Role of TIMPs (tissue inhibitors of metalloproteinases) in pericellular proteolysis: the specificity is in the detail


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Abstract

Pericellular proteolysis represents one of the key modes by which the cell can modulate its environment, involving not only turnover of the extracellular matrix but also the regulation of cell membrane proteins, such as growth factors and their receptors. The metzincins are active players in such proteolytic events, and their mode of regulation is therefore of particular interest and importance. The TIMPs (tissue inhibitors of metalloproteinases) are established endogenous inhibitors of the matrix metalloproteinases (MMPs), and some have intriguing abilities to associate with the pericellular environment. It has been shown that TIMP-2 can bind to cell surface MT1-MMP (membrane-type 1 MMP) to act as a ‘receptor’ for proMMP-2 (progelatinase A), such that the latter can be activated efficiently in a localized fashion. We have examined the key structural features of TIMP-2 that determine this unique function, showing that Tyr36 and Glu192-Asp193 are vital for specific interactions with MT1-MMP and proMMP-2 respectively, and hence activation of proMMP-2. TIMP-3 is sequestered at the cell surface by association with the glycosaminoglycan...
glycan chains of proteoglycans, especially heparan sulphate, and we have shown that it may play a role in the regulation of some ADAMs (a disintegrin and metalloproteinases), including tumour necrosis factor α-converting enzyme (TACE; ADAM17). We have established that key residues in TIMP-3 determine its interaction with TACE. Further studies of the features of TIMP-3 that determine specific binding to both ADAM and glycosaminoglycan are required in order to understand these unique properties.

Introduction

Cell–matrix and cell–cell interactions influence a diverse range of cellular functions, including proliferation, differentiation, migration and survival. Proteolytic degradation or activation of cell surface and extracellular matrix proteins can mediate rapid cellular responses to their microenvironment, and hence modulate cell behaviour. Members of the metzincin clan of zinc endopeptidases (metalloproteinases) are key players in such activities, and have been the subject of intense study in relation to the biology of the cell. Of particular interest are the strategies adopted by the cell to focus and control proteolytic activities, which range from directed trafficking and cell surface localization to extracellular matrix binding. Proteolytic activation cascades are also important in the regulation of functional activity at the cell surface, as well as control by natural inhibitors.

Both secreted and membrane-bound forms of metalloproteinases have been implicated in pericellular proteolysis, including the matrix metalloproteinases (MMPs), the adamalysin proteinases with both metalloproteinases and disintegrin-like domains [ADAM (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin motifs)] and the astacins [1]. Comprehensive descriptions of all the known members of these families have been published recently [2–4] and will not be repeated here.

The tissue inhibitors of metalloproteinases (TIMPs)-1 to -4 were originally identified as natural regulators of the MMPs in mammalian cells. The four TIMPs have many basic similarities, but they exhibit distinctive structural features and biochemical properties, suggesting that each has specific roles in vivo [5,6]. They have an N-terminal domain of three disulphide-bonded loops, and a C-terminal subdomain that also comprises three disulphide-bonded loops that has specific functions in binding to some MMPs. The ability of these proteins to inhibit the MMPs is due largely to the interaction of a wedge-shaped ridge on the N-terminal domain which binds within the active-site cleft of the target MMP, allowing the co-ordination of the catalytic Zn$^{2+}$ of the MMP by the α-amino and carbonyl groups of the N-terminal Cys, while the side chain of the Ser/Thr at position 2 occupies the S1’ pocket. Variations in TIMP binding to MMPs can also occur, e.g. TIMP-1 is a poor inhibitor of a number of the membrane-type MMPs (MT-MMPs) and MMP-19.

This review will summarize our contributions towards the understanding of the functions of TIMP-2 and TIMP-3 in regulating pericellular proteolysis through the analysis of features that form the structural basis of their specificity. The implications of our findings regarding the regulation of cellular function are discussed.
Activation of proMMP-2 and the role of TIMP-2

