Characterization of the AB Loop Region of TIMP-2

IN INVOLVEMENT IN PRO-MMP-2 ACTIVATION

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Tissue inhibitor of metalloproteinases-2 (TIMP-2) is unique as it is the only member of the TIMP family that is involved in the cellular activation of proenzyme metalloproteinase-2 (pro-MMP-2) by virtue of forming a trimolecular complex with membrane type 1 matrix metalloproteinase (MT1-MMP) on the cell surface. TIMP-4 is similar in structure to TIMP-2 but is unable to support the activation of the proenzyme. Several reports have highlighted the importance of the TIMP-2 C-terminal domain in the pro-MMP-2 activation complex; however, very little is known about the role of the extended AB loop of TIMP-2 in this mechanism even though it has been shown to interact with MT1-MMP. In this study we show by mutagenesis and kinetic analysis that it is possible to transfer the MT1-MMP binding affinity of the TIMP-2 AB loop to TIMP-4 but that its transposition into TIMP-4 does not endow the inhibitor with pro-MMP-2 activating activity. However, transfer of both the AB loop and C-terminal domain of TIMP-2 to TIMP-4 generates a mutant that can activate pro-MMP-2 and so demonstrates that both regions of TIMP-2 are important for the activation process.

Matrix metalloproteinases (MMPs)2 have important and diverse roles in the remodeling of the extracellular matrix during development and disease (reviewed in Refs. 1–3). MMP activity is associated with tumor growth, metastasis, and neovascularization. The activities of various MMPs are controlled at the protein level via inhibition and activation by the following four homologous tissue inhibitors of metalloproteinases (TIMPs): TIMP-1, TIMP-2, TIMP-3, and TIMP-4, the most recent member of the family. TIMPs inhibit the MMPs in a 1:1 stoichiometry by tightly binding to the active site of the enzyme primarily through their N-terminal domain (4). However, TIMP binding and inhibition are improved by contacts with sites on the C-terminal domain of both the enzyme and the inhibitor (5). All four TIMPs inhibit active forms of most MMPs, but some differences in their inhibitory properties have been reported. For example, TIMP-2, TIMP-3, and TIMP-4 are effective inhibitors of the membrane-bound MMPs, whereas TIMP-1 is a very poor inhibitor of these enzymes (6). TIMPs have no inhibitory activity against astacins, but TIMP-3 and to some extent TIMP-1 have been shown to be active against the ADAMs. TIMP-3 inhibits ADAM-10, -12, and -17 and the aggrecan-degrading enzymes ADAM-TS4 and ADAM-TS5, whereas TIMP-1 inhibits ADAM-10 (7–9). The association of TIMPs with pro-MMP-2 and pro-MMP-9 is also specific. TIMP-2 binds to pro-MMP-2 through the hemopexin-like domain of the enzyme and facilitates enzyme activation, an action that is unique to TIMP-2; TIMP-1 binds to pro-MMP-9, whereas TIMP-3 binds to both proenzymes (10–12). TIMP-4 also binds to pro-MMP-2 through the hemopexin-like domain (13); however, it is unable to promote the activation of thezymogen by MT1-MMP (14, 24, 28), although it is an excellent inhibitor of the latter. Recent mutagenesis studies by several groups have identified residues Glu192 and Asp193 of the C-terminal “tail” of TIMP-2 as the key residues for binding to pro-MMP-2 (15, 16). The lack of these residues in the TIMP-4 C-terminal tail probably reduces the stability of complex formation with the hemopexin-like domain of pro-MMP-2 leading to the inability of TIMP-4 to promote activation of pro-MMP-2. These findings were substantiated by the structural study of the pro-MMP-2–TIMP-2 complex by Morgunova et al. (17).

