Dynamic Interdomain Interactions Contribute to the Inhibition of Matrix Metalloproteinases by Tissue Inhibitors of Metalloproteinases*\textsuperscript{5}

Albert G. Remacle, Sergey A. Shiryaev, Ilian A. Radichev, Dmitri V. Rozanov, Boguslaw Stec, and Alex Y. Strongin

From the Cancer Research Center, Sanford-Burnham Medical Research Institute, La Jolla, California 92037

Because of their important function, matrix metalloproteinases (MMPs) are promising drug targets in multiple diseases, including malignancies. The structure of MMPs includes a catalytic domain, a hinge, and a hemopexin domain (PEX), which are followed by a transmembrane and cytoplasmic tail domains or by a glycosylphosphatidylinositol linker in membrane-type MMPs (MT-MMPs). TIMPs-1, -2, -3, and -4 are potent natural regulators of the MMP activity. These are the inhibitory N-terminal and the non-inhibitory C-terminal structural domains in TIMPs. Based on our structural modeling, we hypothesized that steric clashes exist between the non-inhibitory C-terminal domain of TIMPs and the PEX of MMPs. Conversely, a certain mobility of the PEX relative to the catalytic domain is required to avoid these obstacles. Because of its exceedingly poor association constant and, in contrast with TIMP-2, TIMP-1 is inefficient against MT1-MMP. We specifically selected an MT1-MMP-TIMP-1 pair to test our hypothesis, because any improvement of the inhibitory potency would be readily recorded. We characterized the domain-swapped MT1-MMP chimeras in which the PEX of MMP-2 (that forms a complex with TIMP-2) and of MMP-9 (that forms a complex with TIMP-1) replaced the original PEX in the MT1-MMP structure. In contrast with the wild-type MT1-MMP, the diverse proteolytic activities of the swapped-PEX chimeras were then inhibited by both TIMP-1 and TIMP-2. Overall, our studies suggest that the structural parameters of both domains of TIMPs have to be taken into account for their re-engineering to harness the therapeutic in vivo potential of the novel TIMP-based MMP antagonists with constrained selectivity.

There are 24 individual MMPs\textsuperscript{2} in humans. MMPs cleave multiple extracellular matrix components, growth factors, cytokines, and cell signaling adhesion receptors. Aberrant performance of MMPs plays a role in a plethora of diseases, including cancer (1–3). There are 18 soluble and 6 membrane MMPs. The structure of soluble MMPs includes a prodomain (PRO), a catalytic domain (CAT) that contains the active site zinc, a hinge, and a PEX. Additionally, membrane-type MMPs (MT-MMP) contain a transmembrane domain followed by a short cytoplasmic tail (CYTO) (MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP) or a glycosylphosphatidylinositol moiety (MT4-MMP and MT6-MMP) that anchors the proteinase to the cell surface (1, 4, 5).

MMPs are synthesized as zymogens, which require the proteolytic processing of the N-terminal inhibitory PRO to generate the active enzymes (4). It is accepted that secretory tissue inhibitors of MMPs (TIMPs) play an important role in the regulation of the proteolytic activity of MMPs (6, 7). Four TIMPs (TIMP-1, -2, -3, and -4) are present in humans (8). There is at least a 25% sequence identity among all TIMPs, including 12 conserved Cys residues that form 6 disulfide bridges resulting in 6 loop regions. There are two domains, N-terminal and C-terminal, in TIMPs. An N-terminal domain (NT-TIMP) binds the CAT, carries the MMP-inhibitory activity, and encompasses the three first loops. A non-inhibitory C-terminal domain (CT-TIMP) binds, albeit with distinct affinities, the PEX of MMPs. TIMP-1 forms a stoichiometric complex with the MMP-9 proenzyme via the binding of its CT-TIMP with the PEX (9). In turn, TIMP-2 forms a complex with the MMP-2 proenzyme (10, 11).

Truncated NT-TIMP folds correctly and exhibits a significant level of the MMP-inhibitory activity. Crystal structures of multiple TIMP-MMP inhibitory complexes have been determined. They demonstrate similar inhibitory mechanisms, involving a region surrounding the conserved Cys\textsuperscript{1}–Cys\textsuperscript{70} disulfide bond of TIMPs and especially the N-terminal Cys\textsuperscript{1}–Pro\textsuperscript{6} and Glu\textsuperscript{67}–Cys\textsuperscript{70} segments (numbering is given according to TIMP-1) that approach the MMP active site (PDB 1UEA, 1BUV, 1GXD, 1BR9, and 2E2D) (12–18). The Cys\textsuperscript{3} coordinates, via bidentate interactions, the catalytic zinc of the MMP active site. Normally, TIMPs perform as nanomolar or even femtomolar range inhibitors of MMPs (8, 19–22).

