Transformed human fibroblasts secrete two structurally and functionally related inhibitors of matrix metalloproteinases, tissue inhibitor of metalloproteinases (TIMP) 1 and 2. In assays measuring the relative inhibitory capability of TIMP-1 and TIMP-2 against autoactivated 72-kDa gelatinase, which consists of two major active peptides and several inactive fragments, TIMP-2 was more effective than TIMP-1. The isolated 42.5-kDa active fragment that formed as a result of the autoactivation of 72-kDa gelatinase showed the greatest preference for TIMP-2; at half-maximal inhibition, TIMP-2 was >10-fold more effective than TIMP-1. TIMP-2 was also >2-fold more effective than TIMP-1 at inhibiting 72-kDa gelatinase-TIMP-2 complexes activated with 4-aminophenylmercuric acetate, and >7-fold more effective than TIMP-1 at inhibiting 92-kDa gelatinase activated with 4-aminophenylmercuric acetate. Furthermore, these active gelatinases preferentially bound 125I-TIMP-2 when incubated with equal amounts of radiolabeled TIMP-1 and TIMP-2. The ratios of 125I-TIMP-2/125I-TIMP-1 binding to 92-kDa gelatinase, autoactivated 72-kDa gelatinase, and 42.5-kDa fragment were 4.4, 10, and 33, respectively. On the other hand, interstitial collagenase was inhibited by TIMP-1 >2-fold more effectively than TIMP-2 in assays measuring cleavage of loose collagen fibrils.

Cultured cells derived from connective tissue secrete an array of metalloproteinases that, upon activation, degrade collagen, proteoglycans, and other extracellular matrix proteins. These proteinases are thought to be pivotal in such cellular activities as migration, invasion, and remodeling (1-3). Among the metalloproteinases found in tissues and conditioned medium are interstitial collagenase, which cleaves fibrillar collagen (4), and two known gelatinases, which migrate at 72 and 92 kDa in SDS gels. 72-kDa progelatinase has been purified and sequenced (5-7) and has been shown to be particularly prevalent in the conditioned medium of transformed cells (8, 9). Human 92-kDa progelatinase has also been purified, and the primary structure has been determined (10, 11). The presence of this metalloproteinase is inducible by transformation or treatment with phorbol esters (12, 13). Cells also secrete metalloproteinase inhibitors that regulate the actions of the proteinases. One inhibitor, TIMP-1 (originally known as TIMP), is secreted by several cell types in culture, including fibroblasts (14), osteosarcomas (15), promyelocytic leukemia cells (16), endothelial cells (17), bone explants (18), and astrocytes and gliomas (19), and can be extracted from cartilage (20), connective tissue, serum, and amniotic fluid (21-23). Several studies suggest that TIMP-1 acts as a physiologic inhibitor of metalloproteinase action in tissues. The addition of anti-TIMP-1 immunoglobulin G to cultured chondrocytes results in the degradation of collagen films because of the disruption of collagenase inhibition by TIMP-1 (24). The addition of TIMP-1 to cultured B16 melanoma cells interferes with invasion of basement membranes by these cells (25, 26). Reduced TIMP-1 concentrations in cells transfected with anti-sense TIMP-1 mRNA result in increased invasiveness (27). These and other studies suggest that the regulation of TIMP-1 expression and activity may play an important role in the regulation of proteolysis.

TIMP-1 is a secreted glycoprotein that forms stable complexes with target metalloproteinases (28, 29). Unlike serine proteinase inhibitors, complexes with TIMP-1 are apparently not covalent and are not stable in the presence of SDS. However, TIMP-1 retains its activity after disruption of the proteinase-inhibitor complex (30). TIMP-1 has been cloned and sequenced and has a molecular mass of ~28.5 kDa, which can be decreased to 20 kDa by deglycosylation (31). TIMP-1 contains 12 cysteine residues, all of which are thought to be involved in disulfide links, making TIMP-1 a very stable protein resistant to many forms of denaturation.

Recently, several TIMP-like inhibitors have been identified. TIMP-2, derived from human melanoma and transformed human fibroblasts (32), has been sequenced, as has metalloproteinase inhibitor, a similar inhibitor derived from bovine aortic endothelium (33). These proteins are gene products distinct from TIMP-1. TIMP-2, which copurifies with 72-kDa progelatinase during gelatin affinity chromatography (34), migrates near 25 kDa in SDS-polyacrylamide gels under reducing conditions. Reverse zymography of culture medium conditioned by rabbit endothelial cells and fibroblasts indicates that several other inhibitors, referred to as IMPs, might exist (35). Similar inhibitors have been observed in cultured human gliomas (19).