Another common feature of TIMP-2 and TIMP-4 is the extended hairpin between strands A and B. We had shown previously that Tyr56 at the tip of the hairpin in TIMP-2 makes unique and specific interactions with MT1-MMP at a site away from the catalytic site of the enzyme (18, 19). Our aim in this paper was to investigate the importance of this region in TIMP-4 for MT1-MMP inhibition and whether the extended AB loop region of TIMP-2 is required for the pro-MMP-2 activation complex. First, we created a number of N-TIMP-4 constructs, one without the AB loop and others where the AB loop of TIMP-4 had been replaced with elements of the TIMP-2 loop. Kinetic analyses of the mutants showed that they were superior inhibitors of MT1-MMP compared with N-TIMP-2 and N-TIMP-4. Second, we then fused these N-TIMP-4 mutants to the C-terminal domain of either TIMP-2 or TIMP-4 and investigated whether they potentiate activation of pro-MMP-2 in a dose-dependent manner. For this study we used an in vitro cell membrane-based assay derived from TIMP-2−/− cells.
EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were purchased from Sigma unless otherwise stated. Restriction enzymes and *Pwo* DNA polymerase were obtained from Roche Applied Science. For the kinetic experiments the fluorometric substrate QF24 (7-methoxycoumarin-4-yl-acetyl-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) was used (33). Kinetic assays were performed at 27 °C in fluorescence assay buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM CaCl$_2$, 0.05% Brij-35, 1% Me$_2$SO, 0.02% NaN$_3$) with a PerkinElmer Life Sciences LS-50B spectrofluorimeter equipped with thermostatically controlled cuvette holders.

Preparation of N-terminal and Full-length TIMPs—Construction of point and deletion/insertion mutants into either N-TIMP-2 or N-TIMP-4 sequences was carried out by a two-step PCR protocol. In step 1, the desired construct was amplified by PCR in two parts by using oligonucleotides coding for the mutation at their translated C and/or N termini. The PCR primers coding for the sequence where the two parts of the construct were to be joined were 5’-phosphorylated in order to allow subsequent ligation. The PCR fragments were gel-purified and ligated using T4 ligase. In step 2, nonmutagenic primers were used to amplify the final construct using as template the ligation mixture from step 1.

Full-length TIMP-2 constructs were generated by ligation of the appropriately engineered N-terminal domain with the C-terminal domain of wild-type TIMP-2 using the available BamH1 site at position 125 in the coding sequence. Full-length TIMP-4 constructs were generated in a similar way using the Xcm1 site at position 376 in the coding sequence. This restriction site was also used to generate the chimeric T2AB-NT4#CT2 and IYG-NT4#CT2 mutants by taking the wild-type C-terminal domain from the previously described NT4#CT2 construct (16). All constructs were inserted into pET23a (Novagen) and sequenced to confirm their identity. The sequences for human TIMP-2 and murine TIMP-4 were used in this study.

Protein Production—Active TIMP proteins were prepared from inclusion bodies after expression in *Escherichia coli* BL21 (DE3) cells and refolded as described previously (20, 21). In some cases it was found that addition of 0.5 M l-arginine to the refolding buffer was necessary for efficient renaturation of the protein. All proteins were purified by ion exchange chromatography, and their activities were determined by titration against the protein. All proteins were purified by ion exchange chromatography. Active TIMP proteins were prepared with 0.5 mg/ml gelatin. TIMP concentrations.

Trimolecular Complex Formation between Pro-MMP-2, TIMP-2/TIMP-4, and MT1-MMPcat —The catalytically inactive pro-MMP-2 mutant E375A (25) was incubated with equal amounts of wild-type and mutant TIMPs in TCAB buffer (50 mM Tris, pH 7.4, 10 mM CaCl$_2$, 0.02% NaN$_3$, 0.05% Brij-35) for 5 h at 4 °C. Active MT1-MMP$_{cat}$ was then added at 2-fold molar excess, and the mixture was incubated for a further 4 h at 4 °C to allow trimolecular complex formation. The mixtures were then applied to gelatin-agarose mini columns (50 µl), and the columns were washed with TCAB buffer containing 1 M NaCl (wash buffer). Pro-MMP-2 and pro-MMP-2-bound proteins were eluted with 10% Me$_2$SO in wash buffer, and the elution fractions were analyzed by SDS-PAGE. Western blots were developed using polyclonal antibodies to MT1-MMP, TIMP-4, TIMP-2, pro-MMP-2, and a peroxidase-conjugated donkey anti-sheep antibody (Jackson ImmunoResearch). Antibody staining was visualized by ECL.