Multiple studies have been performed to constrain the specificity of TIMPs and transform TIMPs into selective rather than wide-ranging inhibitors (14, 23–26). The inhibitory NT-TIMP alone and the individual CAT of MMPs were predominantly used in these studies. Differences in contacts and chemistry of the interfaces of TIMP-MMP complexes provide a basis for re-
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MATERIALS AND METHODS

General Reagents and Antibodies—All reagents are purchased from Sigma-Aldrich unless indicated otherwise. A murine monoclonal antibody against the CAT of MT1-MMP (clone 3G4) and a hydroxamate inhibitor of MMPs (GM6001) were purchased from Chemicon. A SuperSignal West Dura Extended Duration Substrate kit, EZ-Link sulfo-NHS-SS-biotin (sulfo succinimidyl-2-(bifarnitamido) ethyl-1,3-dithiopropionate), and EZ-Link sulfo-NHS-LC-biotin (sulfo succinimidyl-6-(bifarnitamido)hexanoate) were from Pierce. The secondary species-specific antibodies conjugated with HRP were purchased from Jackson ImmunoResearch. The secondary species-specific antibodies conjugated with Alexa Fluor 594 (red) were obtained from Molecular Probes. A proteinase inhibitor mixture set (Set III) was from Calbiochem.

Recombinant Proteins—Recombinant human TIMP-1 was obtained from Invitrogen. Recombinant TIMP-2 was purified from the medium conditioned by the recombinant CHO cells (30). The secretory N-end appended TIMP-2 mutant (A-TIMP-2) that exhibited the Glu-Ala-Glu-Ala-Tyr-Val-Glu-Phe sequence attached to the N-terminal Cys was purified from the recombinant Pichia pastoris yeasts using FPLC on a Mono-Q column (31). The TIMP-2 free MMP-2 proenzyme was isolated from p2AH2A72 cells derived from the fibrosarcoma HT1080 cell line sequentially transfected with the E1A and MMP-2 cDNAs (32). The individual CAT of MT1-MMP and MT6-MMP was expressed in Escherichia coli, purified from the inclusion bodies, and refolded to restore its catalytic activity (33, 34). The concentrations of the purified, catalytically active MT1-MMP and MT6-MMP were measured by titration against a standard GM6001 solution of a known concentration and Mca-PLGL-Dpa-AR-NH₂ as a fluorescent peptide substrate (35).

Cloning of the MT1-MMP Chimeras—To design both chimeras, we used the cDNA coding for the full-length human wild-type MT1-MMP (WT), MMP-2, and MMP-9 cDNAs. The sequences coding for the PEX of MMP-2 (470–659) and MMP-9 (517–703) were each inserted between Cys³¹⁹ and Cys⁵⁰⁸ of the MT1-MMP sequence to generate the PEX/MMP-2 and PEX/MMP-9 chimeras, respectively. The authenticity of the recombinant constructs was confirmed by DNA sequencing.

Cells—Human breast carcinoma MCF-7 cells were obtained from ATCC (Manassas, VA). The full-length human β₂ integrin subunit and MT1-MMP cDNAs were each cloned into the pcDNA3.1-neo and pcDNA3.1-zeo vectors, respectively (Invitrogen) (36). The catalytically inactive MT1-MMP mutant (E240A) was generated by replacing the essential Glu²⁴⁰ of the proteinase active site with Ala (37). The C-end-truncated MT1-MMP (ΔCYTO) mutant lacking the 563–582 CYTO sequence was described earlier (38). The inert mutant lacking the 112–284 sequence of the CAT (ΔCAT) and the mutant lacking the 319–508 sequence of the PEX (ΔPEX) were described earlier (39). Cell transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. To generate double transfectants, the parental MCF-7 cells were first transfected with the full-length β₂ integrin chain cDNA in the pcDNA3.1-neo plasmid (MCF7-β₂ cells) as described earlier (40). MCF7-β₂ cells were next transfected with the original pcDNA3.1-zeo vector or the pcDNA3.1-zeo plasmid encoding for the WT, E240A, ΔCAT, ΔPEX, ΔCYTO, PEX/MMP-2, and PEX/MMP-9 constructs to generate the respective stably transfected cell lines. Routinely, transfected cell lines were maintained in DMEM-10% FBS supplemented with G418 and/or Zeocin (0.2 mg/ml each).

In addition, to quantitatively assess the inhibitory potency of TIMP-1 and TIMP-2 against the WT and PEX/MMP-9 constructs, we employed MCF7-β₂ cells transiently transfected engineering of the inhibitory selectivity. Because of its exceedingly poor association constant, TIMP-1 is incapable of efficiently inhibiting the full-length MT1-MMP enzyme, albeit a certain level of inhibition was observed with the individual catalytic domain of MT1-MMP (21, 23, 24). A single point mutation of Thr⁹⁸ to Leu (T98L) at a distal site from the inhibitory loop, however, increased the association constant and transformed the NT-TIMP of TIMP-1 into a tight-binding inhibitor of MT1-MMP (14, 27).