We have previously shown that human 72-kDa progelatinase can be separated from its copurifying TIMP-2 component, yielding active proteinase. This purified gelatinase autoactivates into two major active fragments and several less active...
The purpose of this study was to determine the relative inhibitory capability of TIMP-1 and TIMP-2 against isolated human matrix metalloproteinases.

**Experimental Procedures**

**Materials**—Human 72-kDa progelatinase-TIMP-2 complexes, as well as isolated gelatinase and TIMP-2, were purified from the conditioned, serum-free medium of AT2SF-395 cells (provided by John Murnane, University of California, San Francisco), as described previously. Briefly, 72-kDa progelatinase-TIMP-2 complexes were isolated by gelatin affinity chromatography. After further purification with lentil lectin affinity resin to remove 92-kDa progelatinase, the 72-kDa progelatinase was separated from TIMP-2 by RP-HPLC in the presence of 3% trifluoroacetic acid. Upon dialysis in column buffer (20 mM Hepes, 500 mM NaCl, 1 mM CaCl₂, 10% glycerol, 0.05% Brij-35, 0.02% NaN₃, pH 7.5), 72-kDa progelatinase autocatalytically activates to two major gelatinolytically active fragments, migrating at 42.5 and 62 kDa on SDS-polyacrylamide gels. Peaks containing these fragments were separated by additional RP-HPLC.

TIMP-1 and collagenase were purified from the human fibroblast-conditioned medium that did not bind to the gelatin affinity resin. TIMP-1 was recirculated over affinity matrix (Duolight Chemical Co.; iminodiacetic acid immobilized to TSK HW-65F), which had been prepared with 3 mg/ml ZnCl₂, washed with water, and equilibrated in column buffer. Bound material, which contained collagenase, was eluted with acetate buffer (50 mM sodium acetate, 500 mM NaCl, 10% glycerol, 0.02% NaN₃, pH 4.5). The material that did not bind to the Zn⁺⁺ affinity column. This material was recirculated over lentil lectin Sepharose (Pharmacia Biotechnology Inc.), washed with column buffer, and eluted with 500 mM methyl β-D-mannopyranoside in column buffer. Eluted material was concentrated, and pure TIMP-1 was obtained by RP-HPLC as described for TIMP-2.

Samples of conditioned medium were concentrated by cooled 13071

**Results**

Presumably, the functional roles of both TIMP-1 and TIMP-2 are to inhibit proteolysis by metalloproteinases in tissues. TIMP-1 has been shown to inhibit several metalloproteinases. In addition, inhibitory kinetics have been reported for TIMP-1 and other metalloproteinase inhibitors. To determine if the inhibitors are selective for particular proteases, we assayed the inhibitory effects of TIMP-1 and TIMP-2 against purified metalloproteinases. A previous study showed that several forms of 72-kDa gelatinase can be isolated and that material purified from conditioned medium contains TIMP-2 bound to inactive 72-kDa progelatinase, possibly at a stabilization site. Upon activation with APMA, 72-kDa gelatinase-TIMP-2 complexes remain intact, the propeptide of gelatinase is cleaved, and the preparation will degrade gelatin (34). Removal of TIMP-2 from 72-kDa progelatinase by RP-HPLC in the presence of 0.1% trifluoroacetic acid, followed by dialysis in neutral pH buffer, results in the autodegradation of 72-kDa progelatinase. This autodegradative event yields two active and several inactive fragments. The two active fragments migrate at 62 and 42 kDa on SDS-polyacrylamide gels or gelatin zymograms (Fig. 1). The two activities can be separated by an additional RP-HPLC step.

