Tissue Inhibitor of Metalloproteinases-4 Inhibits But Does Not Support the Activation of Gelatinase A via Efficient Inhibition of Membrane Type 1-Matrix Metalloproteinase

Heather F. Bigg, Charlotte J. Morrison, Georgina S. Butler, Marie A. Bogoyevitch, Zhiping Wang, Paul D. Soloway, and Christopher M. Overall

Faculty of Dentistry and Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; [H. F. B., C. J. M., C. M. O.]; Department of Biochemistry, University of Western Australia, Nedlands, Perth, W.A. 6007 Australia [M. A. B.]; and Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263 [Z. W. P. D. S.]

ABSTRACT

The tissue inhibitors of metalloproteinases 1–4 (TIMPs) have discrete regulatory roles in the activation of matrix metalloproteinase (MMP)-2 (gelatinase A), an important basement membrane-degrading MMP pivotal to tumor metastasis and angiogenesis. TIMP-2 binds to both the hemopexin C domain of progelatinase A and the active site of membrane type-1 (MT1) MMP. This trimeric complex presents the cell surface-bound gelatinase Azymogen to a free MT1-MMP molecule for activation. To investigate the role of TIMP-4 in the activation process, we developed a new procedure for the expression and purification of recombinant human TIMP-4 from baby hamster kidney cells. The recombinant TIMP-4 was a potent inhibitor of gelatinase A (apparent $K_i$ for $V_{app}$, $i$ is the rate constant), 9 ps; $k_{on}$ (association rate constant), 4.57 $\pm$ 0.13 $10^{9}$ m$^{-1}$s$^{-1}$) and was less dependent upon hemopexin C domain interactions than TIMP-2 in its mode of binding and inhibition. Unlike TIMP-1, TIMP-4 strongly inhibited MT1-MMP ($K_i$ for $V_{app}$, $i$ is the rate constant), 100 ps; $k_{on}$ 3.49 $\pm$ 0.34 $10^{6}$ m$^{-1}$s$^{-1}$) and blocked the concanavalin A-induced cellular activation of gelatinase A. In concanavalin A-stimulated homozygous $Timp2$ mice, fibroblasts or unstimulated MT1-MMP-transfected $Timp2$ cells, which cannot activate gelatinase A, was restored by the addition of 0.3–5 nM TIMP-2 but not by TIMP-4, unequivocally showing the TIMP-2 dependency of MT1-MMP-induced activation of gelatinase A and the fact that TIMP-4 cannot support activation. The dominance of TIMP-2 in the activation process was further supported by the preferential binding of TIMP-2 compared with TIMP-4 to the hemopexin C domain of progelatinase A in inhibitor mixtures and by the ability of TIMP-2 to displace TIMP-4 from the hemopexin C domain. Hence, TIMP-4 regulates gelatinase A activity by efficient inhibition of MT1-MMP-mediated activation and by inhibiting the activated enzyme and, thus, is a tumor resistance factor in the peritumor stroma.

INTRODUCTION

MMP$^1$ activity enhances tumor growth, metastasis, and neovascularization (1), playing pivotal roles in the degradation of extracellular matrix and processing of biologically active molecules (2, 3). MMP activity is regulated by four homologous TIMPs, TIMP-1 (4, 5), TIMP-2 (6, 7), TIMP-3 (8), and TIMP-4 (9–11). The TIMPs are tight activity is regulated by four homologous TIMPs, TIMP-1 (4, 5), TIMP-2 (6, 7), TIMP-3 (8), and TIMP-4 (9–11). The TIMPs are tight association and inhibition is improved by contacts with stabilization sites on the hemopexin C domain (12). In crude assays, the TIMPs are largely interchangeable in their capacities as MMP inhibitors except for TIMP-1, which is a poor inhibitor of MT1-MMP (17). Subtle differences in inhibition are detected by kinetic analysis, but such studies of TIMP-4 have not been reported, attributable in part to difficulty in purifying this protein.

TIMP-2 expression is mainly constitutive (18, 19), whereas TIMP-4 shows a highly restricted tissue distribution. cDNA encoding TIMP-4 was first cloned from a human heart cDNA library (9), and TIMP-4 appears to be normally expressed at high levels only in the heart (9, 10) but is induced upon vascular injury (20) and is an angiogenesis inhibitor (21). TIMP-4 expression is also high in peritumor stroma (11), indicating an important role in regulating tumor progression. Association with the MMPzymogens is TIMP-specific. TIMP-2 binds to progelatinase A (MMP-2) and facilitates enzyme activation (6, 22–24); TIMP-1 binds to progelatinase B (MMP-9; Ref. 25), whereas TIMP-3 binds to both (26). We have shown previously (27) that TIMP-4 can bind to progelatinase A, but its function in this complex is unknown.

Activation of progelatinase A is a cell membrane-mediated process (28–30) in which the activator has been identified as MT1-MMP (31, 32). Active MT1-MMP binds the TIMP-2 NH$_2$-terminal domain in an inhibitory complex (33) that functions as a receptor for the 72-kDa progelatinase A. A trimeric complex then forms on the cell surface by an interaction between the TIMP-2 C domain and the hemopexin C domain of progelatinase A (30, 32–36). This enhances the rate of proenzyme cleavage at Arg$^16$-Leu$^{18}$ by free MT1-MMP to generate a 68-kDa intermediate form (17, 28, 30, 34). The fully activated 66-kDa gelatinase A is then autocatalytically generated in vivo using TIMP-2 knockout mice, but the role of TIMP-4 in gelatinase A activation and in regulating MT1-MMP activity is unknown.

Previously (27), we demonstrated the specific binding of TIMP-4 to progelatinase A on the hemopexin C domain leading us to postulate that TIMP-4 might also play a role in regulating activation. Dissecting the relationship of TIMP-4 and TIMP-2 and their roles in gelatinase A activation is important in understanding tumor cell invasion and angiogenesis where both inhibitors are present in the stroma (11, 18, 21, 42). To understand the regulatory role of TIMP-4 in the activation process, it is critical to determine its MT1-MMP-binding, kinetic, and inhibitory properties. If TIMP-4 inhibits MT1-MMP, TIMP-4 may block activation or potentially support this process, like TIMP-2, within a critical concentration range. Alternatively, if the inhibitory properties of TIMP-4 were similar to TIMP-1, which does not inhibit...
MT1-MMP (17), TIMP-4 would have little role in regulating gelatinase A activation in tumor cell invasion and angiogenesis. We report in this study that TIMP-4 inhibits the TIMP-2-mediated cellular activation of gelatinase A by efficiently inhibiting MT1-MMP. However, TIMP-4 does not form an alternative trimeric complex and so is unable to support MT1-MMP-mediated activation of progelatinase A.