The activation of MMPs by sequential proteolysis of the propeptide blocking the active-site cleft is regarded as one of the key levels of regulation of these proteinases. Current thinking, based on many studies, is that these events may be orchestrated in a controlled fashion close to the cell surface, with membrane-associated proteinases such as urokinase-type plasminogen activator and MT-MMPs playing key initiating roles (Figure 1) [7]. The activation of the secreted MMP, proMMP-2 (progelatinase A), has proved particularly interesting since, although a soluble MMP, it was found to occur after binding to the surface of the cell. This led to the identification of the membrane-associated MT-MMPs as potential mediators of proMMP-2 activation (reviewed in [8]). It was originally found that proMMP-2 could be activated by soluble forms of MT1-MMP or MT2-MMP in a two-step activation mechanism analogous to the activation of other MMPs by plasmin. The initial propeptide cleavage in proMMP-2 effected by MT-MMP activity is at the Asn37–Leu38 peptide bond. The second propeptide cleavage is autoproteolytic, since an inactive proMMP-2 mutant (proE375A-MMP-2) is only processed by MT1-MMP to the Leu38 intermediate. Processing by MT1-MMP is inhibited by either TIMP-2 or TIMP-3.

**Figure 1** Pericellular activation cascades for MMPs. Pericellular proteolysis is highly regulated by activation cascades initiated by the generation of plasmin by receptor-associated urokinase-type plasminogen activator (uPA) and by the action of MT1-MMP (reviewed in [7]). uPAR, uPA receptor; ECM, extracellular matrix.
TIMP-3, but not by TIMP-1. This agrees with the observation that TIMP-1 is a very poor inhibitor of MT1-MMP, but TIMP-2 and TIMP-3 are extremely efficient [9]. ProMMP-2 activation by cells expressing MT1-MMP also involves a two-step activation mechanism, with an MT1-MMP-mediated ‘initiation’ cleavage followed by autolytic cleavage of MMP-2 (Figure 2). The process appears to involve binding of proMMP-2 to an MT1-MMP–TIMP-2 complex, which forms a ‘receptor’ at the surface of the cell through interaction of the haemopexin domain of proMMP-2 with the C-terminal subdomain of TIMP-2. By establishing a trimolecular complex consisting of MT1-MMP, TIMP-2 and proMMP-2, the components of the ‘activation cascade’ are concentrated on the cell surface. Processing of proMMP-2 to the Leu38 intermediate may then be effected by an adjacent functional MT1-MMP molecule. This initial cleavage event destabilizes the structure of the propeptide, and autolytic cleavage to generate the fully mature enzyme proceeds in an MMP-2-concentration-dependent manner. In cell culture studies, the enzyme concentrations in solution are very low, and deletion of either the proMMP-2 haemopexin domain or the transmembrane domain of MT1-MMP abrogates

![Figure 2 Mechanism of pericellular activation of proMMP-2. ProMMP-2 binds via interaction of its C-terminal haemopexin domain with the C-terminal subdomain of TIMP-2, which is in turn bound by its N-terminal domain to cell surface MT1-MMP, forming a trimolecular complex. An adjacent free molecule of MT1-MMP initiates cleavage of the proMMP-2 propeptide, generating an activation intermediate of proMMP-2. This is susceptible to autoproteolytic processing to a fully active form. The activation mechanism is subject to tight regulation by the level of TIMP-2: a low concentration of TIMP-2 promotes efficient activation of proMMP-2, but an excess is inhibitory [7].](image-url)
proMMP-2 processing. This emphasizes that the binding mechanism involving the MT1-MMP–TIMP-2 complex on the cell surface acts as a focusing mechanism for the reactants in this cascade that is crucial for the efficiency of activation (Figure 2).

Addition of small amounts of TIMP-2 to cells expressing MT1-MMP can enhance proMMP-2 activation, because this increases the concentration of the MT1-MMP–TIMP-2 receptor for proMMP-2 on the cell surface. However, at high TIMP-2 concentrations, all of the MT1-MMP molecules are complexed to TIMP-2 and, although binding of proMMP-2 can occur, no active MT1-MMP is available to initiate propeptide processing [8]. This suggests that activation of proMMP-2 is regulated by the amount of TIMP-2 relative to that of MT1-MMP displayed at the cell surface. The importance of TIMP-2 for proMMP-2 activation by MT1-MMP has been highlighted by the finding that TIMP-2−/− mice do not show activation of proMMP-2 [10,11].

