex as shown in Figs. 4 and 5.

The enzyme–inhibitor complex was activated by treatment with 1 mM APMA for 1 hr at 37°C with (Fig. 5, lanes 8–12) or without (Figs. 4 and 5, lanes 2–7) further addition of the inhibitor in the amounts indicated. At the end of the activation reaction, inhibitor was added to the samples, which were activated in the absence of the inhibitor (Figs. 4 and 5, lanes 2–7). Each sample was divided into two aliquots, and each aliquot was assayed for activity by using [^{14}C]gelatin as a substrate (see *Methods*), or analyzed on $NaDodSO_4$/PAGE. The gels were then scanned with a laser densitometer to quantitate the amount of TIMP-2 relative to the amount of enzyme present in the reaction. This permitted us to assess the extent of enzyme activation (by a shift in the apparent molecular weight) independent of the activity assay. The addition of a molar excess of TIMP-2 to the activated type IV collagenase–TIMP-2 complex completely inhibited its gelatinolytic activity (Figs. 4 and 5). Furthermore, presence of the same amounts of TIMP-2 during the APMA treatment inhibited activation, since no conversion of the enzyme into a lower molecular mass form was observed, compared with the control (Fig. 5, lane 1). In agreement with this result, the gelatinolytic activity of the complex treated with APMA in the presence of excess TIMP-2 was completely inhibited.

Inhibition of both AMPA-induced activation and gelatinolytic activity of the 72-kDa type IV collagenase–TIMP-2 complex required addition of stoichiometric quantities of free TIMP-2, rather than small amounts as compared to the amount of enzyme complex in the reaction mix. Although the 72-kDa type IV procollagenase was found complexed only to TIMP-2, the addition of recombinant TIMP to the complex resulted in complete inhibition of activity (Fig. 4, lanes 2–4). This result suggests the possibility that binding of two mol of TIMP-2 or 1 mol each of TIMP and TIMP-2 per 1 mol of the enzyme is required to achieve complete inhibition of activation and catalytic activity. These results are also consistent with the autoproteolytic mechanism of metalloprotease activation (21).

DISCUSSION

We have reported the substrate specificities and the primary sequences of two type IV collagenases. The 72-kDa type IV collagenase is secreted by *HRAS*-transformed TBE-1 cells and normal skin fibroblasts (16). This metalloprotease is identical to the human tumor cell enzyme secreted by A2058 melanoma cells, SV40-transformed human lung fibroblasts and HT1080 fibrosarcoma cells and most probably is the human homolog of the 68-kDa murine type IV collagenase (22). The 92-kDa type IV collagenase, although very similar to the 72-kDa type IV collagenase in structure and substrate specificity, is strikingly different in tissue specificity and regulation of its expression (15). The 92-kDa enzyme is secreted by some transformed cell lines of fibroblast origin, such as SV40-transformed human lung fibroblasts and HT1080 cells. Under normal circumstances it is expressed by macrophages and epidermal keratinocytes in preference to the 72-kDa enzyme (15).

Interaction of the extracellular matrix metalloproteases with the specific inhibitor TIMP is essential for regulation of their activity in the extracellular space. TIMP has been reported to inhibit interstitial collagenase, stromelysin (23, 24), and rabbit bone gelatinase (25) by forming an enzyme–inhibitor complex with activated enzymes. The formation of a complex between TIMP and the latent enzymes has not been previously reported to our knowledge. We recently have demonstrated that the purified 92-kDa type IV procollagenase and TIMP exist in a noncovalent complex that is stable in the presence of 1.0 M NaCl or 2.0 M urea (15). The 92-kDa enzyme isolated from polymorphonuclear leukocytes