There are, however, additional important structural elements, which are outside of the inhibitory and catalytic domains of the inhibitor and the proteinase, respectively. Thus, the contacts of the CT-TIMP of TIMP-1 and TIMP-3 are required with the C-terminal, non-catalytic domain to achieve an efficient inhibition of a disintegrin and metalloprotease-10 (ADAM10). The NT-TIMP alone is insufficient for the inhibition of ADAM10 (28). Conversely, the full-length TIMP-4 is a week inhibitor of ADAM17 (tumor necrosis factor-alpha converting enzyme; TACE), whereas the C-terminal truncation significantly increases the inhibitor’s potency (29). Overall, both the CT-TIMP and its counterpart, the C-terminal non-catalytic PEX in MMPs, play a likely important, albeit unidentified, role in the mechanisms of inhibition of MMPs by TIMPs.

To highlight the effect of the PEX on the interactions of MT1-MMP with the full-length TIMPs, we designed and characterized the MT1-MMP mutants with the truncated domains and also the MT1-MMP chimeras. In these chimeras the original PEX was substituted in the MT1-MMP molecule by the PEX derived from MMP-2 and MMP-9. We specifically selected MMP-2 and MMP-9 because of their contrasting proenzyme complex formations with TIMP-2 and TIMP-1, respectively. In addition, we specifically selected TIMP-1 for our experiments because of its exceedingly low ability to interact with MT1-MMP and because any improvement of the affinity of TIMP-1 would be readily recorded. Based on our experiments, we now suggest that the interactions between the PEX of the full-length MT1-MMP and the CT-TIMP also contribute to the inhibitory efficacy of TIMPs. As it appears now, the global fold of the PEX, the interdomain dynamics, and the intrinsic protein flexibility of both MMPs and TIMPs play a role in defining the binding interface in the course of inhibition of the full-length cellular MMPs by the full-length TIMPs. From the practical perspectives, it is likely that constraining the specificity of TIMPs toward the individual full-length MMPs also requires the modification in the non-inhibitory CT-TIMP and that the modifications in the inhibitory NT-TIMP alone are insufficient for the re-design of the inhibitor specificity.
with the pcDNA3.1-zeo plasmid encoding for WT and PEX/MMP-9. The cells were transfected with the recombinant plasmids using Lipofectamine LTX and the Plus reagent (Invitrogen) according to the manufacturer’s protocol. The cells were used in our inhibitory experiment in 48 h post-transfection.

**Cell Surface Biotinylation and 2-Mercaptoethane Sulfonic Acid (MESNA) Treatment**—Cells were grown in wells of a 6-well plate in the presence of GM6001 (50 μM). Cells were then surface-biotinylated using non-cleavable membrane-impermeable EZ-Link sulfo-NHS-LC-biotin (0.3 mg/ml in Sorensen phosphate buffer, pH 7.8, containing 14.7 mM KH₂PO₄, 2 mM Na₂HPO₄, and 120 mM sorbitol) (41). Cells were next lysed using 50 mM N-octyl-β-D-glucopyranoside in Tris-buffered saline supplemented with 1 mM PMSE, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM EDTA, and proteinase inhibitor mixture set III (N-octyl-β-D-glucopyranoside buffer). The biotin-labeled plasma membrane proteins were pooled down using streptavidin beads (42). The precipitates were dissolved in SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.005% Bromphenol Blue) with 100 mM DTT and analyzed by Western blotting with the MT1-MMP antibody followed by the secondary HRP-conjugated antibody and a SuperSignal West Dura Extended Duration Substrate kit.

For the uptake experiments, cells were surface-biotinylated using the cleavable membrane-impermeable EZ-Link sulfo-NHS-SS-biotin. Immediately following the biotinylation procedure, cells were incubated for 25 min at 37 °C in serum-free DMEM supplemented with 1% insulin-transferrin-selenium to allow the internalization of biotin-labeled MT1-MMP (41, 42). To remove the residual cell surface biotin, cells were incubated for 25 min on ice in Sorensen phosphate buffer containing membrane-impermeable MESNA (150 mM). Cells were next extensively washed using Sorensen phosphate buffer and lysed, and the lysates were precipitated using streptavidin beads and analyzed as above.

**Cell Treatment with MMP Inhibitors**—Cells (2 × 10⁵) were grown in DMEM-10% FBS for 18 h. Where indicated, the medium was replaced with serum-free DMEM containing TIMP-1 (100 nM), TIMP-2 (100 nM), A-TIMP-2 (100 nM), or GM6001 (50 μM). Cells were washed with PBS and lysed in the N-octyl-β-D-glucopyranoside buffer. Insoluble material was removed by centrifugation (10,000 × g; 15 min). The supernatant aliquots (3 μg of total protein) were analyzed under reducing conditions by Western blotting with the MT1-MMP antibodies as above.