**MATERIALS AND METHODS**

**Antibodies.** Rabbit polyclonal α-C-TIMP-4, α-C-TIMP-2, and α-N-TIMP-2 antipeptide antibodies were raised and affinity-purified against the following synthetic peptides: CCGGLRKEFIDVQ-CONH₂, CCGAPPKQEFLIED-CONH₂, and Ac-AGKAEAGDGKMHITLC, respectively, using methods described previously (39). Underlined residues represent a double glycine spacer and terminal cysteine for peptide coupling. The α-C-TIMP-4 and α-N-TIMP-2 antibodies did not cross-react with TIMP-2 or TIMP-4, respectively (data not shown). A rabbit antipeptide antibody, α-C-TIM1-MMP, has been characterized previously (39), and a mouse monoclonal α-N-TIM1-MMP antipeptide antibody (MAB918; R&D Systems, Inc.) and rabbit mTIM1-MMP antibody (AB815; Chemicon International, Inc.) were purchased.

**Recombinant TIMP-4 Expression Vector.** The coding region of hTIMP-4, including the signal sequence and stop codon, was amplified from cDNA of hTIMP-4 in pBluescript (kindly supplied by Dr. E. Shi, Department of Pediatrics, Albert Einstein College of Medicine, New York, NY; Ref. 9) by PCR using the primers 5'-AGTTACGGGCCCAGCTGGGAAGCCCTGGGCCC-3' (5' primer) and 5'-AGTTAGGCAGCTCTACTGGACTGGAACGATGTC-3' (3' primer), which added ApaI restriction enzyme sites to both extensions. The 702-bp amplicon was gel-purified, digested with ApaI, ligated into the mammalian expression vector pNUT (42), and then fully sequenced.

**Expression of Recombinant TIMP-4 from Baby Hamster Kidney Cells.** pNUT/TIMP4 or pNUT vector (as control) were linearized with EcoRI and transfected into baby hamster kidney cells by electroporation. Methotrexate (50–500 μM; Faulin, Inc.) resistant foci were picked 27 days after transfection, expanded, and screened for TIMP-4 production by Western blotting. A hTIMP-4 standard was kindly provided by Dr. E. Shi (11). For production of recombinant protein, typically 12 confluent roller bottle cultures were incubated in DMEM/Ham’s F-12 (Life Technologies, Inc.) with 1% (v/v) newborn bovine serum (Life Technologies, Inc.) with or without 2 mM sodium butyrate (Sigma Chemical Co.). The conditioned medium was collected every 2–3 days for 15 days and clarified by centrifugation, and then NaN₃ and phenylmethasulfonfyl fluoride were added to 0.02% (v/v) and 1 mM, respectively.

**Purification of Recombinant TIMP-4.** Protease inhibitor tablets (Boehringer Mannheim), EDTA (1 mM), and benzamidine HCl (1 mM) were added to the TIMP-4-containing conditioned medium, which was then applied to a Red Sepharose CL-6B column (Amersham Pharmacia Biotech; total column bed volume (Vₜ) = 50 ml) equilibrated in PBS [0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 1.5 mM KH₂PO₄ (pH 7.4)] followed by washes with PBS. All of the purification buffers contained 1 mM EDTA, 1 mM benzamidine HCl, and 0.02% (v/v) NaN₃, unless otherwise stated. TIMP-4 was eluted first with 4 mM NaCl, phosphate buffer (2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 1.5 mM KH₂PO₄ (pH 7.4)) followed by 0.5 M NaCl, 0.1 M Tris-HCl (pH 8.5). The pH 8.5 eluate was dialed at 4°C against 4 mM NaCl, phosphate buffer (pH 7.4). Both eluates were then separately applied to a phenyl Sepharose CL-4B column (Amersham Pharmacia Biotech; Vᵣ = 50 ml) equilibrated in the same buffer, followed by buffer washes. The column was eluted in three steps, first with 4-fold diluted buffer, then with distilled water, and finally with PBS containing 20% (v/v) DMSO. The TIMP-4-containing 20% DMSO eluate was.govdialed at 4°C against 2-fold diluted PBS, 0.05% (w/v) Brij 35, and then applied to a Q Sepharose Fast Flow column (Amersham Pharmacia Biotech; Vᵣ = 5 ml) equilibrated in this buffer. Flow-through fractions that contained the TIMP-4 were pooled and dialed at 4°C against 0.5 M NaCl, 0.05% (w/v) Brij 35, phosphate buffer (pH 7.4) without EDTA and applied to a Zn²⁺-charged chelating Sepharose Fast Flow column (Amersham Pharmacia Biotech; Vᵣ = 1 ml). The column was washed with this buffer and then with 1 M NaCl, phosphate buffer (pH 6.0; without EDTA), and the TIMP-4 was eluted with 50 mM EDTA in PBS, 0.05% (w/v) Brij 35. After dialysis at 4°C against PBS, 0.05% (w/v) Brij 35, the purified protein was stored at −70°C. For cell assays, Brij 35 was removed using Extracti-Gel D (Pierce) minicolumns. Protein yields were calculated by the BCA assay (Pierce) and by spectroscopy using an extinction coefficient determined from the absorbance of protein samples measured using a Cary 219 spectrophotometer immediately before amino acid analysis (n = 3) using norleucine as an internal standard.

**Electrophoretic Techniques.** Proteins were analyzed by SDS-PAGE on 13% polyacrylamide gels stained with Coomassie Brilliant Blue R-250 or silver nitrate. Samples analyzed by zymography were electrophoresed nonreduced on 10% polyacrylamide gels copolymerized with 1 mg/ml or 160 μg/ml gelatin. Reverse zymography (43) and Western blotting were performed as described previously (39). TIMP-1, TIMP-2, and TIMP-3 protein standards were kindly provided by Dr. D. R. Edwards (University of East Anglia, Norwich, United Kingdom).

**Cell Culture.** Primary fibroblasts were prepared from Timp2 homozygous (-/-) and heterozygous (+/-) 13.5-day mouse embryos after removal of the head and internal organs (40). Cells were maintained in DMEM with 10% (v/v) FBS (Hyclone Laboratories, Inc.) to passage 2. Myocytes were dissociated from the ventricles of neonatal (1 to 2-day-old) Sprague Dawley rat hearts (44). To remove fibroblasts, the cells were preplated for 30 min in DMEM/Medium 199 (4:1, v/v) supplemented with 10% (v/v) equine serum, 5% (v/v) FBS, and antibiotics. The unattached myocytes were then plated at 4 × 10⁵ cells/25-cm² flasks and maintained in DMEM/Medium 199 (4:1, v/v), 20% (v/v) equine serum, 10% (v/v) FBS, and antibiotics with 0.1 mM bromodeoxyuridine added to prevent proliferation of any fibroblasts.

