Domain Interactions in the Gelatinase A-TIMP-2-MT1-MMP Activation Complex

THE ECTODOMAIN OF THE 44-kDa FORM OF MEMBRANE TYPE-1 MATRIX METALLOPROTEINASE DOES NOT MODULATE GELATINASE A ACTIVATION*

On the cell surface, the 59-kDa membrane type 1-matrix metalloproteinase (MT1-MMP) activates the 72-kDa progelatinase A (MMP-2) after binding the tissue inhibitor of metalloproteinases (TIMP)-2. A 44-kDa remnant of MT1-MMP, with an N terminus at Gly285, is present on the cell after autolytic shedding of the catalytic domain from the hemopexin carboxyl (C) domain, but its role in gelatinase A activation is unknown. We investigated intermolecular interactions in the gelatinase A activation complex using recombinant proteins, domains, and peptides, yeast two-hybrid analysis, solid- and solution-phase assays, cell culture, and immunocytochemistry. A strong interaction between the TIMP-2 C domain (Glu153-Pro221) and the gelatinase A hemopexin C domain (Gly446-Cys569) was demonstrated by the yeast two-hybrid system. Epitope masking studies showed that the anionic TIMP-2 C tail lost immunoreactivity after binding, indicating that the tail was buried in the complex. Using recombinant MT1-MMP hemopexin C domain (Gly285-Cys569), no direct role for the 44-kDa form of MT1-MMP in cell surface activation of progelatinase A was found. Exogenous hemopexin C domain of gelatinase A, but not that of MT1-MMP, blocked the cleavage of the 68-kDa gelatinase A activation intermediate to the fully active 60-kDa enzyme by concanavalin A-stimulated cells. The MT1-MMP hemopexin C domain did not form homodimers nor did it bind the gelatinase A hemopexin C domain, the C tail of TIMP-2, or full-length TIMP-2. Hence, the ectodomain of the remnant 44-kDa form of MT1-MMP appears to play little if any role in the activation of gelatinase A favoring the hypothesis that it accumulates on the cell surface as an inactive, stable degradation product.

The rapid pathological destruction of connective tissues is most readily achieved by secreted proteinases that can diffuse into the extracellular matrix and function at distant sites. In contrast, physiological remodeling processes are subtler and occur in a highly regulated manner to maintain the pericellular environment, tissue integrity, and function. These homeostatic requirements are likely achieved through cell-directed focal proteolysis in which plasma membrane-associated proteinases play key roles (1, 2). Being anchored to the cell membrane, the six known membrane-type matrix metalloproteinases (MT-MMPs)3 (3) are well suited to this function, with MT1-MMP known to cleave collagen and other matrix proteins (4). In addition, two secreted MMPs, MMP-2 (gelatinase A) and MMP-13 (collagenase-3), can be recruited to the cell surface and activated (5, 6) to change the balance of the cellular proteolytic profile (2). The mechanisms by which these MMPs bind to the cell membrane are important for focal proteolysis and in the function of the cell. Cell association of gelatinase A occurs before (7), during (6, 8–10), and after (11) cellular activation of thezymogen, but several aspects of the assembly of the gelatinase A activation complex remain unclear.

The induction of cell-mediated activation of progelatinase A was first described in normal cells in response to concanavalin A (ConA) treatment (12). Collagen is a physiological activator in normal cells (13–15), whereas many tumors constitutively activate progelatinase A (16, 17). Activation is proposed to occur after the formation of a ternary complex of gelatinase A linked to cell-surface MT1-MMP via a tissue inhibitor of metalloproteinases (TIMP)-2 bridge (9, 18, 19). Here, the TIMP-2 inhibitory amino (N) domain binds to the active site of MT1-MMP inhibiting proteolytic activity (20, 21). TIMP-2 also binds progelatinase A outside the catalytic domain (22–26) on the outer rim of the hemopexin carboxyl (C) domain at the junction of hemopexin modules III and IV (27). Deletion of the anionic 9-amino acid, COOH-terminal tail of TIMP-2 decreases the association rate (28). However, direct localization of the progelatinase A-binding site on TIMP-2 has not been reported since the TIMP-2 C domain has not been expressed in isolation. Although the loss of binding following deletion of this domain demonstrates its important role (10), these experiments do not show that the progelatinase A-binding site resides exclusively on the TIMP-2 C domain. Defining this site is important for understanding gelatinase A activation and cell surface localization.

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¶ The abbreviations used are: MT, membrane-type; MMP, matrix metalloproteinase; BSA, bovine serum albumin; C domain, carboxyl-terminal domain; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TIMP, tissue inhibitor of metalloproteinases; N-T2, peptide present in the N domain of TIMP-2; C-T2, peptide representing the anionic carboxyl-terminal tail of TIMP-2; ConA, concanavalin A.
The transition from gelatinase A zymogen to the activation intermediate is critical for activation. Cleavage at Asp<sup>66</sup>-Leu<sup>67</sup> in the gelatinase A prodomain (29, 30) by a free MT1-MMP molecule produces a 68-kDa activation intermediate (8, 12). The fully active gelatinase A with a NH<sub>2</sub>-terminus of Tyr<sup>110</sup> (29, 30) is then produced by <i>in trans</i> autocatalytic intermolecular cleavage. Activation is enhanced by MT1-MMP clustering, but the binding interactions that occur in the cell surface rafts of gelatinase A activation complexes are not resolved. Evidence indicates that cell surface-bound active gelatinase A, rather than soluble active enzyme, is needed to efficiently perform the degradation to the 44-kDa form may release the activated TIMP-2 complex from the cell surface (7). However, the potential functions of the 44-kDa form are unknown. It is unclear which properties of the enzyme this remnant form of MT1-MMP retains. It is not known if the 44-kDa form binds TIMP-2, whether it directly participates in binding MT1-MMP or gelatinase A, or whether it can modulate gelatinase A activation.