In assays measuring the relative inhibitory capability of TIMP-1 and TIMP-2 versus activated gelatinase-TIMP-2 complexes, TIMP-2 was more effective than TIMP-1 (Fig. 2A). Activated 72-kDa gelatinase-TIMP-2 complexes contain one primary gelatinolytic moiety (62 kDa) and are not as gelatinolytically active as gelatinase separated from TIMP-2. Fig. 2B illustrates the inhibition by TIMP-1 and TIMP-2 of autodeactivated 72-kDa gelatinase that is no longer complexed with TIMP-2.
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with TIMP-2. At higher inhibitor concentrations, TIMP-2 was more effective than TIMP-1. Previously, it was determined that approximately 7% of the protein in samples of autoactivated 72-kDa gelatinase consists of active peptides.\(^2\) This would yield approximately 0.5 pmol of active proteinase. Because of the heterogeneous population of peptides in the gelatinase samples, the dissection of the binding capabilities of TIMP-1 and TIMP-2 is difficult. It has been shown that an additional binding domain for TIMP-2 may exist on 72-kDa progelatinase.\(^3\) To reduce this complexity, assays were performed with the 42.5-kDa active fragment of autoactivated gelatinase, which was obtained free of the 62-kDa active fragment but still contained inactive peptides that were byproducts of autoactivation. Fig. 2C shows the results of assays with this preparation versus TIMP-1 and TIMP-2. At a concentration at which TIMP-2 reduced gelatinase activity by approximately 90%, TIMP-1 was ineffective at inhibiting gelatinolytic activity. The preparation containing 42.5-kDa active gelatinase fragment contained no additional TIMP-2-binding domain other than the active site.\(^3\) Purified human 92-kDa gelatinase was also assayed for its interaction with TIMP-1 and TIMP-2. Incubation of activated 92-kDa gelatinase with TIMP-1 or TIMP-2, followed by addition of labeled gelatin, resulted in a ~7-fold better inhibition by TIMP-2 in gelatin degradation assays (Fig. 2D).

The inhibition of both activated 72- and 92-kDa gelatinases was measured over time to determine if the inhibition of gelatinolysis reaches saturation. TIMP-1 and TIMP-2 were preincubated with either enzyme for up to 3 h, followed by

\(^{2}\) E. W. Howard and M. J. Banda, manuscript submitted.

\(^{3}\) E. W. Howard and M. J. Banda, manuscript submitted.
the addition of labeled gelatin. Extended preincubation time did not alter the inhibition of activated 72-kDa gelatinase-TIMP-2 complexes (Fig. 3A). A slight increase in the inhibition of 92-kDa gelatinase was noted, but the relative inhibition by TIMP-1 and TIMP-2 was unchanged (Fig. 3B).

The binding of both TIMP-1 and TIMP-2 to 92-kDa gelatinase and two forms of 72-kDa gelatinase was investigated next. Activated 92-kDa gelatinase, autoactivated 72-kDa gelatinase, and 42.5-kDa gelatinase fragment were each incubated with a mixture of equal amounts of radiolabeled TIMP-1 and TIMP-2. The resulting $^{125}$I-inhibitor-proteinase complexes were separated from free inhibitor on gelatin affinity resin. Fig. 4 shows the resulting autoradiograph of the SDS-polyacrylamide gel. The ratio of $^{125}$I-TIMP-2 to $^{125}$I-TIMP-1 that bound to 92-kDa gelatinase was 4.4, which reflected the relative ability of TIMP-2 and TIMP-1 to inhibit this metalloproteinase in gelatin degradation assays. The same was true for the 42.5-kDa active gelatinase fragment ($^{125}$I-TIMP-2: $^{125}$I-TIMP-1, 33) and autoactivated 72-kDa gelatinase ($^{125}$I-TIMP-2: $^{125}$I-TIMP-1, 10). These results indicate that the inhibitory capability of TIMP-1 and TIMP-2 against gelatinases probably derives from their relative binding to the active sites of these proteinases and does not involve binding to a second site involved in stabilization.

Collagen degradation experiments were performed with human interstitial collagenase activated with APMA. This enzyme preparation showed only collagenase activity when analyzed by zymography but was not homogeneous when visualized on Coomassie-stained gels (Fig. 1). Nevertheless, the relative inhibitory capabilities of TIMP-1 and TIMP-2 could be determined. Both TIMP-1 and TIMP-2 were preincubated with collagenase, followed by incubation with diffuse collagen fibrils to determine remaining collagenolytic activity (Fig. 5). TIMP-1 was the preferred collagenase inhibitor by >2-fold.

**DISCUSSION**

It is apparent that a complex cascade of serine proteinases and metalloproteinases is used by cells to influence their surroundings, and especially to effect connective tissue turnover. Proteinase inhibitors serve to control this influence. To date, studies on metalloproteinase inhibitors have focused on TIMP-1. It now appears that another inhibitor, TIMP-2, inhibits gelatinases more effectively than does TIMP-1.