**Gelatin Zymography**—Cells (2 × 10⁵) were seeded for 24 h in DMEM-10% FBS in wells of a 24-well plate. The medium was then replaced with fresh serum-free DMEM (0.225 ml/well) supplemented with pro-MMP-2 (0.5 nM). Where indicated, TIMP-1 (1–1,000 nM), TIMP-2 (1–1,000 nM), A-TIMP-2 (100 nM), or GM6001 (50 μM) was added to the samples. In 24 h the medium aliquots (20 μl) were analyzed by gelatin zymography in 0.1% gelatin-10% acrylamide gels. Alternatively, to concentrate the samples 3- to 4-fold and, as a result, to increase the sensitivity of the procedure (e.g. with the MT1-MMP chimera), the 150-μl medium aliquots were precipitated at 4 °C for 16 h using gelatin-Sepharose 4B beads (20 μl of a 50% slurry), eluted using 50 μl of SDS sample buffer, and a half of the eluted material was analyzed by gelatin zymography.

**Enzymatic Assay**—MMP activity was measured in triplicate in wells of a 96-well plate in 0.2 ml of 50 mM HEPES, pH 7.5, containing 10 mM CaCl₂ and 50 μM ZnCl₂, Mca-PLGL-Dpa-AR-NH₂ (10 μM) was used as a fluorescent substrate. The concentration of MT1-MMP and MT6-MMP in the reactions was 5 nM. The steady-state rate of substrate hydrolysis was monitored continuously (λₘ₄ = 320 nm and λₑ₄ = 400 nm) at 37 °C for 3–25 min using a fluorescent spectrophotometer. Where indicated, TIMP-1 (25–125 nM) and TIMP-2 (25–125 nM) were co-incubated for 30 min at 20 °C with the MMP samples prior to adding the substrate.

**Immunostaining of Cells**—Cells grown on 15-mm glass coverslips were fixed for 20 min with 4% formaldehyde. Where indicated, cells were permeabilized for 4 min using 0.1% Triton X-100 or left untreated. Cells were then blocked for 1 h at ambient temperature using 10% BSA in PBS and then stained overnight at 4 °C with the MT1-MMP 3G4 antibody (dilution 1:1000) or the polyclonal rabbit MT1-MMP AB815 antibody (dilution 1:200) followed by a 1-h incubation with the secondary species-specific antibody (dilution 1:200) conjugated with Alexa Fluor 594. The slides were mounted in the Vectashield medium containing DAPI for the nuclear staining. The slides were analyzed using an Olympus BX51 fluorescence microscope equipped with a MagnaFire digital camera.

**In Situ Gelatin Zymography Using FITC-gelatin**—FITC-gelatin was prepared as described earlier (33). Cells (1 × 10⁶) were seeded onto the gelatin-coated coverslips and incubated for 16 h at 37 °C in serum-free DMEM supplemented with TIMP-1 (100 nM), TIMP-2 (100 nM), or GM6001 (50 μM). The cells were then fixed with 4% formaldehyde for 16 min, permeabilized for 4 min using 0.1% Triton X-100, and stained for MT1-MMP as described above. The dark regions of degraded FITC-gelatin can be readily detected using a fluorescent microscope.

**Structural Modeling**—The structural coordinates of the porcine full-length MMP-1 enzyme complexed with a specific inhibitor N-[3-N-hydroxy carbamoyl]-2-(2-methylpropyl)-propanoyl-O-methyl-L-tyrosine-N-methylamide (PDB 1FBL) (43), the human MMP-1 E200A mutant proenzyme (PDB 1SU3) (44), the human MMP-2 E385A mutant proenzyme complexed with TIMP-2 (PDB 1GXD) (17), the human full-length MMP-2 E404A mutant proenzyme (PDB 1CK7) (45), the individual CAT of human MMP-3 in complex with human mutant (N30A and N77A) TIMP-1 (PDB 1UEA) (13), the individual CAT of MT1-MMP complexed with bovine TIMP-2 (PDB 1BUV) (12), the individual CAT of MT1-MMP complexed with the C-terminally truncated, V4A, P6V, and T98L triple mutant TIMP-1 (PDB 3MA2) (14, 23), the individual PEX of human MMP-2 (PDB 1RTG) (46), and the individual PEX of MT1-MMP (PDB 3C7X) were used in our study. The structures were analyzed and superimposed using PyMOL. The images were prepared also in PyMOL.