Inhibition Constant Measurement ($K_i^{app}$) —MMP-2 (0.05 nM) and MT1-MMP (0.15 nM) were preincubated with increasing concentrations of the N-terminal or the full-length TIMP mutants. Incubation was performed at room temperature for 3 h before steady-state measurement ($V_s$). Reactions were initiated by addition of the quenched fluorescent substrate QF24 to a final concentration of 1 µM. The $K_i^{app}$ was determined from the steady-state rates plotted against TIMP concentration. All data were fitted into the tight binding equation using the computer program Grafit to obtain an estimation of $K_i^{app}$ values using Equation 1 (27).

\[
V_s = \left(\frac{V_e}{2E_t}\right) \times \left\{ \left( E_t - I_t - K_i^{app}\right) + \left( K_i^{app} + I_t - E_t\right)^2 + 4E_t K_i^{app} \right\}^{1/2}
\]  

(Eq. 1)

where $V_e$ is the rate in the absence of inhibitor; $E_t$ is the total enzyme concentration, and $I_t$ is the total inhibitor concentration.

Association Rate Constant Measurement ($k_{on}$) —Association rate measurements ($k_{on}$) were performed by adding different concentrations of the TIMP mutants to MMP-2 (0.015 nM) or MT1-MMP (0.05 nM). The rate of inhibition was followed using a continuous fluorometric assay at 27 °C until steady state had been reached. The progress curves were analyzed using the program Grafit and Equation 2 (27).

\[
P = \left( V_o - V_e\right) \left( 1 - e^{-kt}\right) / k
\]  

(Eq. 2)

where $P$ is the product concentration; $V_o$ is the initial velocity; $V_e$ is the steady-state velocity, and $k$ is the apparent first order rate constant of equilibrium between enzyme and TIMP complex. $k_{on}$ values were calculated by linear regression of $k$ on TIMP concentrations.

MT1-MMP-containing Membranes—TIMP-2$^{-/-}$ mouse fibroblasts stably transfected with MT1-MMP were grown in the presence of 1 µM of the MMP inhibitor BB94 to stabilize cell surface MT1-MMP (16, 36). When 80% confluent, the cells were washed three times with Dulbecco's modified Eagle's medium, pH 9.0, to remove MT1-MMP-associated BB94, scraped from the flasks, and pelleted by centrifugation (1200 × g). Lysates were prepared by resuspending cells in 5 mM Tris-HCl, pH 7.6, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, and 1 µg/ml trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane, and incubated on ice before homogenization through a narrow gauge needle (26-gauge) as described by Worley et al. (16). Cell debris and nuclei were removed by...
centrifugation at low speed (28,000 × g), and the crude plasma membranes were diluted into the same buffer but containing only 10 mM Tris-HCl, pH 7.9. Brains were diluted into the same buffer but containing only 0.02% (w/v) NaN₃. For subsequent experiments, the membranes were diluted into the same buffer but containing only 10 mM Tris-HCl, pH 7.9.

RESULTS

Design of AB Loop Mutants—In our previous kinetic study (19), we showed that the side chain of Tyr³⁶ in the AB loop of TIMP-2 was responsible for a specific interaction with the catalytic domain of MT1-MMP (Table 1). Deletion of the entire tip of the AB loop of TIMP-2 (Pro³⁵–Ala³⁶) with that from TIMP-2 (Ile³⁵–Gly³⁷); and (iv) the exchange of the tip of the AB loop of TIMP-4 (Pro³⁵–Ala³⁶) with IYG from the tip of the TIMP-2 AB loop; (ii) the exchange of the entire AB loop of TIMP-4 (Pro³⁰–Lys⁴¹) with that from TIMP-2 (Asp³⁰–Lys⁴¹); (iii) the exchange of the tip of the AB loop of TIMP-4 (Pro³⁵–Ala³⁶) with IYG from the tip of the TIMP-2 AB loop; and (iv) the exchange of the tip of the AB loop of N-TIMP-2 but associates with the proteinase at a 20-fold slower rate (Table 1). To determine whether the extended AB loop of TIMP-4 is similarly important for MT1-MMP interaction and to further investigate the properties of the TIMP-2 AB loop, we constructed a series of mutations where the extended AB loop of N-TIMP-4 was either deleted or replaced with the corresponding region of TIMP-2 (Fig. 1). Four N-TIMP-4 mutants were constructed and expressed as follows: (i) the deletion of the entire extended AB loop of TIMP-4 (residues Lys³³–Gln³⁹); (ii) the exchange of the entire AB loop of TIMP-4 (Pro³⁰–Lys⁴¹) with that from TIMP-2 (Asp³⁰–Lys⁴¹); (iii) the exchange of the tip of the AB loop of TIMP-4 (Pro³⁵–Ala³⁶) with that of TIMP-2 (Ile³⁵–Gly³⁷); and (iv) the exchange of aspartic acid for asparagine at position 37. These four N-TIMP-4 mutants are referred to as ΔAB-NT4, T2AB-NT4, IYG-NT4, and D37N-NT4 respectively.