**Kinetic analysis of the binding of TIMP-2 to MMP-2**

Kinetic studies of the TIMP-2-mediated inhibition of various MMPs have demonstrated specific interactions with both MMP-2 and MT1-MMP. We initially analysed the interactions of wild-type TIMP-2, a mutant lacking the C-terminal subdomain (Δ128–194-TIMP-2) and a mutant from which the C-terminal nine-amino-acid extension had been removed (Δ186–194-TIMP-2) with both full-length MMP-2 and a mutant of MMP-2 from which the haemopexin domain had been deleted. Binding of all forms of TIMP-2 to all the forms of MMP-2 was very tight, with binding constants in the low picomolar range, which precluded accurate measurement and the achievement of steady-state kinetic techniques using quenched fluorescent peptide cleavage assays [12]. We employed techniques to overcome this problem, in order to establish a more accurate value for the binding constant, $K_i$ [13]. We found that measurements of the rate of dissociation of MMP-2 and TIMP-2 gave a half-life of approx. 400 days, i.e. binding is effectively irreversible. Using rapid reaction techniques, we were able to measure the rate constant for the inhibition of MMP-2 by TIMP-2, and deduced that the overall $K_i$ value was 0.6 nM. The data suggested that binding of TIMP-2 to MMP-2 occurs in two phases, since the rate-limiting step for inhibition is dependent on TIMP-2 concentration at low inhibitor concentrations, whereas at high concentrations the rate-limiting step is independent of free TIMP-2. Due to the lack of availability of large amounts of the TIMP-2 mutants, most subsequent kinetic analyses were confined to the determination of rate constants for the interactions using steady-state kinetics. We noted that inhibition of MMP-2 was markedly affected by the removal of the C-terminal domain of TIMP-2, with a decrease in $k_{on}$ of 50–100-fold (Table 1a) when the truncated form of enzyme or inhibitor was used. The existence of at least two sites of interaction between TIMP-2 and proMMP-2 through the haemopexin domain has been confirmed in the recently published crystal structure of the proMMP-2–TIMP-2 complex [14]. This showed that the tail sequence of TIMP-2 established five salt bridges with residues within blades III and IV of the haemopexin domain of proMMP-2 that flank hydrophobic interactions established by Phe188 with a hydrophobic groove within the enzyme.
Table 1 TIMPs: role of domain structures and motifs in (a) rate of binding to and (b) determination of apparent binding constants for MMP-2 and MT1-MMP. Studies were carried out at 25°C, apart from °37°C. nd, not determined.

(a) $10^{-6} \times k_{on} \text{ (M}^{-1} \text{s}^{-1})$

<table>
<thead>
<tr>
<th>TIMP construct</th>
<th>MMP-2</th>
<th>Δ418–631-MMP-2</th>
<th>MT1-MMP</th>
<th>Δ269–559-MT1-MMP</th>
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</thead>
<tbody>
<tr>
<td>TIMP-2</td>
<td>11.9</td>
<td>0.3*</td>
<td>3.0</td>
<td>2.4</td>
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<tr>
<td>Δ128–194-TIMP-2</td>
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<td>0.3*</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Δ186–194-TIMP-2</td>
<td>7.2</td>
<td>nd</td>
<td>3.9</td>
<td>nd</td>
</tr>
<tr>
<td>TIMP-2-(1–127)–TIMP-4-(129–195)</td>
<td>0.48</td>
<td>nd</td>
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<tr>
<td>Glu^{192}Ile/Asp^{193}Gln-TIMP-2</td>
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<tr>
<td>TIMP-4</td>
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<td>nd</td>
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</tr>
<tr>
<td>Δ129–195-TIMP-4</td>
<td>0.043</td>
<td>nd</td>
<td>0.17</td>
<td>0.095</td>
</tr>
</tbody>
</table>

(b) $K_{app} \text{ (pM)}$

<table>
<thead>
<tr>
<th>TIMP construct</th>
<th>MMP-2</th>
<th>Δ418–631-MMP-2</th>
<th>MT1-MMP</th>
<th>Δ269–559-MT1-MMP</th>
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<td>1200</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>nd</td>
<td>nd</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Δ129–195-TIMP-4</td>
<td>300</td>
<td>nd</td>
<td>nd</td>
<td>1070</td>
</tr>
</tbody>
</table>
A role for the C-terminal ‘tail’ of TIMP-2 was also established by kinetic analysis (Table 1). By studying the effect of salt concentration on the rate of association $k_{on}$, it was shown that ionic interactions are predominant in the association of the TIMP-2 tail with the haemopexin domain of MMP-2 [15]. Indeed, mutation of Glu$^{192}$-Asp$^{193}$ to Ile$^{192}$-Asn$^{193}$ (the sequence found in TIMP-4; see below and Figure 4) reduced the $k_{on}$ for MMP-2 by 20-fold (Table 1a). In contrast, when the interaction of TIMP-2 with MT1-MMP was analysed similarly, it was found that only the N-terminal domains of both enzyme and inhibitor were critical for initial binding (Table 1a) [16].