**RESULTS**

**TIMP-MMP Inhibitory Complex**—Like all of the other members of the MMP family, the active MT1-MMP proteinase is regulated by TIMPs. The association constant of TIMP-1 with
the MT1-MMP enzyme, however, is exceedingly poor. This association constant is significantly less efficient compared with those of TIMP-2 and TIMP-3 (23, 24). As a result, TIMP-1 is not capable of inhibiting the cellular MT1-MMP activity, especially under a physiological range of inhibitor concentrations and especially if compared with TIMP-2 (8, 21). Our work and multiple publications by others suggested that the PEX is involved in the MMP homodimerization and the association of the individual MMPs with TIMPs as well as in the interactions of MMPs with their cleavage substrates (9, 10, 31, 47–53). Based on this general assumption, we hypothesized that the structure of the PEX also contributes to the inhibitory interactions of active MMPs with TIMPs.

To support our hypothesis, we performed a thorough superimposition analysis of the available MMP and TIMP structures, including PDB 1FBL, 1SU3, 1GXD, 1CK7, 1UEA, 1BUV, 3MA2, 1RTG, and 3C7X. Thus, the overall fold of the PEX in the MMP-1 proenzyme (PDB 1SU3) is highly similar with that of the individual PEX of MMP-2 (PDB 1RTG) and MT1-MMP (PDB 3C7X). The PEX position, however, is shifted relative to the CAT in the MMP-1 enzyme (PDB 1FBL) compared with the MMP-1 proenzyme (PDB 1SU3). Similarly, there is a difference in the relative positions of the PEX and the CAT in the MMP-2 proenzyme alone (PDB 1GXD) and the MMP-2 proenzyme-TIMP-2 complex (PDB 1CK7). Our estimate suggests that the mobility range of the PEX relative to the CAT exceeds 10° as measured from the extreme conformational states in the MMP-1 (PDB 1FBL and 1SU3) and MMP-2 (PDB 1GXD and 1CK7) structures we analyzed (supplemental Fig. S1). These structural differences are distinct and additional to those local differences that were observed in the interface between the PRO and the PEX (44).

In a similar fashion, the overall fold of TIMP-1 and TIMP-2 is highly similar in their respective complexes with MMP-3 (PDB 1UEA) and MT1-MMP (PDB 1BUV and 3MA2). Notably, a significant difference is in the AB loop of NT-TIMP because of the protruding six-amino acid insert in TIMP-2 relative to TIMP-1 (54). There is, however, an ~15° difference in the position of TIMP-1 and TIMP-2 bound to the CAT of MMP-3 (PDB 1UEA), and MT1-MMP (PDB 1BUV and 3MA2), respectively (supplemental Fig. S2).

Taken together, our analysis suggests that a certain level of motion of the PEX is required to permit an inhibitory TIMP-CAT complex. In the absence of this motion, the CT-TIMP collides with the PEX moiety. As a result, the NT-TIMP appears incapable of the productive inhibitory interactions with the CAT active site (Fig. 1; supplemental Fig. S3). Naturally, molecular packing interactions in the crystal may affect the relative orientation of domains in multi-domain proteins. The mobility of the PEX relative to the CAT domains we recorded, however, significantly exceeds these parameters. To determine experimentally if the PEX affects the inhibitory characteristics of TIMPs, we performed an extensive mutagenesis of the MT1-MMP sequence followed by a characterization of the mutants, including the analysis of their sensitivity to the inhibition by TIMP-1 and TIMP-2.

**Expression and Analysis of the MT1-MMP Constructs**—To identify the role of the individual structural domains in the functionality of MT1-MMP, we constructed mutants in which the CAT, the PEX, and the CYTO were truncated in the MT1-MMP sequence (ΔCAT, ΔPEX, and ΔCYTO, respectively). We also constructed the MT1-MMP chimeras in which the PEX of MMP-2 and MMP-9 replaced the original PEX in the MT1-MMP sequence (PEX/MMP-2 and PEX/MMP-9, respectively). As controls, we used the wild-type MT1-MMP construct and the E240A mutant in which Ala substituted for the catalytically essential Glu240 residue of the proteinase active site (Fig. 2A).

The constructs were then stably co-expressed with the β3 integrin subunit in human breast carcinoma MCF-7 cells. We
specifically selected $\beta_3$ integrin-transfected MCF-7 cells as the host for our experiments. Similarly to the parental MCF-7 cells, $\beta_3$ integrin-transfected cells express neither MT1-MMP nor MMP-2. The $\beta_3$ integrin-transfected cells, however, exhibit high levels of the fully functional $\alpha_v\beta_3$ integrin (36, 40). As a result, $\beta_3$ integrin-transfected cells are easy to handle compared with MCF-7 cell transfected with MT1-MMP alone.