To investigate the role of the AB loop of TIMP-2 in pro-MMP-2 activation, full-length MMP-2 mutants and TIMP-4/TIMP-2 chimeras were constructed and expressed. These mutants include the following: (i) deletion of the extended AB loop from full-length TIMP-2 (residues Asp³⁴–Ile⁴¹); (ii) the exchange of the tip of the AB loop of TIMP-4 (Pro³⁵–Ala³⁶) with IYG from the tip of the TIMP-2 AB loop; (iii) the same AB loop exchange as ii above but with the C-terminal subdomain of TIMP-4 replaced with that from TIMP-2, and (iv) the same N-TIMP-4/C-TIMP-2 construct as in iii above but with the entire AB loop of TIMP-4 (Pro³⁰–Lys⁴¹) exchanged with that of TIMP-2 (Asp³⁰–Lys⁴¹) exchanged with that of TIMP-2. These mutants are referred to as ΔAB-T2, IYG-T4, IYG-NT4#CT2, and T2AB-NT4#CT2, respectively. The boundaries of the N- and C-terminal domain are shown in Fig. 2, and the mutations made in the AB loop regions are shown in Fig. 1.

Expression and Refolding of N-TIMP and Full-length TIMP Constructs—All proteins were expressed in E. coli and refolded from inclusion bodies according to the procedure developed for N-TIMP-2 (21). The ΔAB mutants of N-TIMP-4 and TIMP-2 required the addition of 0.5 M l-arginine in the refolding buffer to achieve appreciable yields of renatured protein. The mutant TIMPs were purified by ion exchange chromatography and analyzed on reducing and nonreducing SDS-polyacrylamide gels. No evidence of band smearing was seen on nonreducing gels, which is taken to be a good indicator of correct disulfide bond formation (data not shown). The structural authenticity of the constructs was confirmed by electrospray mass-spectrometry, and the constructs were found to be between 70 and 80% active by active site titration against MMP-2 (data not shown). Previous structural work has shown that the AB loop of TIMP is highly solvent-exposed and can accommodate changes to its sequence without any perturbation of the rest of the protein structure (19).

Kinetic Analysis of N-TIMP-4 Mutants with Structural Features from the AB Loop of TIMP-2—Each of the N-TIMP-4 mutants was assessed for its rate of association ($k_\text{on}$) and the final equilibrium constant ($K_\text{eq}$) with MMP-2 and the catalytic domain of MT1-MMP (Table 1). Deletion of the entire tip of
The AB Loop of TIMP-2

The AB loop of the molecule (ΔAB-NT4) resulted in a 3-fold decrease in the association rate and a 5-fold increase in the apparent inhibition constant compared with full-length TIMP-2 (Fig. 3A). These values were not as dramatic as the values obtained when the AB loop of N-TIMP-2 was deleted (200-fold decrease in $k_{o_n}$ and 100-fold increase in $K_d$ (18)). It therefore appears that the extended AB loop region in N-TIMP-4 is not as important for the MT1-MMP interaction as the corresponding region in N-TIMP-2. This finding was supported by analysis of the loop replacement mutant (T2AB-NT4). The association rate for MT1-MMP binding for this mutant was increased 30-fold, and the binding affinity for MT1-MMP was 22-fold lower than that for full-length TIMP-2 for MT1-MMP (both catalytic and soluble constructs) (19). Deletion of Asp$^{37}$ as part of the extended AB hairpin from N-TIMP-4 (ΔAB-NT4, where only a modest 5-fold increase on $K_d$) was seen and exchange of Asp$^{37}$ for Asn (the corresponding TIMP-2 residue) has disproved this hypothesis. The D37N-NT4 mutant showed only a small change in the association rate (3-fold increase) and binding affinity (2-fold decrease) for MT1-MMP. Therefore, there is no evidence to suggest that Asp$^{37}$ of N-TIMP-4 is responsible for any binding interactions with MT1-MMP, and the effect of the Asn to Asp change in N-TIMP-4 appears to be unique to this inhibitor.