Like TIMP-1, TIMP-2 forms stable complexes with target metalloproteinases. These complexes are not covalent, as is the case with macromolecular serine proteinase inhibitors. In fact, dissociation of TIMP-1 or TIMP-2 from bound proteinase yields fully active inhibitor and proteinase (30). Both TIMP-1 and TIMP-2 are stable to heat, SDS, and several organic solvents; however, reducing agents destroy their activity. Sequence analysis of TIMP-like inhibitors shows the conservation of the 12 cysteine residues thought to be involved in intramolecular disulfide bonds (31, 32). While TIMP-1 contains two sites of glycosylation, however, TIMP-2 has none. This explains why TIMP-1 is also able to bind several lectins, whereas TIMP-2 is not. The reported sequences of
TIMP-1 and TIMP-2 show 41% homology (32), suggesting that, while not identical, these proteins may share similar domains. Many of their properties indicate that this may be so. However, their immunologic cross-reactivity appears minimal. Anti-bovine cartilage-derived inhibitor (a gift of Gillian Murphy, Strangeways Laboratory, Cambridge, United Kingdom), raised against bovine TIMP-2, recognizes human TIMP-2 but not TIMP-1. Similarly, anti-TIMP-1 does not precipitate TIMP-2 (35).

In fibroblasts, the 72-kDa progelatinase copurifies as a stable gelatinase-TIMP-2 complex (34). Activation of this complex with APMA yields active enzyme, although it appears that TIMP-2 remains bound at some site other than the active site. Removal of TIMP-2 from progelatinase results in an autocatalytic activation of gelatinase via cleavage of the propeptide at its amino terminus, as well as cleavage at the carboxyl terminus, which results in the active 42.5-kDa fragment. Our earlier results indicated that TIMP-2, but not TIMP-1, was able to form a complex with 72-kDa progelatinase in the presence of 1,10-phenanthroline, an inhibitor of metalloproteinases. We hypothesize that TIMP-2 acts to stabilize 72-kDa gelatinase. In the present study, we analyzed the relative inhibitory capability of TIMP-1 and TIMP-2 against several forms of human 72-kDa gelatinase, and in all cases TIMP-2 was more effective than TIMP-1 at inhibiting gelatinolysis. This relative effectiveness was most pronounced against the 42.5-kDa active fragment of 72-kDa gelatinase, which results from the autolytic activation of 72-kDa progelatinase when it is separated from TIMP-2. Why inhibition by TIMP-2 is especially superior to TIMP-1 in the case of this fragment is not understood. Autoactivation of 72-kDa gelatinase results in several peptides representing all the domains of the holoenzyme. Previous results indicated that only TIMP-2, but not TIMP-1, could bind to 72-kDa gelatinase in the presence of 1,10-phenanthroline. This suggests that isolated gelatinase may bind TIMP-2 at more than just the active site, whereas TIMP-1 would bind only the active site. Because TIMP-2 is able to bind 72-kDa gelatinase at two sites, the kinetics of inhibition by TIMP-2 are complex, as seen in Fig. 2B. It is likely that much of the TIMP-2 incubated with autoactivated 72-kDa gelatinase was able to bind to the putative stabilization site, which would result in less TIMP-2 binding to the active site. Knowledge of the binding affinities of each site would help clarify these interactions.

It has been reported that 92-kDa progelatinase copurifies with TIMP-1 (11). We have observed the copurification of some TIMP-1 with 92-kDa gelatinase, but it is not clear if TIMP-1 is acting as a stabilizer, as is the case with TIMP-2 and 72-kDa progelatinase. Incubation of 92-kDa gelatinase with APMA produced a gelatinolytically active protein that was preferentially inhibited by TIMP-2 rather than TIMP-1. This result is substantiated by evidence that activated 92-kDa gelatinase binds preferentially to \(^{125}\)I-TIMP-1 rather than \(^{125}\)I-TIMP-1 when it is incubated with equal quantities of each inhibitor. The role of the association of TIMP-1 with 92-kDa progelatinase remains to be established.

The data presented here suggest that TIMP-2 is likely to be the physiologic inhibitor of 72-kDa gelatinase, and possibly of 92-kDa gelatinase, whereas TIMP-1 is the primary collagenease inhibitor. These data suggest that in conditioned medium of several cell types there exist at least two similar but functionally distinct metalloproteinase inhibitors that preferentially interact with different target proteinases. Other inhibitors have been identified by reverse zymography (19, 35). If these inhibitors are shown to be unique, then their specificity toward a range of proteinases might further indicate the specific role or roles each inhibitor plays in regulating tissue degradation. The availability of the various metalloproteinases may play a significant role in proteinase regulation. It is not clear to what degree the various inhibitors are complexed to different proteinases, both active and zymogen. Regulation of proteolysis might be a function of the relative biosynthetic rates of proteinases and protease inhibitors. In the case of 72-kDa gelatinase, a minor change in the synthesis of TIMP-2 might have significant effects on the activity of the proteinase. These data support the contention that the regulation of matrix metalloproteinase activity is achieved by members of a family of specific metalloproteinase inhibitors consisting of at least TIMP-1 and TIMP-2.

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