**Recombinant TIMP-2, Gelatinase A, and MT1-MMP Expression.** Human TIMP-2 was expressed in CHO-K1 cells (American Type Culture Collection) using the pGWI1GH mammalian expression vector (generously provided by Dr. J. C. Clements, British Biotech Pharmaceuticals Ltd., Oxford, United Kingdom). Transfected cells were grown in roller bottles in CHO-S-SFM medium (Life Technologies, Inc.) with the medium harvested every second day. Conditioned medium was loaded onto a Reactive Green 5 column (Vᵣ = 30 ml; Sigma Chemical Co.), washed in PBS, and eluted in 0.5 M NaCl, phosphate buffer (pH 7.6). The eluate was adjusted to 1.0 M NaCl and loaded onto gelatin Sepharose 4B (Vᵣ = 10 ml; to remove gelatinases) and phenyl Sepharose CL-4B (Vᵣ = 30 ml) columns (Amersham Pharmacia Biotech) in tandem. The columns were washed in 1.0 M NaCl, phosphate buffer (pH 7.6), and the TIMP-2 was eluted from the phenyl Sepharose in phosphate buffer (pH 7.6) and applied to a heparin Sepharose column (Vᵣ = 10 ml). TIMP-2 was collected in the flow-through fractions. Protein purity was assessed by silver staining, amino acid analysis, and mass spectroscopy, and the extinction coefficient was calculated.5 Functionality was confirmed by reverse zymography.

**TIMP-2-free gelatinase A was prepared from Ras-transformed Timp2 −/− cells (40) transfected with human gelatinase A cDNA in pGWI1GH.6 Vaccinia-expressed human progelatinase A and N-gelatinase A lacking the hemopexin domain were generously supplied by Dr. R. Fridman, Department of Pathology, Wayne State University, Detroit, MI (45). Importantly, these sources of enzyme were TIMP-2-free and entirely in the latent form. Human MT1-MMP was expressed on the surface of Ras-transformed Timp2 −/− cells after transfection with the hMT1-MMP cDNA in pGWI1GH. Stable, transfected cells were selected in 25 μg/ml mycophenolic acid. Clones expressing MT1-MMP on the cell surface were identified by flow cytometry using antihuman MT1-MMP (AB815)5 specific antibody. A soluble form of fully active human MT1-MMP (sMT1-MMP) comprised of the catalytic and hemopexin C domains but lacking the transmembrane domain was generously provided by Dr. J. C. Clements. Recombinant hemopexin C domains of human MT1-MMP (39) and gelatinase A (46) were expressed in Escherichia coli with an NH₂-terminal (His)₉ tag and purified as described previously (39, 46).

**Measurement of the Association Rate Constant (kₐ)** Concentrations of 2 mM p-aminophenylmercuric acetate-activated gelatinase A and N-gelatinase A were determined by active-site titration against a standard preparation of TIMP-1 (kindly provided by Prof. G. Murphy, University of East Anglia, Norwich, United Kingdom). MT1-MMP, gelatinase A, or N-gelatinase A were inhibited by appropriate concentrations of TIMP-1, TIMP-2, or TIMP-3 in fluorometry assay buffer (0.1 M NaCl, 0.1 M Tris-HCl, 10 mM CaCl₂, and 0.05% (w/v) Brij 35 (pH 7.5)) at 37°C for TIMP-1-MMP or at 25°C for gelatinase A (33). Activity was monitored using 1 μM quenched

---

5 C. J. Morrison, H. F. Bigg, G. S. Butler, and C. M. Overall, unpublished data.
6 G. S. Butler, H. F. Bigg, C. J. Morrison, and C. M. Overall, unpublished data.
flourescent peptide (7-methoxycoumarin-4-ylacetyl-Pro-Leu-Gly-Leu-3-(2,4-dinitrophenyl)-2,3-diaminopropliny)-Ala-Arg-NH$_2$ (kindly supplied by Dr. C. G. Knight, Cambridge University, United Kingdom; Ref. 33). The $k_{\text{on}}$ was calculated from the progress curves (47), measured using a LS50B fluorescence spectrophotometer (Perkin-Elmer; Ref. 33). Assays were typically performed three to four times. For competition assays, increasing concentrations of the recombinant gelatinase A hemopexin C domain were added with the enzyme, and the $K_{\text{m}}$ was measured (26).

Estimation of the Apparent Overall Inhibition Constant $[K_{\text{app},i}]$.

1. sMT1-MMP (0.25 $\mu$M) was incubated with DMEM, and then 0.005–52.1 nM TIMP-2 or TIMP-2, or PBS was added in DMEM, with or without 20 $\mu$g/ml ConA (Sigma Chemical Co.; Ref. 28), and 1 mg/ml ovalbumin (as carrier) in a final volume of 100 $\mu$L. Conditioned medium was removed 36–65 h later, and the cell layers were washed twice with PBS and solubilized in 130 $\mu$L of 2.0 M urea, 2% (w/v) SDS, and 0.125 M Tris-HCl (pH 6.8) for analysis by zymography.

2. Residual enzyme activity was measured for 2 h at 4°C and then applied to gelatin Sepharose minicolumns (Molecular Devices) using a 320-nm and 405-nm filter pair. $K_{\text{app},i}$ was estimated by fitting a plot of rate on TIMP concentration to a tight binding equation (12). Assays were typically repeated 3–5 times.

Cell Assays. $T_{\text{app}}$ $\rightarrow$ $t$ and $+/-$ primary embryonic fibroblasts or primary rat ventricular myocytes were seeded into 96-microwell tissue culture plates at 4°C for 2 h with a limiting quantity of progelatinase A (0.3 $\mu$g) pretreated with TIMP-2-free gelatinase A in DMEM (prepared from gelatinase A-transfected cells and then incubated with TIMP-2-free gelatinase A in DMEM (prepared from gelatinase A-transfected cells and then expressing human MT1-MMP on the cell surface were seeded at 2.5 × 10$^4$ cells/well. After a 24-h incubation, cells were washed 2× with PBS and then incubated with TIMP-2-free gelatinase A in DMEM (prepared from gelatinase A-transfected $T_{\text{app}}$ $→$ $t$ cell) without serum or ConA. Alternatively, TIMP-4 or TIMP-2 (0.003–81 nM) was added with TIMP-2-free gelatinase A. Medium was harvested after 24 h and analyzed by zymography.

Affinity Chromatography. To characterize TIMP binding to progelatinase A, 1.5 $\mu$g of proenzyme in PBS buffer (PBS, 0.05% (w/v) Brij 35, and 0.02% Na$_2$EDTA (w/v)) was added to 0.25 $\mu$g of TIMP-4 or TIMP-2 in a final volume of 50 $\mu$L (molar ratio of progelatinase A:TIMP $≈ 2:1$). The mixtures were incubated for 2 h at 4°C and then applied to gelatin Sepharose minicolumns ($V_{\text{i}} = 20 $µL). Bound proteins were eluted with 10% DMSO in PBS buffer and analyzed by silver-stained SDS-PAGE gels and Western blotting.