In this study we have investigated the potential role of the 44-kDa form of MT1-MMP in gelatinase A activation by analysis of the interactions of recombinant MT1-MMP hemopexin C domain with TIMP-2, gelatinase A, and MT1-MMP. We report experiments that exclude any direct involvement of the ectodomain of the 44-kDa form of MT1-MMP in gelatinase A activation. In addition, we have refined the location of the gelatinase A-binding site on the TIMP-2 C domain and demonstrate the stability of the gelatinase A activation intermediate.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—The cDNA encoding the hemopexin C domain of human MT1-MMP was PCR amplified and cloned into the pGMYX expression vector (32, 36). Recombinant NH<sub>2</sub>-terminal His-tagged protein either with or without the 30-amino acid residue catalytic domain linker (Gly<sup>285</sup>-Cy<sup>290</sup>, Gly<sup>315</sup>-Cy<sup>320</sup>, respectively) and without the COOH-terminal membrane linker was expressed in *Escherichia coli* strain Le392. The hemopexin C domain of human gelatinase A (Gly<sup>446</sup>-Cy<sup>460</sup>) including the catalytic domain linker was expressed in *E. coli* after transformation with the expression construct pGMYX-13 (32). Recombinant protein expression conditions in shaker flasks and a 1-liter L & H fermenter were as described (27). After cell lysis, the recombinant proteins were refolded and purified under conditions that generate correctly folded and biologically functional hemopexin C domain<sup>2</sup> (27, 32, 36). Purified protein in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 7.0 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), with or without 0.05% Brij 35 was snap frozen in liquid N<sub>2</sub> and stored at −80 °C. By quantitative amino acid analysis, electrospray mass spectroscopy, and optical density measurements using a Cary 219 spectrophotometer, the extinction coefficients at 280 nm of 2.78, 3.02, and 1.97 mg ml<sup>−1</sup> cm<sup>−1</sup> were calculated for the hemopexin C domain protein.

<sup>2</sup>E. Tam, C. R. Roberts, and C. M. Overall, manuscript in preparation.

**Peptides and Polyclonal Antipeptide Antibodies**—Peptides were synthesized on a PE-Applied Biosystems 432 Synthesizer, high performance liquid chromatography purified, and analyzed by matrix-assisted laser desorption-time of flight mass spectroscopy (Ciphergen) to confirm fidelity. All peptides terminated with a cysteine, for efficient disulfide coupling to keyhole limpet hemocyanin and chromatography resin. Antipeptide antibodies were raised against keyhole limpet hemocyanin-coupled peptide in two rabbits for each peptide. Antiserum was purified for the antipeptide specific antibodies by affinity chromatography over peptide coupled to Affi-Gel 10 (Bio-Rad) or activated cyanogen bromide resin (Amersham Pharmacia Biotech) and assayed for specificity and concentration by enzyme-linked immunosorbent assay against the peptide and recombinant protein. Antibodies were designated αHis<sub>80</sub>, α2ex12, oproMT1, αMT1-CD, αN-T2, and αC-T2, respectively.

**Microwell Binding Assay**—96-Well microtiter plates were coated with 0.2–0.6 μg of the coating protein in 15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 0.02% (w/v) NaN<sub>3</sub>, pH 9.6, overnight at 4 °C as described (27, 32). Anti-αH<sub>2</sub> [H<sub>6</sub>IEGRGGC] (36); (ii) part of a surface-exposed β-strand of hemopexin module III of the human gelatinase A hemopexin C domain (Ac-RYNEVKKMDPDCG) as described (27); (iii) an anionic COOH-terminal tail of TIMP-2, without the C-terminal-proline sequence in the N domain of human MT1-MMP (CG-GAEEIKANVRKRY-CONH<sub>2</sub>); (iv) a predicted surface-exposed strand in the MT1-MMP hemopexin C domain (Ac-KHIKELGRGLPTGGC); (v) a sequence in the N domain of TIMP-2 (peptide N-T2) (Ac-AGKAEDDGK-MHTLTC); and (vi) the anionic COOH-terminal tail of TIMP-2, but without the COOH-terminal proline since peptide chain elongation from a COOH-terminal proline is poor (peptide C-T2) (CGGA-PPKPQELFDIE-CONH<sub>2</sub>).

Antipeptide antibodies were raised against keyhole limpet hemocyanin-coupled peptide in two rabbits for each peptide. Antiserum was purified for the antipeptide specific antibodies by affinity chromatography over peptide coupled to Affi-Gel 10 (Bio-Rad) or activated cyanogen bromide resin (Amersham Pharmacia Biotech) and assayed for specificity and concentration by enzyme-linked immunosorbent assay against the peptide and recombinant protein. Antibodies were designated αHis<sub>80</sub>, α2ex12, oproMT1, αMT1-CD, αN-T2, and αC-T2, respectively.

Electrophoresis and Zymography—Heat-denatured protein samples were separated under reducing (65 mM dithiothreitol) or nonreducing conditions by SDS-PAGE using 15% gels and stained with silver nitrate or Coomassie Brilliant Blue R-250. For zymography, aliquots of conditioned culture medium were separated under nonreducing conditions on 10% polyacrylamide gels copolymerized with 100 μM gelatin.