To assess the expression level and the catalytic activity of MT1-MMP, the obtained stably transfected cells were then examined by Western blotting and gelatin zymography (Fig. 2B). The 3G4 and AB815 antibodies (against the CAT and the hinge, respectively) were used in Western blotting. MT1-MMP immunoreactivity was not detected in the mock cell control transfected with the original plasmid lacking the MT1-MMP insert, whereas other cell types expressed the comparable level of MT1-MMP. Naturally, the 3G4 antibody did not detect the CAT construct and the degraded, 40-kDa, MT1-MMP species, which were lacking the CAT. In turn, the degraded species were absent in the inert E240A and CAT mutants. As expected, the size of the degraded CAT construct was ~20 kDa lower compared with the WT construct.

The functional activity of cellular MT1-MMP was measured using the ability of cells to activate the latent MMP-2 zymogen, a direct downstream target of MT1-MMP (4, 47, 55). Because MCF7 cells do not synthesize MMP-2 naturally, the purified MMP-2 proenzyme was added to the cells. As expected, mock, E240A, and CAT cells did not activate MMP-2, whereas all other cell types, including PEX, PEX/MMP-2, and PEX/MMP-9 cells, readily activated MMP-2 and transformed the latent 68-kDa zymogen into the 64-kDa intermediate and, predominantly, the 62-kDa mature enzyme of MMP-2 (Fig. 2B). These results directly suggest that the original PEX of MT1-MMP is not crucial for the MMP-2-processing function of MT1-MMP, and they agree well with the results of others (41, 56–58).

**Mutations Do Not Affect the Internalization Rate of MT1-MMP**—To determine whether the mutations affected the cell surface presentation and internalization rate of MT1-MMP, we used cell immunostaining. To inactivate cellular MT1-MMP and block its self-degradation, prior to immunostaining procedures the cells were cultured in the presence of GM6001 (42). The cells were then fixed and permeabilized or left untreated. The cells were next stained with the 3G4 antibody that recognizes the CAT and, as a result, reacts with the full-length proenzyme-enzyme species but not with the degraded forms of MT1-MMP. Because the CAT was missing in the CAT construct, CAT cells were stained with the AB815 antibody against the hinge region, respectively (two upper panels).

![Figure 2](http://www.jbc.org/)

**FIGURE 2. Expression of the MT1-MMP constructs.** A, domain structure of MT1-MMP mutants. S, signal peptide; PRO, prodomain; CAT, catalytic domain; H, hinge region; PEX, hemopexin domain; ST, stalk region; TM, transmembrane domain; and CYTO, cytoplasmic tail. WT, wild-type full-length MT1-MMP; $\Delta$CAT, $\Delta$PEX, and $\Delta$CYTO represent mutants lacking the sequence regions 112–284, 319–508, and 563–582 (dotted lines), respectively; E240A, the catalytically inert mutant; PEX/MMP-2 and PEX/MMP-9 are the chimeras in which the original PEX was replaced by the PEX of MMP-2 and MMP-9, respectively. B, Western blotting and gelatin zymography of mock, WT, E240A, $\Delta$CAT, $\Delta$PEX, $\Delta$CYTO, PEX, PEX/MMP-2, and PEX/MMP-9 cells. Purified pro-MMP-2 (0.15 nM) was added to the cells. Following a 24-h incubation in serum-free DMEM, gelatin beads were added to the medium aliquots to capture MMP-2. The captured samples were analyzed by gelatin zymography (bottom panel). The cells were lysed, and the lysate aliquots were analyzed by Western blotting with the MT1-MMP 3G4 and AB815 antibodies, which recognize the CAT and the hinge region, respectively (two upper panels).
Cell surface-associated MT1-MMP expression was especially evident in the non-permeabilized cells, whereas the predominantly vesicular MT1-MMP immunoreactivity was observed in the permeabilized cells. In agreement with our previous observations (39) and in contrast to other constructs, the immunoreactivity pattern of the permeabilized and non-permeabilized /H9004 CYTO cells was similar. Predominant association with the caveolin-enriched lipid rafts and the resulting slow internalization rate via the caveolae pathway explain this immunostaining pattern of the /H9004 CYTO construct (41, 59). Based on these results, we conclude that all of the MT1-MMP constructs we designed are efficiently trafficked through the cell compartment and presented on the plasma membrane of MCF-7 cells.

To corroborate this conclusion, we compared the internalization rate of cellular MT1-MMP (Fig. 3B). The cells were surface-biotinylated with membrane-impermeable, cleavable, EZ-Link NHS-SS-biotin. Biotinylation was followed by incubation of the cells at 37 °C to initiate protein uptake. Cells were next transferred on ice to arrest protein trafficking and then treated with MESNA to release the biotin moiety from the cell surface-associated proteins. Control WT cells were treated as above but at 0 °C to demonstrate the quantitative removal of the biotin label by MESNA.