The ability of TIMP-4 to form a trimeric complex with progelatinase A and MT1-MMP was determined by affinity chromatography. Progelatinase A (1.5 $\mu$g) was incubated with TIMP-4 (0.25 $\mu$g) in PBS buffer at 4°C for 2 h. sMT1-MMP (0.25 $\mu$g) was then added, and the 50-$\mu$L mixtures were incubated for 2 h at 4°C (molar ratio of progelatinase A:TIMP:sMT1-MMP $≈ 2:1:0.5$). Alternatively, sMT1-MMP was incubated first with TIMP-4, followed by incubation with progelatinase A. The progelatinase A, TIMP-4, sMT1-MMP mixtures were applied to gelatin Sepharose minicolumns. After column washes, gelatin A and gelatin A-bound proteins were eluted with 10% DMSO in PBS buffer. In parallel experiments, TIMP-2 was substituted for TIMP-4. TIMP-4, TIMP-2, and sMT1-MMP alone did not bind gelatin Sepharose.

To examine the TIMP binding preference of progelatinase A in the presence of both inhibitors, a TIMP-4 and TIMP-2 mixture (0.1 $\mu$g each) was incubated at 4°C for 2 h with a limiting quantity of progelatinase A (0.3 $\mu$g) pretreated with 1,10-phenanthroline (to prevent binding to the active site of any small metalloproteinase, Ref. 48) in a final volume of 50 $\mu$L. The mixture was added to gelatin Sepharose minicolumns equilibrated in PBS buffer, 0.5 mm 1,10-phenanthroline and then washed before elution with 10% DMSO, 0.5 mm 1,10-phenanthroline in PBS buffer. Chromatography fractions were analyzed by Western blotting with Co-C-TIMP-4 and Co-C-TIMP-2 antibodies.

To assess the ability of TIMP-2 to displace TIMP-4 from progelatinase A, the mixture (1.5 $\mu$g) was applied to gelatin Sepharose minicolumns ($V_{\text{i}} = 150 $µL) in PBS, 0.5 mm 1,10-phenanthroline. The columns were washed, and 10-fold molar excess of TIMP-4 (5 $\mu$g) was applied and incubated for 2 h. After thorough washes, 10-fold molar excess of TIMP-2 (5 $\mu$g) was applied. To the control column, PBS alone was added. After column washes, bound proteins were eluted in 10% DMSO and analyzed as above.

**Solid Phase Binding Assays.** Gelatinase A hemopexin C domain (0.42 $\mu$M) was preincubated with TIMP-4 (0.054–5.4 $\mu$M) in 100 $\mu$L of PBS for 1–2 h at room temperature and then added to TIMP-2-coated microwell plates (0.13 $\mu$g of TIMP-2/well) for 1 h. After washing with PBS, 0.05% (v/v) Tween 20, the bound gelatinase A hemopexin C domain was quantitated using affinity purified C-TIMP-2 antibodies (39, 46).

**RESULTS**

Expression and Purification of Recombinant TIMP-4. A single ~24-kDa band that comigrated with a hTIMP-4 standard was detected by Western blotting with Co-C-TIMP-4 antibody in conditioned medium from pNUThtTIMP-4-transfected, but not vector-transfected, baby hamster kidney cells (data not shown). Protein expression and cell maintenance were markedly improved by 1% newborn bovine serum and the general transcription stimulator, sodium butyrate. Developing an optimized purification scheme for TIMP-4 proved extremely difficult. Red Sepharose separated TIMP-4 from the majority of the serum proteins, and many of the remaining impurities were removed by phenyl Sepharose such that TIMP-4 was the major protein in the 20% DMSO eluate. Brij 35 was included in all of the buffers after phenyl Sepharose chromatography, without which considerable losses of protein occurred. Q Sepharose improved purity with the final Zn$^{2+}$-chelating Sepharose column mainly being used to concentrate the purified protein. Without protease inhibitors, degration of TIMP-4 occurred, giving rise to a 23-kDa (reduced) fragment. This band was detectable by reverse zymography but not by Western blotting with Co-C-TIMP-4, showing that cleavage occurred in the C domain (data not shown). The yield of purified TIMP-4 was 0.65 mg/liter of conditioned culture medium. Purified TIMP-4 electrophoresed as a single major band with an apparent molecular mass of 24–25 kDa (reduced) or 21–21.5 kDa (nonreduced; Fig. 1A) consistent with the presence of disulfide bonds in the protein. Western blotting with Co-C-TIMP-4 confirmed the identity of the protein as TIMP-4 with an intact COOH-terminal tail and the absence of dimers (Fig. 1B). Reverse zymography revealed a prominent inhibitory band at ~21 kDa (Fig. 1C), thereby confirming the protein as a functional MMP inhibitor. The molar extinction coefficient of TIMP-4 was determined to be 2.60 × 10$^4$ M$^{-1}$ cm$^{-1}$.

**TIMP-4 Efficiently Inhibits Gelatinase A and MT1-MMP.** TIMPs are extremely tight binding MMP inhibitors, and the difficulties in determining accurate dissociation constants for the interaction between TIMPs and full-length MMPs are compounded because of the effect of multiple domain interactions (13, 14, 47). We estimated the overall inhibition constant, the apparent $K_{\text{on}}$, of TIMP-4 for gelatinase A to be $≈ 9$ pm, which is consistent with estimates for TIMP-1 and TIMP-2 reported here (Table 1) and previously (12, 15, 47). A more precise value of $K_{\text{on}}$ for the inhibition of gelatinase A by the three TIMPs could not be determined because the standard fluorescence assay is not sensitive enough to measure MMP activity at fs enzyme concentrations that are below these apparent $K_{\text{on}}$s. Indeed, the actual $K_{\text{on}}$ of the active gelatinase A/TIMP-2 interaction is controversial with published values differing by several orders of magnitude (12–14). Nonetheless, the inhibition curves for TIMP-4 were similar to those obtained with TIMP-2, indicating that in biological systems TIMP-4 is as efficient an inhibitor of gelatinase A as TIMP-2. Indeed, the $K_{\text{on}}$ for TIMP-4 and gelatinase A of 4.57 ± 0.13 × 10$^8$ M$^{-1}$ s$^{-1}$ was of the same order of magnitude as for TIMP-2 and TIMP-1.