<sup>3</sup>C. M. Morrison, H. F. Bigg, G. S. Butler, and C. M. Overall, unpublished data.
Yeast Two-hybrid Analysis—The vectors pGBT9 and pGAD424 (CLONTECH Laboratories Inc.) were used to make the Gal4 DNA-binding domain and Gal4 activation domain fusion constructs, respectively, for use in the yeast two-hybrid system (38). To each of these constructs was fused the human TIMP-2 C domain residues Gly258-Cys260, Pro253 or the His/HindIII fragment containing agar and tested for expression of reporter genes. The strain H7Fc contains the HIS3 and the LacZ gene under the control of a Gal4 responsive promoter. β-Galactosidase activity resulting from up-regulation of the LacZ reporter gene was quantitated by a colorimetric assay. In brief, yeast transformants containing fusion constructs were grown in 5 ml of the appropriate selection medium at 30 °C for 16 h and then transferred to YPD medium and grown to mid-log phase. Cells were lysed and assayed for β-galactosidase activity with the synthetic substrate o-nitrophenyl-β-D-galactopyranoside (Sigma) (39).

Gelatinase A Activation in Cell Culture—Rat osteosarcoma cells (ROS 17/2.8) and early passage human gingival fibroblasts (7) were grown and maintained in a minimum-essential medium (Life Technologies, Inc.) supplemented with 10% (v/v) newborn calf serum (Life Technologies, Inc.). For experiments, cells were seeded at 1.8–2.0 × 10^4 cells/microwell at 10% in newborn calf serum/α-minimum essential medium. At confluency, cells were grown serum-free for 24 h, then stimulated with ConA (20 μg/ml, 16–30 h) in serum-free α-minimum essential medium to induce the cellular activation of endogenous gelatinase A (12). MT1-MMP-hemopexin C domain (λ linker), gelatinase A hemopexin C domain, or PBS vehicle alone, was added to the ConA-treated or untreated cells in a total volume of 100 µl of 50% PBS, 50% serum-free medium. After 24-h incubation, the conditioned medium was assayed by zymography to determine the effects of the addition of the exogenous hemopexin C domains on ConA-induced gelatinase A activation.

Immunocytochemistry—For immunocytochemical studies, ROS 17/2.8 cells were harvested and seeded onto chamber slides (Nunc). Semi-confluency was typically reached after 96 h of growth whereupon the cultures were washed with PBS and growth continued for 16 h serum-free with or without ConA (20 μg/ml) (12). Purified gelatinase A hemopexin C domain in PBS was diluted to 0.1, 1, 10, and 100 ng/ml serum-free medium in the presence of 1% (v/v) BSA. Recombinant protein was incubated with the live cells for 2 h at 37 °C. Serum-free medium with 1% (v/v) BSA served as a control. The treated cells were then fixed in 3.7% (v/v) formaldehyde for 15 min at room temperature and washed, first in PBS containing 1% (v/v) ovalbumin (2 × 3 min) and then in PBS alone (2 × 3 min). Primary antibodies (α7ex12, α9, α31) were added to the fixed, nonpermeabilized cells in PBS containing 1% (v/v) ovalbumin and incubated for 1 h at 37 °C. Sequential washes were performed with PBS PBS + 1% (v/v) ovalbumin, and then PBS alone (2 × 3 min each) followed by the addition of goat anti-rabbit Texas Red-conjugated secondary antibody (Molecular Probes). To stain cell membranes, cell surfaces were oxidized with 4.2 mM periodate (BDH Inc.) in PBS for 30 min on ice, followed by three PBS washes and incubation with 10 mM fluorescein-5-thiosemicarbazide (Molecular Probes) in 1% (w/v) ovalbumin, PBS, 2 h, 37 °C (40). The wells were washed (4 × 2 min) in PBS and mounted with 10% (w/v) 1,4-diazobicyclo(2,2,2)octane (DABCO) (Sigma) in 90% glycerol, 10% PBS and viewed using a Zeiss Axioskop fluorescence microscope with Plan-Neofluar objectives and a MC80 camera. Representative fields were photographed with Ektachrome P1600 film (Kodak, Eastman) to show gelatinase A hemopexin C domain bound to cell membranes using standard filters for Texas Red and fluorescein isothiocyanate, respectively.

In a second series of experiments, ConA-treated and control ROS 17/2.8 cells were stained for MT1-MMP using a Protein A-purified mouse monoclonal antibody directed against the sequence A^145SIREGHKEQG of human MT1-MMP (R & D Systems), for gelatinase A using the affinity purified rabbit MT1-MMP (z2/12 antibody, and for TIMP-2 (H2/10) using the rabbit affinity purified c-T2 antibody. After fixation as above, wells were washed, blocked in 10% goat serum, 2% BSA in PBS for 2 h and then incubated with 25 μg/ml of the primary antibodies as indicated in 2% BSA/PBS or 2% BSA/PBS for 2 h. Wells were washed as above and primary antibodies localized using 100 μg/ml goat anti-mouse IgG-Texas Red conjugate (Molecular Probes) in 2% BSA/PBS and/or 100 μg/ml goat anti-rabbit IgG-Alexa 488 conjugate (Molecular Probes) for 1 h. They were then incubated for 2 min with 1 μM Hoechst 33342 (Molecular Probes) as a nuclear counterstain. Secondary antibodies were highly preabsorbed against other species’ IgG by the supplier and further screened in our laboratory to verify lack of nonspecific binding to cultured ROS 17/2.8 cells. Preparations were washed, mounted, and viewed by fluorescence microscopy as above, using appropriate filter sets. 8-bit high-resolution monochrome images were captured at a single sitting with a Princeton CCD camera and Northern Eclipse software package on a Pentium II computer. Exposure times for control (no primary antibody) were standardized at 4 s, to obtain representative control images. Images were colorized with Photoshop 5.0 on a Macintosh G4 using identical settings.