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Inhibition of MT1-MMP by TIMP-1 and TIMP-2
MMP (except the ΔCYTO construct) was already internalized following a 25-min incubation. In contrast, only a small fraction of the Δ/H9004 CYTO construct was protected from MESNA, thus consistently suggesting that the Δ/H9004 CYTO MT1-MMP was inefficiently internalized (39, 41, 59).

Inhibition of MT1-MMP by TIMPs—We next evaluated the inhibitory effect of TIMP-1 and TIMP-2 on the MT1-MMP-mediated activation of MMP-2. The similarly high inhibitory activity of the TIMP-1 and TIMP-2 samples we used was confirmed using the purified individual CAT of MT6-MMP and the Mca-PLGL-Dpa-AR-NH₂ peptide as a substrate (Fig. 4A). According to our earlier data, TIMP-1 and TIMP-2 were similarly potent in the inhibition of the MT6-MMP proteolytic activity (33). In agreement, a 5 molar excess of TIMP-1 or TIMP-2 over MT6-MMP was sufficient in our current tests to achieve a near complete inhibition of the proteolytic activity, thus suggesting the equal inhibitory potency of our inhibitor samples. In sharp contrast, the purified individual CAT of MT1-MMP retained its full proteolytic activity in the presence of the high, 125 nM, TIMP-1 concentrations (at a 1:25 molar ratio of MT1-MMP/TIMP-1), whereas no proteolytic activity was recorded in the presence of a 1:5 molar ratio MT1-MMP/TIMP-2.

In agreement with the MT1-MMP test system, 100 nM TIMP-1 was incapable of affecting the ability of the WT, Δ/H9004 PEX, and Δ/H9004 CYTO MT1-MMP constructs to mediate activation of MMP-2 (Fig. 4B). In turn, 100 nM TIMP-1 performed as a potent inhibitor of the chimeric PEX/MMP-2 and, especially PEX/MMP-9 constructs, in the MT1-MMP/MMP-2 cellular activation system. As expected, TIMP-2 appended A-TIMP-2 (because of its ability to replace wild-type TIMP-2 in the TIMP-2 pro-MMP-2 complex) (60, 61), and GM6001 readily inhibited all of the MT1-MMP constructs.

These results are consistent with the effect of the inhibitors on the self-proteolysis of cellular MT1-MMP and on the level of the degraded forms of the cellular protease (Fig. 4B). As
expected, GM6001 almost quantitatively repressed MT1-MMP self-proteolysis, and, as a result, only insignificant amounts of the degraded forms were generated. TIMP-2 (100 nM) also repressed, albeit less efficiently, the self-degradation of the WT, ΔCYTO, ΔPEX, PEX/MMP-2, and PEX/MMP-9 constructs, whereas 100 nM A-TIMP-2 was without a significant effect. The inhibitory effect of 100 nM TIMP-1 was observed only with both the PEX/MMP-2 and PEX/MMP-9 chimeric constructs. Overall, the ability of TIMP-1 to inactivate the PEX/MMP-2 and PEX/MMP-9 chimeras is contrasting relative to the resistance of the individual CAT of MT1-MMP to the similar concentrations of the inhibitor.

Quantitative Assessment of the Inhibitory Potency of TIMPs—To quantitatively assess the inhibition of MT1-MMP by TIMPs, we used MCF7-β3 cells transiently transfected with the WT and PEX/MMP-9 MFC7/H9252 cells (1 × 10^5; top and bottom panels, respectively) were incubated for 24 h in serum-free medium supplemented with the MMP-2 proenzyme (0.15 nM), TIMP-1, or TIMP-2 (1–1000 nM each). The medium aliquots (0.1 ml each) were precipitated with acetone (1:4 v/v) for 18 h at −20 °C. The precipitates were collected by centrifugation and washed using ice-cold acetone. The samples were then dissolved in 2% SDS and analyzed by gelatin zymography. The gels were scanned, and the images were digitized. The levels of the MMP-2 proenzyme (68 kDa) and the MT1-MMP-generated intermediate form (64 kDa) of MMP-2 were measured in the gel images using ImageJ. The PEX/MMP-9 construct is less efficient in MMP-2 activation compared with WT. As a result, the initial conversion of the MMP-2 proenzyme into the activation intermediate was close to 50% in the PEX/MMP-9 samples rather than 100% as in the WT samples.

WT, PEX/MMP-2, and PEX/MMP-9 cells readily degraded FITC-gelatin, whereas mock cells were clearly negative. GM6001 and TIMP-2 each blocked gelatin degradation by WT and PEX/MMP-9. Because TIMP-1 does not bind the PEX domain of MMP-2, our inhibitory results, especially if combined together, cannot be explained only by the non-inhibitory TIMP-1 binding with the PEX domain in the PEX/MMP-9 and PEX/MMP-2 chimeras.