**Kinetic analysis of binding of Δ128–194-TIMP-2 mutants to MT1-MMP**

We carried out kinetic studies on the interaction of Δ128–194-TIMP-2 mutants with MT1-MMP, and found that residues that had been identified at the enzyme–inhibitor interface in the MMP-3–TIMP-1 crystal structure [17] were also important in this case. Mutation of residues Ser$^2$, Ala$^{72}$, Val$^{71}$ and Gly$^3$, which are located on the surface of the TIMP-2 ridge structure, increased the association rate constants and final $K_{i}$ values for MT1-MMP as well as MMP-2, MMP-7 and MMP-13 (Table 1a) [16]. However, we demonstrated a specific interaction between the hairpin turn of the A and B β-strands of TIMP-2 (Tyr$^{36}$) and MT1-MMP. This was in agreement with the structural data for the complex, where Tyr$^{36}$ of TIMP-2 was seen to fit into a cavity on the surface of MT1-MMP, bordered by the ‘MT loop’ and the side chains of Asp$^{212}$, Ser$^{189}$ and Phe$^{180}$ [18]. The data from the crystal structure indicated that several other significant interactions occurred between the MT1-MMP surface and residues of the TIMP-2 AB loop. Alignments of the β-strands in the AB hairpins of TIMPs-1–4 were made using the NMR and X-ray diffraction data for TIMP-1 and TIMP-2, and based on the known properties of the OB (oligosaccharide/oligonucleotide binding) protein fold [19]. In free TIMP-2, the strand alignment for the β-hairpin is very well defined in both the NMR and crystal structures, and ends in a type I β-turn with a G1-β bulge. In the crystal structure of the TIMP-2–MT1-MMP complex, the strand alignment is less clear, and there is some evidence of strand realignment in the middle section (Glu$^{26}$ to Asp$^{30}$) that may occur as part of the large conformational change seen for this region on binding to MT1-MMP. The structural data for TIMP-1 show no significant differences in strand alignment between the free and MMP-3-bound inhibitor, and the predicted hydrogen-bonding pattern suggests that this hairpin results in a four-residue loop. No structural data are currently available for either TIMP-3 or TIMP-4, but their predicted strand alignments and hydrogen-bonding patterns suggest that both of these hairpins may end in type I β-turns (i.e. two residues in the loop positions). The predicted β-strand alignment of the ΔAsp$^{34}$–Ile$^{42}$ mutant of Δ128–194-TIMP-2 is also shown in Figure 3, and the hydrogen-bonding pattern suggests that this hairpin will end in a longer four-residue loop.

**Contribution of the TIMP-2 AB loop to the conformational stability of Δ128–194-TIMP-2**

NMR-based structural analysis of the Ile$^{35}$Gly/Tyr$^{36}$Gly-TIMP-2 mutant clearly showed that the glycine substitutions had no effect on the overall folding
Figure 3 | β-Strand alignments for the AB hairpins of TIMPs-1 to -4. Cross-strand hydrogen-bonding patterns have been predicted from the available NMR and X-ray diffraction data, and based on the known properties of the OB protein fold. Strong Hα–Hα nuclear Overhauser effects seen in the NMR structure analysis of N-TIMP-2 (the N-terminal domain of TIMP-2) are shown by double-headed arrows, and identify residues adjacent to one another in the β-strands. The hydrogen-bonding pattern is shown by single-headed arrows from NH to O. Residues with side chains internalized into the hydrophobic core of N-TIMP-2 are boxed. Residues conserved within the sequences for human TIMPs are shown in black (other residues are shown in grey). The glycosylated Asn residue of TIMP-1 is labelled ‘cho’. Residues within the AB hairpin that were mutated are underlined. The predicted strand alignment for the ΔAsp34–Ile40 deletion mutant is also shown for comparison [19].
of the protein, except at the tip of the AB hairpin. Although this region is highly solvent-exposed and does not interact with the rest of the protein structure, it was found to make a significant contribution to the conformational stability of the molecule. The change in structure at the tip of the AB loop in the Ile35Gly/Tyr36Gly mutant resulted in a denaturation midpoint shift of 0.39 M guanidinium chloride, whereas the removal of the entire tip region (Δ34–40) resulted in a larger shift in stability of 0.68 M guanidinium chloride. The lower stability of the deletion mutant may be due in part to the new non-native turn between strands A and B, which is unlikely to be as energetically stable as the well-ordered turn found in the wild-type protein. The findings from the structural studies on Ile35Gly/Tyr36Gly-TIMP-2 and Δ34–40-TIMP-2 serve to highlight the structural independence of the AB hairpin, and suggest that quite large changes to the sequence can be made in this region without perturbing the structure of the rest of the inhibitor.