RT-PCR—Total RNA was isolated from ROS 17/2.8 cells using RNeasy™ (Qiagen) and treated with amplification grade DNase I (Life Technologies, Inc.). Reverse transcription was carried out using a poly(dT) primer with Superscript II (Life Technologies, Inc.) as described by Morrison et al. (41). PCR was performed with primers specific for human MT1-MMP (sense primer 5'-GACATGCTAGGCGGC-CAACATCTGTTAGC-3', antisense primer 5'-CGAAGCCTAGCCAGC-CATCAGTCC-3') and human TIMP-2 (sense primer 5'-GGCTCGGG-CCTCTCTGCTG-3', antisense primer 5'-CTTGGATGCAGCGGAAGAAG-TTGTG-3') with 30 cycles of amplification (40 s at 94 °C, 1 min at 55 °C, 2 min at 72 °C per cycle).

RESULTS

TIMP-2 Binding Properties of MT1-MMP and Gelatinase A Hemopexin C Domains—The MT1-MMP and gelatinase A hemopexin C domains were expressed in E. coli and purified (Fig. 1). The absence of dimeric intermolecular disulphide cross-linked forms of the recombinant proteins was confirmed by SDS-PAGE under nonreducing conditions (Fig. 1) and by electrospray mass spectroscopy (not shown). Confirmation that the recombinant MT1-MMP hemopexin C domain proteins were functional was shown by a strong binding interaction with native type I collagen (not shown) consistent with the collagenolytic activity of MT1-MMP (4) and the essential role of the hemopexin C domain of collagenases in triple helicate activity (5).

Compared with the specific and strong binding of the gelatinase A hemopexin C domain to TIMP-2 (apparent K_d 3.0 × 10^{-10} M) as found before (42) the MT1-MMP hemopexin C domain, either with or without the catalytic domain linker, did not bind TIMP-2 coated on microwell plates (Fig. 2A). The MT1-MMP hemopexin C domain also did not bind to coated gelatinase A hemopexin C domain (Fig. 2B). To confirm these results, intact cells were isolated by fine-needle aspiration. Again, neither the gelatinase A hemopexin C domain nor TIMP-2 bound to the MT1-MMP hemopexin C domain and linker immobilized on an Affi-Gel 10 mini-column, with all applied protein being recovered in the unbound and wash fractions (Fig. 2C). Gel permeation chromatography of a mixture of MT1-MMP and gelatinase A hemopexin C domain proteins

![Image](39499)
FIG. 2. Microwell binding assay of the interactions between TIMP-2 and recombinant hemopexin C domain proteins of MT1-MMP and gelatinase A. Panel A, TIMP-2 binding affinity of the hemopexin C domain of gelatinase A compared with the MT1-MMP hemopexin C domain. TIMP-2 was coated on microwells, incubated with hemopexin C domain proteins in PBS as indicated, and bound protein was measured by a His6 antibody. Binding to myoglobin controls for each protein is indicated. Panel B, microwell binding assay of the MT1-MMP hemopexin C domain proteins added in PBS to gelatinase A hemopexin C domain coated on microwells and detected with affinity purified aMT1-CD antibody. Myoglobin controls for each protein are shown. Panel C, MT1-MMP L, C domain, 100-μl mini-columns of MT1-MMP hemopexin C domain with linker coupled to Affi-Gel 10. Gelatinase A hemopexin C domain (Gel A C domain) (30 μg) or TIMP-2 (TIMP-2) (30 μg) in PBS were applied to the columns (B, before chromatography), the unbound fraction collected (U) and the column washed with PBS (W). Any bound protein was eluted with 1.5 M NaCl (N) followed by 5% dimethyl sulfoxide (D). Gel A C domain, hemopexin C domain of human gelatinase A was bound to 200-μl mini-columns of chelating Sepharose 6B charged with Zn2+ as described under “Experimental Procedures.” TIMP-2 (50 μg) was applied to the column and the unbound flow-through collected (U), the column was washed with chromatography buffer (W) before step elution with NaCl (0.5 M and 1.0 M) and 10% (v/v) dimethyl sulfoxide (10% DMSO) as indicated. Bound protein was eluted with 50 mM EDTA in chromatography buffer. Each fraction was analyzed by 15% SDS-PAGE and Coomassie Brilliant Blue R-250 staining. M, molecular mass marker proteins in Da as indicated.

did show no evidence of homo- or heterodimers, with both proteins eluting as single peaks on an FPLC Superdex 75 column (not shown). In contrast, TIMP-2 bound tightly to the gelatinase A hemopexin C domain immobilized on a Zn2+ chelate mini-column (Fig. 2C). No TIMP-2 was recovered in the unbound or wash fractions after loading and neither 1.0 M NaCl nor 10% dimethyl sulfoxide could elute the bound protein. TIMP-2 and the gelatinase A hemopexin C domain were eluted with 50 mM EDTA.

The Binding Site on TIMP-2 for the Gelatinase A Hemopexin C Domain—To localize the binding site on TIMP-2 for the hemopexin C domain, we assessed the interaction of the isolated TIMP-2 C domain with the gelatinase A hemopexin C domain by yeast two-hybrid analysis, since the small size of the TIMP-2 C domain renders this protein difficult to express in E. coli. The C domain of TIMP-2 interacted strongly with the gelatinase A hemopexin C domain. The two reporter genes HIS3 (Fig. 3A) and LacZ (Fig. 3B) were strongly up-regulated when the Gal4 domains were fused with the respective C domains, whereas little or no activation was observed with the control transformants containing only one of the two Gal4 fusion expression plasmids. Thus, these data are a direct demonstration that the TIMP-2 C domain is necessary and sufficient for binding the gelatinase A hemopexin C domain.