Kinetic Analysis of the Full-length TIMP-2 Mutants and TIMP-4#TIMP-2 Chimeras—The association rate constant ($k_{o_n}$) for TIMP-4 binding to MT1-MMP was smaller and the inhibition constant ($K_d$) value obtained slightly larger than that for TIMP-2 (Table 2). Comparison of the full-length and C-terminally truncated constructs showed that the C-terminal subdomains of both TIMP-2 and TIMP-4 make significant interactions with the MT1-MMP catalytic domain. The binding constant for the interaction of full-length TIMP-2 with MT1-MMPcat was 46-fold lower than the N-terminal domain, and a similar comparison for TIMP-4 revealed a 6.7-fold decrease. In contrast, the presence of the C-terminal hemopexin-like domain of MT1-MMP had no effect on the inhibitory binding of TIMP-2 and only a small effect on TIMP-4 (Table 2).

The MT1-MMP binding constants obtained for the loopless TIMP-2 (ΔAB-T2) help to confirm our previous findings with N-TIMP-2 (19). Deletion of the AB loop reduces the affinity of TIMP-2 for MT1-MMP (both catalytic and soluble constructs) by over 200-fold. However, the effect of the AB loop mutations on the rate of association was less marked for full-length TIMP-2 compared with the wild-type. The change in $k_{o_n}$ for ΔAB-NT2 binding to MT1-MMPcat was 200-fold lower than the wild-type compared with only 14-fold lower for ΔAB-T2. This difference must be because of the presence of the C-terminal domain, but it is interesting to note that no elevation of $k_{o_n}$ is seen for the full-length wild-type inhibitor compared with its N-terminal domain.

Kinetic analysis of the full-length TIMP-4 mutant (IYG-T4) and TIMP-4#TIMP-2 chimeras showed that the binding constants and association rates for MT1-MMP were decreased and increased, respectively, by incorporating either the entire AB loop of TIMP-2 or just its tip (IYG). However, as seen for full-length TIMP-2 mutants, these changes were more modest than seen for the same mutations in the isolated TIMP-4 N-terminal domain.
The AB Loop of TIMP-2

The role of the C-terminal domain of TIMP-2 to the IYG-T4 mutant (IYG-NT4#CT2) had only a small effect on $k_{on}$ and $K_{i,app}$ values.

Binding of TIMP-2, TIMP-4, and Mutant Constructs to Pro-MMP-2 and MT1-MMP—It has been well documented that TIMP-4 does not support the activation of pro-MMP-2 by MT1-MMP on the cell surface (14, 16, 28). The formation of a stable trimolecular complex between TIMP-2, pro-MMP-2, and MT1-MMP is believed to be a key step in the process of pro-MMP-2 activation. To investigate the inability of TIMP-4 to participate in the activation of pro-MMP-2, a gel binding experiment was used to compare the stability of the trimolecular complex formed with TIMP-2, TIMP-4, and the full-length TIMP-2/TIMP-4 mutants. Fig. 4A shows that TIMP-4 and all the TIMP mutants were recovered from the column bound to pro-MMP-2, although in significantly lesser amounts than TIMP-2. The column elution fractions were also analyzed by using an MT1-MMP antibody (Fig. 4B). MT1-MMPcat alone does not bind gelatin-agarose or pro-MMP-2 (35); therefore, MT1-MMPcat in the column elution represents the protein complexed with pro-MMP-2 and TIMP. MT1-MMPcat was present in the TIMP-2 experiment as demonstrated previously (Fig. 4B, lane 1) (16, 24). In the case of IYG-NT4#CT2 and T2AB-NT4#CT2 (Fig. 4B, lanes 5 and 6), MT1-MMPcat was also present in the elution fraction, although in much lower amounts than in the TIMP-2 experiment, confirming that these constructs form a trimolecular complex with pro-MMP-2 and MT1-MMP, but the complex is much less stable. In the case of ΔAB-T2, TIMP-4, and IYG-T4 (Fig. 4B, lanes 2–4), MT1-MMPcat was not present in the eluted samples suggesting that these constructs were unable to form a stable trimolecular complex. Previous studies have shown that the domain-swap mutant NT4#CT2 was also able to form the trimolecular complex, whereas NT2#CT4 was not (16). Together with this work, these results show that the C-terminal domain of TIMP-2 is essential for trimolecular complex formation as is the TIMP-2 AB loop when in a TIMP-2 context; however, the N-terminal domain of TIMP-4 can substitute for N-TIMP-2 when the entire domain is replaced. These findings reflect the relative binding affinities for MT1-MMPcat. The $K_{i,app}$ of ΔAB-T2 is 100-fold greater than both wild type N-TIMP-2 and N-TIMP-4 (19) (Table 1), whereas IYG-NT4 and T2AB-NT4 showed $K_{i,app}$ values at least 20-fold less than the wild type N-terminal domains.