Because TIMP-1 is a poor inhibitor of MT1-MMP (17), we could perform the assays above the sensitivity limit and measure the $K_{\text{visapp}}$ of 147 nm for MT1-MMP (Table 1). Like TIMP-2, TIMP-4 is an
Fig. 1. SDS-PAGE, Western blot, and reverse zymographic analyses of purified recombinant human TIMP-4 and TIMP-2. A, purified TIMP-4 (1.8 μg/lane) reduced (+65 mM DTT; +DTT) or nonreduced (−DTT) was electrophoresed on a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue R-250. M, molecular mass markers (kDa). The TIMP-4 preparation was essentially pure with only minor contaminants present that were not immunoreactive with αc-TIMP-4 antibodies as shown on the Western blot in B. After electrophoresis on a 15% SDS-PAGE gel, TIMP-4 and TIMP-2 (100 ng/lane) samples were blotted as described under “Material and Methods.” C, reverse zymogram of purified recombinant TIMP-4 (TIMP-4) and TIMP-2 (TIMP-2; 50 ng/lane). The positions of TIMP-1, TIMP-2, and TIMP-3 standards are shown.

Table 1 Kinetic analysis of TIMP-4 inhibition of MT1-MMP and gelatinase A

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>TIMP-1</th>
<th>TIMP-2</th>
<th>TIMP-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{\text{on}} ) &amp; ±9 pm</td>
<td>±9 pm</td>
<td>±9 pm</td>
<td></td>
</tr>
<tr>
<td>( K_{\text{off}} ) &amp; 5.270 ± 0.462 nm</td>
<td>0.575 ± 0.079 nm</td>
<td>1.222 ± 0.224 nm</td>
<td></td>
</tr>
<tr>
<td>( k_{\text{app}} ) &amp; 147.084 ± 4.003 nm</td>
<td>±150 pm</td>
<td>±100 pm</td>
<td></td>
</tr>
</tbody>
</table>

The Role of the Hemopexin C Domain in TIMP-4 Inhibition

Efficient inhibitor of sMT1-MMP (Table 1). The \( K_{\text{app}} \) was ±100 pm for TIMP-4 and ±150 pm for TIMP-2. TIMP-4 associated with MT1-MMP \( (k_{\text{on}} = 3.49 ± 0.34 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \) slightly more rapidly than TIMP-2 \( (k_{\text{on}} = 1.83 ± 0.16 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \). Hence, TIMP-4 is a potent inhibitor of MT1-MMP like TIMP-2 and TIMP-3 (17). TIMP-4 also efficiently inhibited other MMPs, including MMP-7 and MMP-8, confirming its broad inhibitory properties.

The Role of the Hemopexin C Domain in TIMP-4 Inhibition

Kinetics. TIMPs form tight complexes with full-length active MMPs that are stabilized by contact with the hemopexin C domain of the enzyme (12, 15) at a site proposed to lie on the top rim of the domain at the junction of modules I and II juxtaposed to the active site (39). Indeed, removal of the hemopexin C domain markedly decreased the rate of association of active gelatinase A with TIMP-4 by two to three orders of magnitude. The \( k_{\text{on}} \) of TIMP-4 with N-gelatinase A was \( 8.44 ± 0.70 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) compared with \( 4.57 ± 0.13 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) for full-length gelatinase A, and the \( K_{\text{app}} \) was increased from ±9 pm to 1.22 ± 0.22 nm (Table 1). Hence, as found previously for TIMP-1, TIMP-2, and TIMP-3 (12, 15, 26), our data show that the hemopexin C domain of active gelatinase A also forms stabilizing contacts with TIMP-4.

The rate of association of TIMP-2 and gelatinase A \( (k_{\text{on}} = 7.02 ± 0.55 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \) was decreased dramatically in competition experiments by the addition of increasing amounts of recombinant hemopexin C domain (Fig. 2). The C domain of TIMP-2 binds the exogenous hemopexin C domain (12, 39, 47) at a cationic docking site at the junction of hemopexin modules III and IV (36), thus preventing docking and inhibition of full-length gelatinase A. Consistent with this, the \( k_{\text{on}} \) of TIMP-1, which does not bind gelatinase A, was unaffected by exogenous hemopexin C domain. Surprisingly, the \( k_{\text{on}} \) of TIMP-4 with gelatinase A was also unaffected (Fig. 2), even with 50 μM hemopexin C domain added (data point not shown), so a relative dissociation constant \( K_a \) for the interaction with the hemopexin C domain could not be calculated. Hence, the site involved in TIMP-4 docking to gelatinase A and the hemopexin C domain does not appear to be involved in binding and stabilization of TIMP-4 with the active enzyme. This shows that the mode of binding and inhibition of active gelatinase A by TIMP-4 differs from TIMP-2 and more resembles TIMP-1. Hence, the TIMP-4 inhibitory stabilization site on the hemopexin C domain of active gelatinase A is distinct from the TIMP-2/TIMP-4 docking site on the proenzyme.

TIMP-4 Blocks Cellular TIMP-2-mediated Progelatinase A Activation. Because TIMP-4 inhibits MT1-MMP, we investigated the effects of TIMP-4 on the cellular activation of gelatinase A. The addition of ConA to primary neonatal rat ventricular myocytes containing synchronously beating myocyte clusters or to TIMP2 heterozygous (+/−) primary embryonic fibroblasts resulted in efficient activation of endogenous gelatinase A to the fully active form (Fig. 3, A–D; + ConA, 0 mM TIMP). This effect was comparable to that seen with wild-type fibroblasts (41), thus TIMP-2 synthesis by the TIMP2 +/- cells is adequate to support gelatinase A activation. There was no effect on activation upon addition of up to ~5 nM TIMP-4 or TIMP-2, but higher concentrations of either TIMP-4 or TIMP-2 progressively inhibited this process. Conversion to the intermediate and fully active forms was almost completely abolished above ~30 nM TIMP-4 or TIMP-2 with a comparable effect seen in cell lysates (data not shown). Thus, TIMP-4 can block the TIMP-2-mediated activation of gelatinase A stimulated by ConA in cells that normally do not express TIMP-4 (fibroblasts) and in those that do (heart tissue myocytes) in a nonantigen-specific manner.