To refine the location of the hemopexin C domain-binding site on the TIMP-2 C domain, microwell plates were coated with TIMP-2. Solvent exposure of the COOH-terminal 13-residue tail of the coated TIMP-2 (GAPPKQFELDID) was monitored by reaction with aC-T2 antibody raised against this sequence. The addition of increasing amounts of the gelatinase A hemopexin C domain was accompanied by a progressive loss of immunoreactivity to the C tail (Fig. 4A). Binding of the gelatinase A hemopexin C domain to TIMP-2 was confirmed by reaction with a His6 antibody, which recognized bound hemopexin C domain (Fig. 4A). Essentially identical apparent Kd values were calculated from both approaches. Absence of binding of the hemopexin C domain to BSA confirmed specificity. The masking of the COOH-terminal tail of TIMP-2 upon binding the hemopexin C domain was not complete indicating that some of the tail was exposed.

To determine whether the TIMP-2 COOH-terminal tail was sufficient for binding the hemopexin C domain, microwell

FIG. 3. Yeast two-hybrid analysis demonstrates the interaction between the C domains of gelatinase A and TIMP-2. Plasmids encoding Gal4 DNA-binding protein and Gal4 activation protein fused with the C domains of gelatinase A (Gel A CD) and TIMP-2 (TIMP-2 CD) were introduced into S. cerevisiae strain HF7c, singly and in combination as indicated. Transformants were assayed for histidine independent growth on a His- agar plate as shown in panel A. Yeast colony growth only occurred in cells containing the positive control fusions p53/SV40 T-antigen on either plasmid (A and E) or both the TIMP-2 and gelatinase A C domain fusions on either plasmid as indicated (B and C). Panel B, LacZ reporter gene activity of the transformants (A–H) grown in liquid culture to mid-log phase was quantitated by assaying for β-galactosidase activity.
FIG. 4. The involvement of the TIMP-2 COOH-terminal tail in binding the gelatinase A hemopexin C domain. Panel A, epitope-masking studies. Reactivity of aC-T2 antibody to TIMP-2 coated on microtiter plates was measured after the addition of increasing amounts of gelatinase A hemopexin C domain. Gelatinase A hemopexin C domain binding was measured using αHis6 antibody. BSA served as a control for the gelatinase A hemopexin C domain. Panel B, a control for the gelatinase A hemopexin C domain.

Plates were coated with peptide C-T2 and binding by gelatinase A hemopexin C domain was then assayed. Peptide N-T2 and full-length TIMP-2 were coated as controls. Since the MT1-MMP hemopexin C domain did not bind TIMP-2 (Fig. 2) this protein was also added to peptide-coated wells as a control. As shown in Fig. 4B, peptide C-T2 was insufficient to support hemopexin C domain binding under conditions where the full-length TIMP-2 molecule was tightly bound by the gelatinase A hemopexin C domain. The MT1-MMP hemopexin C domain also did not bind the peptide C-T2. Neither the MT1-MMP nor the gelatinase A hemopexin C domains bound to the peptide N-T2 or to BSA controls. The three-dimensional structure of TIMP-2 reveals the highly flexible nature of the COOH-terminal tail (21). Since peptide binding to plastic microwells would constrain peptide flexibility and so may preclude adoption of a conformation permissive for hemopexin C domain binding, peptide C-T2 was added in increasing amounts to a fixed concentration of gelatinase A hemopexin C domain before addition of the mixture to TIMP-2-coated wells. The gelatinase A hemopexin C domain in the C domain/peptide mixture still bound to TIMP-2 with unaltered affinity (Fig. 4B). Hemopexin C domain was also added to peptide C-T2 coupled to thiols-Sephrose columns. However, all the recombinant protein was recovered in the unbound and wash fraction without evidence for any binding (not shown).

Deletion of the TIMP-2 COOH-terminal tail or NaCl at concentrations as low as 0.5 M is reported to reduce the rates of association of TIMP-2 for inhibition of gelatinase A (28). However, 1.0 M NaCl did not disrupt the gelatinase A hemopexin C domain/TIMP-2 complex (Fig. 2C). When hemopexin C domain was added in the presence of 1.0 M NaCl to reduce electrostatic interactions, no disruption in TIMP-2 binding at equilibrium was observed (Fig. 5). Hence, although the TIMP-2 COOH-terminal tail is necessary for rapid association with the gelatinase A hemopexin C domain (28), our data show that it is not sufficient for binding. Thus, other regions on the TIMP-2 C domain are also needed to form the stable binding site.

The hemopexin C domain of MMPs is stabilized by one or two Ca2+ ions in the central pore of the domain (43, 44). Fibronectin and heparin binding by the gelatinase A hemopexin C domain is Ca2+ ion-dependent, being disrupted by divalent cation chelators (32). Hemopexin C domain incubated with 50 mM EDTA for 18 h prior to assay still bound TIMP-2 in the microwell plate assays (Fig. 5) revealing a Ca2+ ion independent binding site. This also indicates that on the affinity columns shown in Fig. 2C, EDTA eluted the intact hemopexin C domain/TIMP-2 complex by chelation of the ligating Zn2+ ion from the metal chelate resin rather than by disrupting Ca2+ ion-dependent stability or a Ca2+ ion salt bridge.