The kinetic studies of the binding of Δ128–194-TIMP-2 mutants to MT1-MMP (Table 1) clearly showed that the side chain of Tyr36 is the most important feature of the AB loop, in terms of both initial association and final binding. Complete deletion of the tip of the AB hairpin (Δ34–40) of Δ128–194-TIMP-2 did not modify these parameters significantly, suggesting that there is no net binding contribution towards association or final binding from other residues in this region of the inhibitor apart from Tyr36 (results not shown; [19]). This finding further supports our previous suggestion that, although the position of the extended AB hairpin will necessitate its close contact with a proteinase bound at the inhibitory site of TIMP-2, this interaction need not contribute to the overall binding affinity in all cases and could, in some cases, conceivably weaken the overall binding interaction by making unfavourable contacts with the proteinase.

**Kinetic analysis of Δ128–194-TIMP-2 mutants with structural features of TIMP-4**

The C-terminal deletion mutant Δ129–195-TIMP-4 associates with MT1-MMP at a 20-fold lower rate than Δ128–194-TIMP-2 mutants, which may be due to the lack of a residue comparable with Tyr36 in the AB β-turn (see Figure 3). However, Δ129–195-TIMP-4 has been shown to have a similar $K_{\text{app}}$ value for MT1-MMP binding as Δ128–194-TIMP-2 (Table 1b), suggesting that the lack of a binding contribution from Tyr36 is compensated for by other interactions elsewhere. The charged and polar residues Asp34 and Asn38 were considered as potentially important residues for the TIMP-2–MT1-MMP association because they occupy the same positions as Asp34 and Asp37 in TIMP-4. Mutation of both residues to Ala did markedly reduce the rate of initial binding to MT1-MMP (the $k_{\text{on}}$ value was decreased by 180-fold), but had little effect on the final $K_{\text{app}}$ value. Interestingly, the modification of Asn38 to Gln in TIMP-2 (making it comparable with TIMP-4) resulted in a 50-fold decrease in the value of $K_{\text{app}}$ for binding to MT1-MMP. The side chain of Asn38 is involved in making a hydrogen bond to its own backbone (amide O to HN) and in making weaker electrostatic interactions across the hairpin with the side chain of Asp34 and to the proteinase with the side chain of Asn208 and the backbone HN of

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Substitution of Asn\textsuperscript{38} with the negatively charged Asp may cause some structural rearrangement at this site, allowing stronger interactions to form between the tip of the AB hairpin and the catalytic domain of MT1-MMP. It is interesting to speculate that Asp\textsuperscript{37} in \textit{H9004}129–195-TIMP-4 may help to compensate for the lack of a residue equivalent to Tyr\textsuperscript{36}, allowing the overall binding constant for MT1-MMP to be similar to that measured for \textit{H9004}128–194-TIMP-2.

The precise biological significance of the unique interactions between the AB loop of TIMP-2 and MT1-MMP can only be speculated upon, but they may play an important role in the stabilization of the TIMP-2–MT1-MMP complex in which the C-terminal region of TIMP-2 is free to bind to the haemopexin-like domain of MMP-2. This would represent the basis of a cell surface MT1-MMP–TIMP-2–proMMP-2 complex, leading to the activation of proMMP-2. It is clear from this work that TIMP-2 may be engineered to abrogate MT1-MMP binding, whereas its binding properties for many other MMPs, including MMP-2, are maintained.

\textbf{TIMP-2 mutants that affect activation of proMMP-2 by MT1-MMP}

We have begun a study on the effects of mutating TIMP-2 in order to modify its interactions with proMMP-2 in the context of the efficiency of cellular MT1-MMP-mediated activation of the latter. It is well documented that TIMP-4 is unable to promote activation of proMMP-2 by MT1-MMP [20,21]. However, the kinetic constants indicate that TIMP-4 is an excellent inhibitor of MMP-2, with a comparable \(k_{\text{on}}\) value to TIMP-2 or \textit{H9004}128–194-TIMP-2. Furthermore, TIMP-4 binds to proMMP-2 via the C-terminal domain of the enzyme, the binding site being complementary to the TIMP-2 binding site [20,21a]. However, from the crystal structural data of the TIMP-2–proMMP-2 complex, we can deduce that the C-terminal interactions in a TIMP-4–proMMP-2 complex are likely to be considerably weaker, e.g. only three salt bridges could be formed between the C-terminal tail of TIMP-4 and the haemopexin domain of proMMP-2 [21a].