TIMP-4 Cannot Support Activation of Progelatinase A by TIMP2 −/− Cells. Over a precise concentration range that does not saturate all of the cell surface MT1-MMP molecules, we postulated...
that TIMP-4 might substitute for TIMP-2 and support progelatinase A activation by binding progelatinase to MT1-MMP. We investigated this using primary embryonic fibroblasts from homozygous (−/−) TIMP-2-deficient mice (40) where activation via TIMP-2 can be unequivocally excluded. These cells are unable to activate progelatinase A upon ConA stimulation without the addition of exogenous TIMP-2 (40, 41). Gelatinase A in the medium of unstimulated Timp2 −/− cells was in the 72-kDa latent form (Fig. 3E and F; 0 nM TIMP− ConA). Upon ConA stimulation, some 68-kDa-activation intermediate was generated, even in the absence of TIMP-2 (Fig. 3E, F; 0 nM TIMP− ConA), but the 66-kDa fully active form was never observed. Gelatinase A activation did not occur at any concentration of TIMP-4 used (Fig. 3E) and above 5 nM TIMP-4, the low level conversion of progelatinase A to the 68-kDa activation intermediate was inhibited. This is in stark contrast to 5 nM TIMP-2 (Fig. 3F) where maximal activation of gelatinase A was observed. Higher amounts of TIMP-2 dose-dependently inhibited cleavage to the 68-kDa intermediate and 66-kDa active forms, both of which were completely absent at more than 75 nM TIMP-2. This was consistent with the Timp2 +/- cell studies (Fig. 3D) and analysis of the cell lysates (data not shown), confirming that TIMP-4 was unable to substitute for TIMP-2 in the activation process.

The identity of the MT-MMP in the Timp2 −/− cells responsible for progelatinase A activation upon addition of TIMP-2 is uncertain because ConA can stimulate the expression of multiple MT-MMPs. To analyze the role of TIMP-4 and TIMP-2 specifically in MT1-MMP, we investigated whether TIMP-4 might substitute for TIMP-2 and support progelatinase A activation by binding progelatinase to MT1-MMP. We investigated the activation deficiency, the binding of TIMP-4 and TIMP-2 to recombinant proenzyme in solution by zymography. The positions of the proenzyme and fully active gelatinase are indicated. In the heart myocytes, the upper endogenous gelatinase B band is shown.

activation of either endogenous or exogenous TIMP-2-free progelatinase A by the MT1-MMP transfectants was observed over an extended TIMP-4 concentration range (Fig. 4C), even when 800 nM TIMP-4 was added (data not shown). This confirmed the primary embryonic fibroblast Timp2 −/− results (Fig. 3), hence unequivocally showing that TIMP-4 cannot substitute for TIMP-2 in progelatinase A activation by MT1-MMP.

Binding of TIMP-4 to Progelatinase A and MT1-MMP. With our previous demonstration that TIMP-4 binds progelatinase A (27) and our present work showing that TIMP-4 binds and inhibits sMT1-MMP, it appeared that TIMP-4 has all of the potential binding properties necessary to form a ternary complex that would bridge MT1-MMP to progelatinase A, and yet TIMP-4 did not support gelatinase A activation. To investigate the activation deficiency, the binding of TIMP-4 and TIMP-2 to recombinant proenzyme in solution was compared by capturing the TIMP-progelatinase A complexes on gelatin Sepharose. Like TIMP-2 (Fig. 5B), when TIMP-4 was preincubated with a 2:1 molar excess of progelatinase A, TIMP-4 was eluted with the enzyme, but some TIMP-4 was also recovered in the gelatin Sepharose unbound and wash fractions (Fig. 5A). Because
TIMP-4 or TIMP-2 alone do not bind gelatin Sepharose (data not shown). TIMPs recovered in the gelatin Sepharose eluates represent progelatinase A-bound TIMP. The zinc chelator 1,10-phenanthroline binds the active site of activated enzyme and prevents TIMP complexation here (48) but not to the hemopexin C domain (24, 46). There was no effect of 1,10-phenanthroline on TIMP levels in the eluates (data not shown), confirming that TIMP-4 binds progelatinase A at sites distinct from the active site.

Next, we investigated the interactions of progelatinase A, TIMPs, and MT1-MMP using a soluble form of MT1-MMP. The sMT1-MMP preparation consisted of a predominant 45-kDa form and minor 29-kDa species (Fig. 5C). The molecular integrity of the 45-kDa sMT1-MMP was confirmed by recognition with both α5N-MT1-MMP and αC-C(MT1-MMP antibodies (Fig. 5C). When electrophoresed, nonreduced sMT1-MMP showed an increase in mobility compared with the reduced form, consistent with a disulfide-bonded hemopexin C domain. The 29-kDa forms represented fragments of the MT1-MMP catalytic domain because these were recognized by α5N-MT1-MMP, but not by αC-C(MT1-MMP, and did not show a reduction-sensitive shift in electrophoretic mobility (Fig. 5C).

Whether TIMP-2 was preincubated with progelatinase A, followed by sMT1-MMP, or with sMT1-MMP first, followed by progelatinase A, all of the TIMP-2 and almost all of the sMT1-MMP (both the full-length and catalytic domain forms) were recovered in the gelatin Sepharose DMSO eluate (Fig. 5D). Because sMT1-MMP alone did not bind to gelatin Sepharose (data not shown) and MT1-MMP does not bind gelatinase A (39), sMT1-MMP in the eluate represents a complex with progelatinase A and TIMP-2. Thus, TIMP-2 forms a trimolecular complex with progelatinase A and sMT1-MMP, regardless of the order of complex assembly.

When TIMP-4 was used in parallel experiments, sMT1-MMP was only found in the gelatin Sepharose unbound and wash fractions and never in the DMSO eluates with gelatinase A and TIMP-4 (Fig. 5E). This occurred whether TIMP-4 and progelatinase A were initially combined or if TIMP-4 was added first to sMT1-MMP. Because TIMP-4 inhibits sMT1-MMP (Table 1), the sMT1-MMP and TIMP-4 recovered in the unbound and wash fractions most likely represents a sMT1-MMP/TIMP-4 complex. Of note, less TIMP-4 was recovered bound to progelatinase A when sMT1-MMP and TIMP-4 were combined first, compared with when progelatinase A and TIMP-4 were combined first (Fig. 5E) or when sMT1-MMP was not present (Fig. 5A). This suggests that binding of TIMP-4 to sMT1-MMP prevents subsequent interaction with progelatinase A. Moreover, the TIMP-4/hemopexin C domain complexes must also differ from those of TIMP-2, because TIMP-4 binding precludes subsequent interactions of TIMP-4 with MT1-MMP, but TIMP-2 can simultaneously interact with both enzymes. Hence, TIMP-4 cannot form a trimolecular complex with progelatinase A and sMT1-MMP, which explains why TIMP-4 does not support gelatinase A activation.