Cellular Response to Recombinant MT1-MMP and Gelatinase A Hemopexin C Domains—To determine the potential effect of the ectodomain of the 44-kDa form of MT1-MMP on gelatinase A activation, recombinant MT1-MMP hemopexin C domain was added for 24 h to ROS17/2.8 cells and human fibroblasts stimulated with ConA. ConA induced the cellular activation of gelatinase A from the 66-kDa zymogen to the fully activated 59-kDa form via the 62-kDa intermediate (Fig. 6) (zymography molecular masses are for nonreduced gela-
tinase A and correspond to the 72-, 68-, and 66-kDa reduced forms described in the text). After addition of the MT1-MMP hemopexin C domain protein no effect was observed either on the secretion or activation of progelatinase A, in the presence or absence of ConA (Fig. 6A). In contrast, addition of the gelatinase A hemopexin C domain progressively blocked the activation of progelatinase A in ConA-treated ROS 17/2.8 cells (Fig. 6B) or human gingival fibroblasts (not shown), in a dose-dependent manner. Although zymograms are not quantitative and the specific activity of the different molecular weight forms of the enzyme differ in this assay (12) the ratio of the active band compared with the progelatinase A band decreased as the gelatinase A hemopexin C domain concentration was increased. This was due largely to a marked reduction in the amount of the fully activated form of the enzyme present. There was no difference apparent in the levels of the activation intermediate. Thus, the inability of the MT1-MMP hemopexin C domain to compete for gelatinase A activation suggests that the ectodomain of the 44-kDa form of MT1-MMP and the hemopexin C domain of full-length MT1-MMP, do not modulate the ConA-dependent activation process.

Immunocytochemistry was used to examine if the modulation of gelatinase A activation by the gelatinase A hemopexin C domain may have occurred by binding to cell surface receptors. Although binding of the hemopexin C domain was apparent on the rounded, dendritic shaped ConA-treated ROS 17/2.8 cells (not shown), it was striking to also note the specific and discrete pattern of binding to the plasma membrane of cells grown in the absence of ConA (Fig. 7C). These cells only secreted the latent form of gelatinase A in the absence of ConA (Fig. 6B). Whereas cell binding was detected at 0.1 ng/ml protein on ConA-treated cells, 1 ng/ml was required to produce a characteristic lacunar shaped or punctate immunocytochemical staining pattern on cells not treated with ConA (Fig. 7C). When hemopexin C domain was not added (Fig. 7A, 0 ng control) or where a control antibody was used with a 100-fold higher concentration of the hemopexin C domain (Fig. 7E, 100 ng control), the cells did not show any specific fluorescence. In these fields the presence of cells was confirmed by the clear outlining of plasma membranes after membrane staining (Fig. 7, B, D, and F). Staining conditions and exposure times for these experiments were selected so that endogenous gelatinase A was not visible. However, gelatinase A could be detected in the cells under the appropriate conditions (see below).

It was unexpected that the gelatinase A hemopexin C domain would bind to cells not treated with ConA. Therefore, we assessed the levels of MT1-MMP and TIMP-2 to determine the presence of this receptor complex for gelatinase A. RT-PCR confirmed that both control and ConA-treated ROS17/2.8 cells produced MT1-MMP and TIMP-2 mRNAs (Fig. 8). Immunocytochemistry showed that the MT1-MMP protein was expressed in control cells (Fig. 9A). The slightly retracted ConA-treated cells showed clearer evidence for cell membrane localization of MT1-MMP (Fig. 9D, arrows). Gelatinase A levels were elevated in ConA-treated cells (Fig. 9E) as found before (12), but TIMP-2 expression was relatively unaffected (Fig. 9, H and K). Cells that contained mitotic figures (Fig. 9C, arrows) exhibited higher levels of MT1-MMP (Fig. 9A, arrows) and gelatinase A (Fig. 9B, arrows) than resting cells. Thus, both resting and ConA-treated cells expressed MT1-MMP and TIMP-2 that likely functioned as the cell surface receptor for the exogenous gelatinase A hemopexin C domain. However, despite the abundance of MT1-MMP on the cell surface progelatinase A activation did not occur without ConA stimulation suggesting that the clustering of MT1-MMP, as shown in Fig. 9D, is an essential event in the activation process.
Domain Interactions in the Gelatinase A-TIMP-2-MT1-MMP Complex

Removal of the gelatinase A prodomain during cell surface activation results from two separate proteolytic events that are dependent upon the assembly of an activation complex consisting of cell surface MT-MMPs, TIMP-2, pro- and active gelatinase A molecules. Additional interactions may also occur including binding to α5β1 integrin (45) and matrix components (7, 31, 32). One aim of this study was to determine whether the 44-kDa autolyzed form of MT1-MMP that is present in large amounts during gelatinase A activation interacts with the activation complex. Our characterization of the MT1-MMP hemopexin C domain and linker representing the ectodomain of the 44-kDa form of MT1-MMP discounted several possible domain-domain interactions with other components of the activation complex that could also have been involved in its assembly or modulate gelatinase A activation. We also have used isolated domains in the yeast two-hybrid system to provide direct evidence for the first time that the TIMP-2 C domain binds to the gelatinase A hemopexin C domain. These data are consistent with, and extend the TIMP-2 C domain deletion studies previously reported (10, 28).

Surprisingly, the binding interaction between the TIMP-2 C domain and the gelatinase A hemopexin C domain has not previously been directly demonstrated. Although C domain deletions of TIMP-2 indicate the importance of this domain (10, 28), these experiments do not show that the gelatinase A-binding site resides exclusively on the TIMP-2 C domain. Domain deletion may simply disrupt the integrity of a binding site that spans both the N and C domains (2). We utilized the yeast two-hybrid system to demonstrate directly that the TIMP-2 C domain was sufficient for binding the gelatinase A hemopexin C domain. Colony growth on His - media and high β-galactosidase activity revealed a strong productive interaction between the two C domains when fused to either GAL4 protein that was equal to the positive control. Typically, yeast two-hybrid analyses have been limited to demonstrating intracellular protein interactions and this system has not been generally thought to be suitable for the study of extracellular interactions, particularly those involving proteins with disulfide bridges. Here we have confirmed the utility of this nuclear assay in demonstrating interactions between extracellular, disulfide-rich protein domains. Notably, disulfide bond increase protein stability in harsh conditions. In the milder nuclear environment at 30 °C, thermal and other stresses may be insufficient to destabilize the protein fold to lose function. Thus, the yeast two-hybrid system is suitable for the study of extracellular protein interactions involving MMPs.