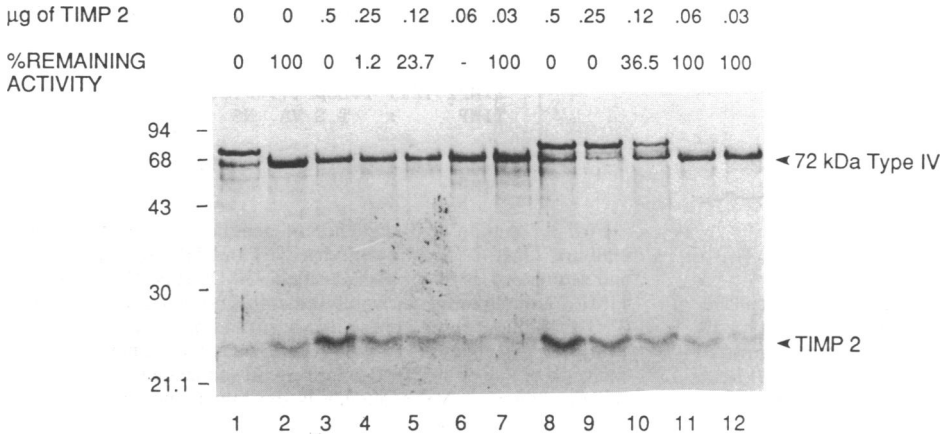

FIG. 5. Activation of the 72-kDa type IV procollagenase–TIMP-2 complex and inhibition of its activity by the further addition of TIMP-2. One microgram of the proenzyme–inhibitor complex purified from TBE cells (lane 1) was activated with 1 mM APMA (lane 2) with (lanes 8–12) or without (lanes 3–7) indicated additional amounts of TIMP-2. Samples in lanes 3–7 were briefly incubated with indicated amounts of purified TIMP-2 after activation. Samples were analyzed as in Fig. 4. (Half of the sample was analyzed on the Coomassie blue-stained gel; the other half was assayed for activity.)

(PMNs) was found in a TIMP-free form, which is consistent with the fact that these cells do not produce TIMP (26) and most likely accounts for the 10-fold higher specific activity it exhibits against both gelatin and type IV collagen.

The presence of a stoichiometric quantity of TIMP in the U937 92-kDa enzyme preparation did not prevent its activation by the organomercurial APMA, resulting in the generation of an 84-kDa enzyme species. This is in agreement with the results of Murphy *et al.* (27), who reported that the presence of TIMP slowed but did not prevent organomercurial-initiated autoactivation of a 97-kDa metalloprotease from pig PMNs. The data presented in this report demonstrate that the 72-kDa type IV procollagenase forms a noncovalent stoichiometric complex with the inhibitor TIMP-2. Purification of the 92-kDa type IV collagenase–TIMP complex and the 72-kDa type IV collagenase–TIMP-2 complex from the same starting material, conditioned medium of SV40-transformed fibroblasts, clearly shows that each of these proenzymes forms a complex exclusively with either TIMP or TIMP-2. The fact that the preparations of the TIMP-2 obtained by dissociation of the 72-kDa type IV collagenase complex did not contain contaminating TIMP is in agreement with this interpretation.

The 72-kDa type IV collagenase–TIMP-2 complex has properties similar to the 92-kDa type IV collagenase–TIMP complex. It is stable in 1 M NaCl and dissociates upon HPLC chromatography in 0.1% trifluoroacetic acid or in the presence of 0.1% $NaDodSO_4$. The complex can be activated with APMA; however, activation does not dissociate TIMP-2 from the enzyme.

The considerable gelatinolytic activity of the enzyme–inhibitor complex resulting from organomercurial activation is in itself surprising. Even more surprising is the fact that further addition of stoichiometric quantities of either recombinant TIMP or purified TIMP-2 blocks this activity. Addition of the TIMP-2 also prevents activation of the complex by blocking APMA-initiated proteolytic processing of the amino-terminal domain of the enzyme. These data suggest that 72-kDa type IV procollagenase can form a partially active complex with TIMP-2. This complex may then be available for interaction with TIMP or TIMP-2 leading to complete inactivation. The effect of the binding of inhibitor on the activity of the free enzyme remains to be determined when inhibitor-free enzyme preparations become available. It is tempting to speculate that formation of a 1:1 molar ratio complex reduces the activity of the enzyme, possibly by preventing a hypothetical second-step activation analogous to the one reported for interstitial collagenase (28, 29).

As mentioned above, interstitial collagenase and stromelysin require enzyme activation for binding to TIMP. The physiological significance of the formation of a TIMP–proenzyme complex in the case of the type IV collagenases is unclear. One can speculate that this phenomenon has to do with binding of the enzyme to its substrate. Indeed, interstitial procollagenase does not bind to the collagen substrate prior to activation (4). In contrast, both type IV procollagenases can bind to their substrates because of the presence of the fibronectin-like collagen binding domain, a structural feature of these enzymes (15, 16). The presence of the inhibitor in such a ternary complex may provide a safety mechanism as well as serve some other function yet to be determined.

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