**Binding of Progelatinase A to TIMP-4 in TIMP-2 Mixtures.** A possible role of TIMP-4 in gelatinase A activation may be to bind the hemopexin C domain and compete for TIMP-2 binding. To assess this, a limiting quantity of progelatinase A was found to bind only TIMP-2 and not TIMP-4 when presented with an equimolar mixture of the two TIMPs. All of the TIMP-4 was found in the gelatin Sepharose unbound fraction, whereas the TIMP-2 was recovered in
TIMP-2 competes for TIMP-4 binding to the gelatinase A hemopexin C domain and full-length progelatinase A. A, progelatinase A (0.3 μg) pretreated with 0.5 mM 1,10-phenanthroline was incubated with a mixture of 0.1 μg each of TIMP-4 and TIMP-2 (molar ratio, ~1:1:1) and applied to gelatin Sepharose (see “Materials and Methods”). A, TIMP sample applied; U, unbound material; W, column washes. Bound proteins were eluted with 10% (v/v) DMSO (DMSO). Chromatography fractions were analyzed by Western blotting using αC-TIMP-4, and then the membrane was stripped and reprobed with αC-TIMP-2. The positions of molecular mass markers in kDa are shown. B, gelatinase A hemopexin C domain was preincubated (2 h) with TIMP-4 at molar ratios of TIMP-4:hemopexin C domain of 12.9, 6.5, 2.6, 1.3, 0.64, 0.26, and 0.13, respectively. The mixtures were applied to a TIMP-2-coated ELISA plate for 1 h. Bound gelatinase A hemopexin C domain was detected using αHis6 antibody as described under “Materials and Methods.” C, TIMP-4 (5 μg) was applied to gelatin Sepharose columns loaded with progelatinase A (1.5 μg) in PBS containing 0.5 mM 1,10-phenanthroline and incubated for 2 h. After washing the columns free of unbound inhibitor, 10-fold molar excess TIMP-2 (5 μg) was applied to Column 2, and the column flow was stopped for a 2-h incubation, after which the drop through was collected (TIMP-2). To the control column (Column 1 Control), PBS without TIMP-2 was applied, and the drop through was collected (PBS). After PBS washes as indicated (WASH), bound proteins from both columns were eluted in 10% (v/v) DMSO (DMSO). Chromatography fractions were analyzed by Western blotting using αn-TIMP-4 (H. Kai and C. M. Overall, unpublished data) or αC-TIMP-2 antibodies as shown. The position of TIMP-4 and TIMP-2 standards (Stf) are indicated.

DISCUSSION

The high yield purification of human TIMP-4 has allowed a detailed analysis of the kinetic and progelatinase A activation properties of TIMP-4 for the first time. The kinetic analyses reported here show that TIMP-4, like TIMP-2, is a potent inhibitor of both gelatinase A (K_{i(app)} ≤ 9 pM) and MT1-MMP (K_{i(app)} ≤ 100 pM). The robust inhibition of MT1-MMP is supported by very recent collaborative cell studies with Toth et al. (49) showing that TIMP-4 inhibits vaccinio- overexpressed MT1-MMP autolysis. Consistent with the MT1-MMP inhibitory properties of TIMP-4 in biochemical assays, we found that TIMP-4 also inhibited the MT1-MMP-mediated initial cleavage of progelatinase A to the 68-kDa activation intermediate in cellular assays. In addition, because TIMP-4 inhibits MT1-MMPs like TIMP-2 and TIMP-3 (17, 26), our study reaffirms that TIMP-1 is unique with regard to sparing MT-MMPs.

The defect in progelatinase A activation in Timp2−/− cells and its restoration by the addition of exogenous TIMP-2 agrees with the recent data of Wang et al. (40) and Caterina et al. (41), who have both reported the generation of a Timp2−/− mouse. The absolute requirement for TIMP-2 in the cellular activation of progelatinase A by MT1-MMP, specifically, was unequivocally demonstrated here by generating stable MT1-MMP-transfected Ras-transformed Timp2−/− cells. TIMP-4 could not substitute for TIMP-2 in promoting gelatinase A activation in these cells nor could TIMP-4 promote activation by ConA-stimulated Timp2−/− embryonic fibroblasts. Notably, we found that ConA-stimulated Timp2−/− embryonic cells generated a small amount of the partially activated form of gelatinase A without any requirement for TIMP-2. It was the conversion from the 68-kDa activation intermediate to the fully activated 66-kDa form that we found to be totally TIMP-2-dependent. As discussed earlier, MT1-MMP makes the first cleavage in the prodomain of gelatinase A, with the final step being performed autocatalytically by active gelatinase A in trans. In agreement with Itoh et al. (37) and by hemopexin C domain competition studies (39), because the final intermolecular autocatalytic activation cleavage is absolutely TIMP-2-dependent, we infer this requires tethering of active gelatinase A to MT1-MMP by TIMP-2 on the plasma membrane, and cannot be efficiently performed by active TIMP-2-free gelatinase A in solution. Using Timp2−/− cells, proenzyme activation was stimulated only over a narrow concentration range of TIMP-2 (0.3 to 5 nM). Too little TIMP-2 did not support activation, and too much TIMP-2 inhibited the process as also found by Butler et al. (33) using hydroxymate inhibitor to deplete TIMP-2 from the cell layer. The comparable suppressive effects of TIMP-4 and TIMP-2 at higher concentrations on progelatinase A activation in Timp2−/− fibroblasts and heart myocytes suggest a similar mechanism of action. We propose that TIMP-4 blocks activation both by inhibition of cell-surface MT1-MMP (inhibiting the first cleavage step) and by inhibition of any cell-bound active gelatinase A (blocking the final cleavage).

Consistent with Murphy et al. (12), the K_{i(app)} for TIMP-2 with gelatinase A was ≤9 pm using the standard fluorometry method. Olson et al. (13) and Hutton et al. (14) measured k_{on} and k_{off}
Gelatinase A is very low and the discrepancy in these values has not been resolved. Thus, although the $K_i$ for TIMP-4 and gelatinase A is very low, it might still be higher than that of TIMP-2 and gelatinase A. Comparing $K_i$-gelatinase A with full-length gelatinase A, the more rapid association of TIMP-2 than TIMP-4 with N-gelatinase A reveals an inherent preference of TIMP-4 for binding to the gelatinase A catalytic domain. As with TIMP-1 and TIMP-2 (12, 15, 24), the principal interaction of TIMP-4 is with the catalytic domain. However, by forming stabilizing contacts with the bound TIMP-4, the hemopexin C domain of active gelatinase A markedly improves the rate of TIMP-4 association by two to three orders of magnitude with a similar improvement in the overall inhibition of the active enzyme.

Compared with TIMP-4, a preferred interaction of TIMP-2 with the hemopexin C domain of progelatinase A occurred, consistent with the slightly lower $K_i$ of TIMP-2 (27). Progelatinase A also preferentially bound TIMP-2 when exposed to an equimolar mixture of both TIMPs. TIMP-4 complexed to recombinant hemopexin C domain did not prevent subsequent TIMP-2 binding, and TIMP-4 was displaced by TIMP-2 from recombinant hemopexin C domain and progelatinase A. The structural basis of the less dominant TIMP-4 interaction may lie in the nature of the C domain of TIMP-4. TIMP-4 has a highly negatively charged COOH-terminal tail with four Asp/Glu residues that increases the rate of inhibition of gelatinase A (47) by initiating binding to a cationic docking site at the junction of hemopexin modules III and IV (36). Upon complex formation, the COOH-terminal tail is masked by the hemopexin C domain (39). However, the TIMP-4 tail has a net charge of only $-1$, which could contribute to the slower $k_{on}$ compared with TIMP-2. In the presence of both inhibitors in solution, this reduced dipole moment should favor the association of TIMP-2 with the hemopexin C domain of progelatinase A at the expense of TIMP-4. This is an important consideration in understanding the interactions that occur in tissues expressing both TIMP-4 and TIMP-2, such as the breast carcinoma/stromal interface (11) and during vascular injury (20) and angiogenesis (21).