In addition to directly demonstrating that the TIMP-2 C domain forms stable complexes with the gelatinase A hemopexin C domain, we refined the location of the binding site by showing that the TIMP-2 anionic COOH-terminal tail was masked on binding. Although the C tail is necessary for rapid association (28), we found it is not sufficient for hemopexin C domain binding. Peptide columns and solid phase binding assays did not support hemopexin C domain binding, nor did the C tail peptide or 1 M NaCl compete for binding in solution. We conclude that the anionic C tail forms only part of the hemopexin C domain docking site and is not sufficient for the interaction. With the strong dipole moment of the hemopexin C domain produced by the lysine clusters at the junction of hemopexin modules III and IV that form the TIMP-2 docking site (27), correct orientation of the binding faces of these two molecules can thereby occur before physical contact. Hence, this electrostatic influence would favor the first contact of TIMP-2 with the docking site, despite the much higher binding affinity between the TIMP-2 N domain and the active site (28, 46, 47).

Kinetic analyses show that TIMP binding and inhibition of MMPs is stronger for full-length MMPs and TIMPs than for hemopexin and TIMP C domain deletion mutants (10, 20, 28, 46). The inhibitory TIMP interaction with the active site of several MMPs forms an “elongated wedge” topography (48) that is stabilized by further contacts with the hemopexin C domain (24). Here we term these contacts, which are found in all MMPs, “stabilization sites” in order to distinguish such sites from the gelatinase A-specific, salt bridge-dominated “docking site.” The TIMP-2 docking site on gelatinase A hemopexin modules III and IV (27) lies on the opposite side of the molecule from where we predict the inhibitory stabilization site will be located at the junction of modules I (Val459, Thr511) and II (Ser546, Gly551), which forms the lower wall of a groove that lies between the catalytic domain and the hemopexin C domain. In TIMP-2 both of the loops that are predicted to contact the stabilization site are flexible in the protein and so could adapt to the surface of the hemopexin C domain stabilization site upon contact.

Adopting the elongated wedge geometry is more complicated for gelatinase A than for other MMPs since TIMP-2 also forms an alternate stable complex with the hemopexin C domain alone or the full-length zymogen at the docking site. When TIMP-2 is bound to the gelatinase A hemopexin C domain the N domain of TIMP-2 is aligned away from the gelatinase A active site and so is available for binding to MT1-MMP in the activation complex (27). As discussed previously (27), the gelatinase A docking site is unlikely to also form the inhibitory stabilization site unless a very large, entropically unfavorable shift in domain position occurs to allow the inhibitory N domain to engage the active site. It is more likely that stable autoinhibition in cis of gelatinase A will only occur upon rearrangement of the molecular interaction to form the archetypal elongated wedge geometry. Hemopexin C domain movements may allow the TIMP-2 N domain to transiently engage the gelatinase A active site, but in order to adopt the inhibitory elongated wedge geometry, the TIMP-2 C domain would need to disengage from the docking site and then bind the stabilization site. Two-step binding, first to the docking site and then to the stabilization site, may also explain the biphasic inhibition kinetics of TIMP-2 (46, 47). Compared with free TIMP-2 in solution, TIMP-2 bound to the docking site via C tail-directed interactions would favor its subsequent interaction with the active site by molecular rearrangement providing an alternative explanation for the reduced $k_{un}$ of gelatinase A inhibition by C-tail-truncated TIMP-2 (28).

FIG. 8. RT-PCR analysis of TIMP-2 and MT1-MMP mRNA expression in ROS 17/2.8 cells. RT-PCR products were electrophoresed on a 1% agarose gel and ethidium bromide stained. The electrophoretic position of the 506-base pair marker is indicated.

Kinetic analyses show that TIMP binding and inhibition of MMPs is stronger for full-length MMPs and TIMPs than for hemopexin and TIMP C domain deletion mutants (10, 20, 28, 46). The inhibitory TIMP interaction with the active site of several MMPs forms an “elongated wedge” topography (48) that is stabilized by further contacts with the hemopexin C domain (24). Here we term these contacts, which are found in all MMPs, “stabilization sites” in order to distinguish such sites from the gelatinase A-specific, salt bridge-dominated “docking site.” The TIMP-2 docking site on gelatinase A hemopexin modules III and IV (27) lies on the opposite side of the molecule from where we predict the inhibitory stabilization site will be located at the junction of modules I (Val459, Thr511) and II (Ser546, Gly551), which forms the lower wall of a groove that lies between the catalytic domain and the hemopexin C domain. In TIMP-2 both of the loops that are predicted to contact the stabilization site are flexible in the protein and so could adapt to the surface of the hemopexin C domain stabilization site upon contact.

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Support for this concept also comes from consideration of the interactions involved in forming the gelatinase A trimeric complex with MT1-MMP on the cell surface. TIMP-2 likely adopts the inhibitory elongated wedge geometry with MT1-MMP. Hence, the TIMP-2 C domain sites in contact with the stabilization site of the MT1-MMP hemopexin C domain are clearly not involved in binding the gelatinase A hemopexin C domain-docking site. Because the TIMP-2 N domain is engaged in the MT1-MMP active site, a gelatinase A inhibitory rearrangement of the TIMP-2 cannot take place in the trimeric complex. Therefore, to inactivate cell surface-bound gelatinase A an additional mole of TIMP must be required. In this manner 2 mol of TIMP would be bound per mole of cell surface gelatinase A for inhibition. The inhibitory time interaction is also likely to be in the elongated wedge topology with interactions to stabilization sites on the hemopexin C domain. Hence it is most unlikely that the cationic docking site also forms the TIMP-2 stabilization.