The Role of the AB Loop in Pro-MMP-2 Activation—An in vitro assay was set up to investigate whether the difference between TIMP-2 and TIMP-4 in their ability to promote activation is a consequence of the differences in the AB loop regions of the proteins. Exogenous TIMP and pro-MMP-2 were added to fibroblast membranes from TIMP-2−/− mouse cells stably expressing MT1-MMP and pro-MMP-2 activation measured by a molecular weight shift seen on gelatin zymogra-

![Figure 3](image-url)

**FIGURE 3.** Inhibition profiles of wild-type N-TIMP-4 and the AB loop mutants against the catalytic domain of MT1-MMP. MT1-MMP (0.4 nM) was incubated with increasing amounts of wild-type N-TIMP-4 and the N-TIMP-4 mutants. The remaining activity of MT1-MMP was measured after 3 h of incubation. A, deletion of the AB loop of N-TIMP-4 (ΔAB-NT4) compromises the binding affinity of the molecule for MT1-MMP when compared with wild-type N-TIMP-4 (N-TIMP-4, B, complete exchange of the AB loop of N-TIMP-4 with that of the N-TIMP-2 molecule, T2AB-NT4) or partial exchange of residues found at the tip of the loop (IYG-NT4, D37N-NT4) dramatically improves the binding affinity. Replacement of Asp37 in N-TIMP-4 with Asn (the equivalent residue in N-TIMP-2) (D37N-NT4) does not further improve the binding affinity of the inhibitor.

**TABLE 2**

| Construct | MT1-MMP soluble, $k_{on}$ | MT1-MMP cat, $k_{on}$ | MT1-MMP soluble, $K_{i,app}$ | MT1-MMP cat, $K_{i,app}$ |
|-----------|--------------------------|----------------------|--------------------------------|-------------------------|
| TIMP2     | 2.44 ± 0.36              | 2.0 ± 0.01           | 0.024                          | 0.026                   |
| ΔAB-T2    | 0.2 ± 0.07               | 0.14 ± 0.07          | 6.5 ± 0.7                      | 5.5 ± 0.4               |
| TIMP4     | 0.543 ± 0.005            | 0.88 ± 0.09          | <0.05                          | 0.016 ± 0.01            |
| IYG-T4    | 1.37 ± 0.33              | 1.86 ± 0.05          | <0.05                          | <0.05                   |
| IYG-NT4#CT2 | 0.4 ± 0.009         | 1.12 ± 0.01          | <0.05                          | 0.08 ± 0.001            |
| T2AB-NT4#CT2 | 3.95 ± 0.4            | 0.9 ± 0.02           | <0.05                          | 0.09 ± 0.002            |

On average each determination was carried out four times, and the standard error is shown. Assays were carried out at 27 °C. Values more than 10-fold different to the respective wild-type TIMP are shown in boldface. $K_{i,app}$ values could only be estimated reliably to 0.05 nM; therefore, values below this limit are expressed as <0.05 nM.
The AB Loop of TIMP-2

DISCUSSION

The activation of pro-MMP-2 is a cell membrane-mediated process in which the activator has been identified as MT1-MMP (29, 30). In the model that is widely accepted to date, active MT1-MMP binds the N-terminal domain of TIMP-2, and this complex functions as a “receptor” for the 72-kDa pro-MMP-2. The hemopexin-like C-terminal domain of pro-MMP-2 interacts with the C-terminal domain of TIMP-2 forming a trimeric complex on the cell surface (31). The formation of the complex enhances the rate of propeptide cleavage by TIMP-2-free MT1-MMP to generate the intermediate 66-kDa form that is then rapidly converted autocatalytically to the fully active 62-kDa species. The concentration of TIMP-2 is crucial in mediating the first step of the activation process; too low a concentration results in insufficient proenzyme localization on the cell surface, and too high a TIMP-2 concentration inhibits the first cleavage step by inhibiting all available MT1-MMP.