**TIMP-1 Inhibits Gelatin Degradation by MT1-MMP Chimeras**—We next measured the ability of the cellular MT1-MMP constructs, including WT, PEX/MMP-2, and PEX/MMP-9 to degrade FITC-gelatin. Mock cells were used as a control. Where indicated, TIMP-1, TIMP-2, or GM6001 were added to the samples. In 16 h the cells were fixed, stained with the MT1-MMP antibody, and observed using a fluorescence microscope. Gelatinolytic activity was detected by the presence of the dark zones of digested gelatin on the fluorescent background of the intact FITC-gelatin (Fig. 6).
**DISCUSSION**

MT1-MMP, the first characterized, archetype member of the MT-MMP family, was discovered as an MMP-2 cellular activator (47, 64). Pro-tumorigenic MT1-MMP is a key proteinase in cell migration, and its inhibitors are urgently required to combat multiple diseases, including malignancies. MT1-MMP is known to be readily inhibited by TIMPs, excluding TIMP-1, whereas other MMPs are similarly sensitive to the inhibition by TIMPs, including TIMP-1, -2, -3, and -4 (8, 14, 21). From practical perspectives, this unique relation between TIMP-1 and MT1-MMP facilitates the discrimination of the latter from other individual MMPs.

Our structural studies provided a basis for the hypothesis that the global fold and the relative positions of the PEX and the CAT manipulate the way TIMPs, including TIMP-1 and TIMP-2, interact with MMPs, including MT1-MMP. Overall, our computational analysis suggests that a noticeable motion of the PEX relative to the CAT is required to allow an inhibitory complex with the TIMP moiety. In the absence of this motion, the loop 6 of the CT-TIMP clashes with the C-terminal region of the first N-terminal propeller blade of the PEX, including MMP-1 and MMP-2 (PDB 1FBL and 1CK7), and, most probably, also MT1-MMP. As a result, the inhibitory NT-TIMP cannot interact in a productive way with the active site of the CAT (Fig. 1 and supplemental Fig. S3). The interdomain dynamics and the intrinsic protein flexibility of both MMPs and TIMPs seem to control their binding interface and play a role in the mechanisms involved in MMP inhibition by TIMPs (14). This suggestion agrees well with the studies by others who suggested that conformational adaptations are required to avoid obstacles for interaction between the full-length TIMP-1 and the CAT of MT1-MMP and MT3-MMP (65).

These previously underexploited structural data allowed us to hypothesize that the nature and the fold of the MMP’s PEX contribute to the selectivity of MMP inhibition by TIMPs. These parameters are distinct and additional to the direct interaction of the NT-TIMP with the CAT of MT1-MMP. Conversely, we suggested that, if the natural PEX is modified in MT1-MMP, the proteolytic activity of the resulting mutant may become sensitive to TIMP-1. To test our hypothesis, we constructed the PEX-swapped constructs. In the latter, the PEX of MMP-2 and MMP-9 replaced the original PEX in the MT1-MMP structure. The wild-type and the catalytically inert E240A constructs of MT1-MMP were used as controls.

We then tested the functionality of the MT1-MMP constructs. These tests included the level of the MT1-MMP presentation on the cell surface and the rate of internaliza-
tion inside the cells. In addition, we measured the ability of the constructs we designed to activate MMP-2, to self-degrade, and to hydrolyze gelatin in situ and also their response to TIMP-1, TIMP-2, and appended A-TIMP-2 with the inactivated inhibitory NT-TIMP (60). A broad-range hydroxamomate inhibitor, GM6001, was used as a control in our inhibitory tests.

We determined that, in contrast to other MT1-MMP constructs, the PEX-MMP-2 and PEX-MMP-9 chimeras became sensitive to the inhibition by both TIMP-1 and TIMP-2. These results provide evidence that the presence of these PEX moieties in the MT1-MMP structure, but not the original PEX, allows both TIMP-1 and TIMP-2 to interact with the active site in the CAT of MT1-MMP.

It is possible that in the chimeras the unnatural PEX stabilizes the interactions of the otherwise weak TIMP-1 inhibitor with the CAT of MT1-MMP. The effects of the PEX are not as prominent for TIMP-2 because of its intrinsic high affinity to the MT1-MMP’s CAT. Overall, it becomes increasingly clear that the inhibitory mechanism of MMPs by TIMPs.

The structural parameters that are involved in these dynamic interactions are not precisely clear as yet. Nevertheless, our biochemical studies suggest that constraining the TIMP specificity appears even more challenging than before and that the structural parameters of the PEX of MMPs should be taken into account for TIMP re-engineering to harness the therapeutic potential of new MMP antagonists with constrained selectivity. The use of the model systems involving the inhibitory NT-TIMP alone and the CAT of the individual MMPs may not satisfy the criteria that are required for the efficient and selective inhibition of the full-length MMPs in vivo.

REFERENCES

Inhibition of MT1-MMP by TIMP-1 and TIMP-2