A separate effect that may also be attributable to the nature of the TIMP COOH-terminal tails was revealed in the hemopexin C domain competition experiments. Recombinant hemopexin C domain could compete with full-length active gelatinase A for binding TIMP-2 (Fig. 2) and also competes for binding to TIMP-3, but the interaction is weaker (26). However, exogenous hemopexin C domain does not compete for binding to TIMP-1 or TIMP-4. TIMP-4 docks to progelatinase A on the hemopexin C domain like TIMP-2 and TIMP-3, but the lack of competition suggests that the mode of TIMP-4 binding the hemopexin C domain and inhibition of active gelatinase A is different. In particular, this suggests that, unlike TIMP-2, TIMP-4 binding to the TIMP-2/TIMP-4 docking site on the hemopexin C domain does not improve the rate of inhibition of active gelatinase A.

In contrast to TIMP-2, we found that TIMP-4 does not support gelatinase A activation. To understand the biological function of the gelatinase A activation complex and the dynamics of its assembly in mixed populations of TIMPs, it is important to understand the nature of this difference. Separately, all of the necessary binding interactions needed to assemble a gelatinase A trimolecular activation complex based on TIMP-4 could be formed; i.e., TIMP-4 binds the hemopexin C domain of progelatinase A, and TIMP-4 binds to and inhibits active MT1-MMP. However, TIMP-4 complexed with progelatinase A did not bind MT1-MMP, and TIMP-4 complexed with MT1-MMP did not bind progelatinase A, indicating that the sequence of binding was not a contributing factor. Indeed, in contrast with previous work (32), we could assemble the TIMP-2 trimolecular complex regardless of the order in which the components were combined. Thus, either the interaction of TIMP-4 with progelatinase A is not sufficiently strong to enable long-term stable complexes to form, or the tight interaction of MT1-MMP with TIMP-4 interferes with the binding of the complexed TIMP-4 to the hemopexin C domain of progelatinase A. This may be because of steric clashes or conformational changes induced upon binding. A definitive answer to this question is only likely to be attained with three-dimensional structures of the individual complexes.

Physiologically, the discrete binding properties of TIMPs regulate gelatinase A activation in a complex but elegant manner. TIMP-4 has a minor role because it cannot bind progelatinase A (24) and is a poor inhibitor of MT-MMPs (with the exception of MT4-MMP; Refs. 17, 50, 51), but it may inhibit the second, autocatalytic activation step, leading to an accumulation of the 68-kDa intermediate form (17, 30, 38). TIMP-2 is absolutely essential for the final activation cleavage step and, although we found that the initial step can occur independently of TIMP-2, possibly indicating a separate receptor, TIMP-2 accelerates this first cleavage by concentrating the proenzyme at the cell surface (33, 40, 41). TIMP-4 and TIMP-3 share some but not all of the characteristics of TIMP-2 in relation to progelatinase A activation; both bind to progelatinase A (26, 27) and are efficient inhibitors of MT1-MMP (17, 26, 27), but neither TIMP-3 (26) nor TIMP-4 supports progelatinase A activation. In addition, we have shown that TIMP-4 can block TIMP-2-mediated activation through inhibition of MT1-MMP. TIMP-3 might be expected to have a similar effect, but this has yet to be specifically reported. Although the role of TIMP-4 in suppressing gelatinase A activation is clear from our data, the role of TIMP-4 binding to progelatinase A is still to be resolved. It remains possible that TIMP-4 might promote progelatinase A activation with other MT-MMPs that could give rise to alternative activation pathways specific to TIMP-4-expressing tissues. Another possibility is that binding of TIMP-4 and TIMP-3 to progelatinase A (and of TIMP-1 to progelatinase B) masks a binding site for other ligands, possibly cell surface receptors, thus introducing another layer of control into the complex biology of these enzymes and inhibitors.

Gelatinase A activation is important in facilitating the invasion and metastasis of cancer cells (42, 52). The ability of TIMP-4 to inhibit these processes has been demonstrated using human breast cancer cells (11, 21). Tumor growth was also slowed by an apparent inhibitory effect of TIMP-4 on angiogenesis (21), and the role of TIMP-4 as an angiogenesis inhibitor has been confirmed recently. We suggest that these suppressive effects of TIMP-4 are attributable to efficient inhibition of MT1-MMP, preventing both progelatinase A activation and MT1-MMP-mediated degradation of extracellular matrix components. High TIMP-4 expression may also protect heart muscle from malignant cancer progression, which rarely occurs in this tissue. Indeed, exogenous TIMP-4 blocks ConA-stimulated progelatinase A activation in heart myocytes, demonstrating that cells from TIMP-4-expressing tissues respond in a similar manner to fibroblasts that do not physiologically express TIMP-4. Hence, the predominant peritumoral stromal expression of TIMP-4 mRNA in fibroblasts surrounding breast carcinomas (11) but not in the majority of breast cancer cell lines (9), together with our data, indicates that TIMP-4 may be an effective host resistance response to invasive cells rather than a tumor-induced mechanism for activation of tumor cell gelatinase A.

REFERENCES


Nguyen, Q., Willenbrock, F., Cocklet, M. I., O’Shea, M., Docherty, A. J. P., and Murphy, G. Different domain interactions are involved in the binding of tissue inhibitors of metalloproteinases to stromelysin-1 and gelatinase A. Biochemistry, 33:20999–20994, 1994.


Nguyen, Q., Willenbrock, F., Cocklet, M. I., O’Shea, M., Docherty, A. J. P., and Murphy, G. Different domain interactions are involved in the binding of tissue inhibitors of metalloproteinases to stromelysin-1 and gelatinase A. Biochemistry, 33:20999–20994, 1994.


Tissue Inhibitor of Metalloproteinases-4 Inhibits But Does Not Support the Activation of Gelatinase A via Efficient Inhibition of Membrane Type 1-Matrix Metalloproteinase


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/9/3610

Cited articles
This article cites 48 articles, 37 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/9/3610.full.html#ref-list-1

Citing articles
This article has been cited by 39 HighWire-hosted articles. Access the articles at:
/content/61/9/3610.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.