Among the four members of the TIMP family, only TIMP-2 has been shown to be actively involved in the activation process of pro-MMP-2 by MT1-MMP. However, TIMP-4 can also bind to pro-MMP-2, and there is considerable sequence identity between the two inhibitors (Fig. 2) (32). In view of these similarities, it was our aim to investigate the specific molecular determinants that make TIMP-2 so unique to the pro-MMP-2 activation process.

Several reports have focused on the importance of the C-terminal domain of TIMP-2 for pro-MMP-2 activation (15, 16, 28). The results show that the charged residues Glu192–Asp193 in the unstructured tail of TIMP-2 are crucial for the activation process. A comparison of the amino acid sequences for TIMP-2 and TIMP-4 leads to the conclusion that the interaction of TIMP-4 with pro-MMP-2 would be considerably weaker than with TIMP-2, because only three of the five salt bridges formed between the TIMP C-terminal tail and pro-MMP-2 are conserved in TIMP-4. Additionally, the hydrophobic interactions with the fourth blade of the hemopexin domain of pro-MMP-2 probably do not exist in TIMP-4 because a hydrophilic threonine replaces the crucial methionine at position 149 (Fig. 2).

We focused our study on the interactions of the N-terminal inhibitory domain of TIMP-2 and TIMP-4 with MT1-MMP...
and, specifically, the extended AB loop region known to be important for the interaction. We created a number of constructs for the N-terminal domain of TIMP-4 (NT4) where the extended AB loop was either deleted (ΔAB-NT4), exchanged (T2AB-NT4), partially exchanged (IYG-NT4), or point mutated (D37N-NT4) with the corresponding region of TIMP-2 (Fig. 1). Kinetic analysis of the above mutants with the catalytic domain of MT1-MMP revealed that it is possible to transplant the binding properties of the TIMP-2 AB loop to the TIMP-4 molecule by simply "swapping" their AB loop regions. The significant increased affinity obtained suggests that the new binding function of the TIMP-2 AB loop does appreciably affect the original N-TIMP-4 binding interaction, showing that by this method it is possible to engineer new molecules with tighter binding properties than those naturally existing. The association rates for the two mutants were also increased making them comparable with N-TIMP-2. This result suggests that the difference in association rate between N-TIMP-4 and N-TIMP-2 (N-TIMP-4 50-fold slower) is because of the lack of a tyrosine residue at the tip of the AB hairpin. These mutations had no effect in the binding affinity and association rate toward MMP-2, indicating that the AB loop interactions are particularly important for MT1-MMP interaction.

Kinetic analysis of the full-length loopless TIMP-2 and the TIMP-4#TIMP-2 chimeras revealed that the C-terminal domains of the inhibitors make significant interactions with the catalytic domain of MT1-MMP, confirming our earlier results (16). We also wanted to investigate whether the full-length inhibitors make specific interactions with the hemopexin-like domain of MT1-MMP (MT1-MMP soluble construct). It appears that the presence of this domain had little effect on TIMP-2 binding; however, TIMP-4 binding was slower (Table 2). The two TIMP-4 chimeras gave inconsistent results with the IYG-NT4#CT2 mutant binding slower to the soluble form of MT1-MMP and T2AB-NT4 binding faster. Bigg et al. (28) reported a higher association rate for TIMP-4 binding to MT1-MMP soluble (3.5 × 10⁻⁶ M⁻¹ s⁻¹) than was determined in this study (0.54 × 10⁻⁶ M⁻¹ s⁻¹), although the values for TIMP-2 were more similar (1.8 × 10⁻⁶ M⁻¹ s⁻¹ compared with 2.44 × 10⁻⁶ M⁻¹ s⁻¹). The difference in TIMP-4 binding may be a result of the different species of TIMP-4 used to conduct the studies. Bigg et al. (28) used human TIMP-4, whereas murine TIMP-4 was used in this study. The two sequences are, however, highly similar (91% sequence identity; Fig. 2), and it is unlikely that serious species differences occur.