Although the TIMP-2 interaction is critical for titrating progelatinase A to the cell surface for activation, other interactions are also necessary. Even though we could demonstrate that cells expressing MT1-MMP and TIMP-2 could bind recombinant gelatinase A hemopexin C domain, activation of progelatinase A did not occur without ConA treatment in the two cell types examined. It was likely that all cell surface MT1-MMP molecules were inhibited with TIMP-2, which therefore functioned as cell surface receptors for the hemopexin C domain of gelatinase A. To induce activation of the bound progelatinase A

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**Fig. 9.** Immunocytochemical localization of MT1-MMP, gelatinase A, and TIMP-2 in ROS 17/2.8 cells. Control (panels A-C and G-I) and ConA-treated (panels D-F and J-L) ROS 17/2.8 cells were stained for MT1-MMP (panels A and D), gelatinase A (panels B and E), and TIMP-2 (panels H and K). Goat anti-rabbit IgG secondary antibody without primary antibody control for gelatinase A and TIMP-2 panels (panel G). Goat anti-mouse IgG secondary antibody without primary antibody control for MT1-MMP panels (panel J). Cell nuclei were counterstained with Hoechst 33342 (panels C, F, I, and L) as described under “Experimental Procedures.” Scale bar in panels C, F, I, and L is 50 μm.
by ConA several events appear to be required: ConA induces the expression of free uninhibited MT1-MMP molecules on the cell surface that can cleave the propeptide of gelatinase A (20). A post-translational effect (49) and ConA-induced clustering of the bound gelatinase A molecules is also needed for efficient activation. In invasive tumors this occurs naturally at invadapodia (16) with a role for integrins indicated in forming these clusters (3). Matrix molecules also appear to modulate this process with heparin or heparin sulfate proteoglycans enhancing clustering and activation (20, 31) by binding the hemopexin C domain of gelatinase A (31, 32). On the other hand, $\beta_2$-integrin-coupled collagen bound to gelatinase A reduces activation (7), despite collagen-mediated induction of MT1-MMP gene expression (15). Binding of MT1-MMP to collagen molecules may also modulate clustering and gelatinase A activation (2).

The first cleavage of the gelatinase A prodomain by MT1-MMP was unaffected by exogenous hemopexin C domains of MT1-MMP or gelatinase A, indicating that cell surface tethering of gelatinase A involving these domains was not absolutely required to initiate activation. The progressive reduction in gelatinase A activation by cells with increasing concentrations of hemopexin C domain of gelatinase A, but not that of MT1-MMP, demonstrated that the exogenous gelatinase A hemopexin C domain can compete with endogenous secreted gelatinase A for soluble TIMP-2 or TIMP-2 bound to cell surface MT1-MMP. The immunocytochemical evidence for exogenous gelatinase A hemopexin C domain bound to cells indicates that this would prevent the subsequent recruitment of progelatinase A-TIMP-2 complexes to the cell surface. The presence of a stable population of activation intermediate shows that autocatalytic cleavage to the fully active form of the enzyme must occur in trans by intramolecular cleavage by an already activated gelatinase A molecule. This is because in cis processing is concentration independent. If the final cleavage to Tyr$^{110}$ was by autocatalysis in cis then a stable population of the intermediate would not be trapped and accumulate.

Two other lines of evidence support the conclusion that the first cleavage of the gelatinase Azymogen is necessary for full activation, but is not sufficient to destabilize the propeptide for intramolecular autolysis in cis. First, use of modified TIMP-2 that is rendered noninhibitory by amino-terminal carboxymethyl (50) or by additional amino-terminal residues (51) blocks activation. However, a pool of activation intermediate was also produced. Second, cells from the Timp2$^{-/-}$ mouse are incapable of activating gelatinase A, even upon treatment with ConA (52). However, we have found that the activation intermediate also accumulates in these cells. The knockout mouse studies are important because they reveal that even though the first cleavage of gelatinase A to generate the activation intermediate can occur in a non-TIMP-2-dependent manner, the final autocatalytic step is absolutely TIMP-2 dependent. Since the gelatinase A hemopexin C domain blocks the conversion of the activation intermediate to the fully active enzyme this indicates that only cell surface TIMP-2-bound active gelatinase A and not soluble active gelatinase A efficiently completes the in trans cleavage of the activation intermediate.

The MT1-MMP hemopexin C domain commencing at Gly$^{285}$ did not bind either TIMP-2 or the gelatinase A hemopexin C domain nor did it modulate ConA-induced enzyme activation on two cell types from rat and human sources. Our data also excludes potential homodimerization involving either the hemopexin C domains from MT1-MMP or gelatinase A, or a heterodimerization interaction between these two domains. Hence, it is unlikely that the ectodomain of the 44-kDa MT1-MMP proteolytic remnant comprised of the hemopexin C domain and linker to Gly$^{285}$ plays any direct role in modulating gelatinase A activation. Moreover, the binding of gelatinase A to MT1-MMP would appear to only occur via the TIMP-2 bridge. Nonetheless, a potential role in modulating activation by the 44-kDa form is still possible by interactions with the cytoplasmic tail, as found for full-length MT1-MMP (53), or through binding cell surface collagen. Recent work reveals the inherent instability of TIMP-2-free MT1-MMP on the cell surface (33). This supports the concept that because of the strong binding interaction between TIMP-2 and MT1-MMP, MT1-MMP autolysis in trans may be one mechanism that releases activated gelatinase A from the cell surface after processing (7), with the accumulation of the 44-kDa MT1-MMP form reflecting this event.

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