FIGURE 5. Reconstitution of pro-MMP-2 activation by MT1-MMP and full-length TIMP mutants. Membranes from TIMP-2−/− fibroblasts expressing MT1-MMP were incubated with increasing concentrations of exogenously added TIMPs in the presence of pro-MMP-2 (1 μg/ml) at 37 °C for 24 h. Processing of pro-MMP-2 was monitored by gelatin zymography. Efficient processing to the fully active form of MMP-2 was obtained only in the presence of wild-type TIMP-2 (panel 1, boxed lanes). Wild-type TIMP-4 and the IYG-T4 construct are unable to activate pro-MMP-2 (panels 2 and 4). Conversion to the intermediate and active form was observed for T2AB-NT4#CT2 (panel 5, boxed lanes). Lane 1, pro-MMP-2; lane 2, pro-MMP-2 with cell membranes; lanes 3–11, as lane 2 but incubated in the presence of increasing amounts of TIMPs.
We investigated the importance of the AB loop of TIMP-2 in the activation of pro-MMP-2 by using MT1-MMP-enriched membranes derived from TIMP-2−/− cells. In this system, the ability of “loopless” TIMP-2 (ΔAB-T2) to promote processing of pro-MMP-2 to the intermediate and fully active form was considerably compromised. The binding affinity of ΔAB-T2 for MT1-MMP was decreased dramatically (270-fold), and the rate by which this mutant associated with the enzyme was also reduced (12 times slower than the wild type). Consequently, the ΔAB-T2-pro-MMP2 complex may not be able to bind tightly enough to the membrane-bound MT1-MMP to generate sufficiently high enough concentrations of proenzyme at the cell surface to allow successful activation of pro-MMP-2 to occur. This hypothesis is supported by the results obtained from the trimolecular complex formation experiment. ΔAB-T2 was unable to form a stable complex with MT1-MMPcat and pro-MMP-2, whereas wild-type TIMP-2 was (Fig. 4B).

Incorporation of the tip of the AB loop of TIMP-2 into TIMP-4 (IVG-T4) was insufficient to bring about processing of pro-MMP-2. This construct was, however, able to reduce background activation by inhibiting MT1-MMP in the same way as wild-type TIMP-4. When the C-terminal domain of TIMP-4 was replaced with that of TIMP-2 (in the T2AB-NT4#CT2 construct), activation to the intermediate and active forms was observed, especially the latter. T2AB-NT4#CT2 was the only TIMP-4 construct out of three tested in the pro-MMP-2 activation experiment to show trimolecular complex formation with pro-MMP-2 and MT1-MMPcat (Fig. 4B), confirming that stable complex formation is an essential part of the activation process. A stable trimolecular complex was also observed in our earlier work with NT4#CT2 (16), but here activation was only seen to the intermediate form, hence full interaction appears to be a property of the inserted TIMP-2 AB loop. It is currently unclear as to why this should be, but it is unlikely to be due simply to differences in the strength of the binding interaction with MT1-MMP. It is possible that the presentation of pro-MMP-2 in the trimolecular complex on the cell membrane is different with the two TIMP constructs acting as the “adaptor” molecule, and this could have a significant effect on the activation process. It is clear that there are differences in the way that the N-terminal domains of TIMP-4 and TIMP-2 bind to MT1-MMP as TIMP-4 achieves a similar binding affinity without receiving a large binding contribution from the AB loop region (Table 1). It is not known what additional interactions TIMP-4 makes to compensate for this loss or how these changes affect the relative orientations of the three molecules in the trimolecular complex. If the presentation of pro-MMP-2 is adversely affected by the TIMP-4 interaction, then the insertion of the TIMP-2 AB loop into the TIMP-4 N-terminal domain may correct the orientation to be more TIMP-2-like.

The findings presented in this study show that the AB loop of TIMP-2 is a contributing factor for the MT1-MMP-mediated activation of pro-MMP-2. When it is absent from TIMP-2, the resulting mutant is unable to form stable trimolecular complexes with pro-MMP-2 and MT1-MMP or enhance pro-MMP-2 activation in the presence of cell membrane. When this TIMP-2 AB region is transferred to the TIMP-4 inhibitor, the resulting mutant is a more potent inhibitor of MT1-MMP activity than either wild-type TIMP-2 or TIMP-4. This inhibitor, however, was still unable to potentiate activation of pro-MMP-2 in the cell membrane activation assay or form a stable trimolecular complex. Complex formation and full activation of pro-MMP-2 were only seen when both the TIMP-2 AB loop and the C-terminal domain of TIMP-2 were inserted into TIMP-4 (the T2AB-NT4#CT2 mutant). These findings therefore suggest that both the AB loop and the TIMP-2 C-terminal domain are required for successful pro-MMP-2 activation via the MT1-MMP/TIMP-2 receptor on the cell membrane.

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