In the case of MT1-MMP, the \(k_{\text{on}}\) values are somewhat smaller and the \(K_{\text{i}}\text{app}\) values slightly larger (Table 1). Using the various domain deletion mutants in the kinetic studies, we found that, interestingly, the C-terminal subdomains of both TIMPs appear to have significant interactions with the catalytic domain of MT1-MMP, but the haemopexin domain of the latter shows no significant interactions with TIMPs (results not shown). We compared the sequences of human TIMP-2 and human and mouse TIMP-4 (Figure 4). Our studies were carried out using mouse TIMP-4, which is 91% identical to the human sequence and therefore unlikely to present significant species differences.

We hypothesized that the interaction between TIMP-2, proMMP-2 and MT1-MMP is tailored to promote the cellular activation of proMMP-2 by MT1-MMP, and that unique features of the TIMP-2 structure relative to TIMP-4 would be a good focus for studies to test this concept. We analysed the ability of modified forms of TIMP-2 to bind both the active catalytic domain of MT1-MMP and the mutant proE\textsuperscript{375}A MMP-2. The use of this catalytically inactive form of proMMP-2 prevents full proteolysis of the proMMP-2 propeptide, but allows binding to gelatin–Sepharose. Species associating with the proE\textsuperscript{375}A MMP-
2 will hence be retained on gelatin–Sepharose, and were identified by immunoblotting of eluates [21a]. TIMP-2, Δ186–194-TIMP-2, a chimaera of the TIMP-2 N-terminal domain and the TIMP-4 C-terminal domain [TIMP-2-(1–127)–TIMP-4-(129–195)], a chimaera of the TIMP-4 N-terminal domain and the TIMP-2 C-terminal domain [TIMP-4-(1–128)–TIMP-2-(128–194)], TIMP-4 and the Ile193Glu/Gln194Asp-TIMP-4 mutant were assessed for their ability to bind to proMMP-2 in a bimolecular complex and to promote the formation of a trimolecular complex with the active catalytic domain of MT1-MMP. All TIMP forms were able to form a bimolecular complex with proMMP-2 (results not shown; [21a]), and only TIMP-4 and TIMP-2-(1–127)–TIMP-4-(129–195) were unable to promote trimolecular complex formation. The ability of Ile193Glu/Gln194Asp-TIMP-4 to promote trimolecular complex formation led us to conclude that the charged tail sequence of TIMP-2, i.e. Glu 192-Asp193, contributes significantly to stability.

Structural determinants of TIMP-2 required for efficient activation of proMMP-2 by MT1-MMP

Finally, we assessed the ability of the different forms of TIMP to promote the cell-based activation of proMMP-2 via the MT1-MMP mechanism. This was effected by the use of membrane fractions from fibroblasts derived from TIMP-2−/− mice stably expressing human MT1-MMP [21a]. These cells do not make detectable levels of TIMP-2 or TIMP-4. Analysis of proMMP-2 processing by zymography showed that the enzyme was processed effectively to the fully active form by TIMP-2 (Figure 5). It was converted only into the intermediate
form, i.e. MT1-MMP-mediated cleavage of the propeptide at the Asn\(^{37}\)–Leu\(^{38}\) peptide bond, in the presence of the chimaera TIMP-4-(1–128)–TIMP-2-(128–194) or the mutant Glu\(^{192}\)Ile/Asp\(^{193}\)Gln-TIMP-2. TIMP-4, the chimaera TIMP-2-(1–127)–TIMP-4-(129–195) and \(\Delta186–194\)-TIMP-2 did not promote the MT1-MMP-mediated processing of the proMMP-2 peptide at all (Figure 5). This substantiates the concept that the charged amino acid residues Glu\(^{192}\) and Asp\(^{193}\) within the TIMP-2 tail sequence are major determinants of the specificity of this TIMP in the establishment of the MT1-MMP–TIMP-2 receptor required for proMMP-2 activation at the cell surface. Removal of this motif does not prevent assembly of the MT1-MMP–TIMP-2–proMMP-2 trimeric complex or intermediate cleavage of the proMMP-2 propeptide, but the final cleavage of the propeptide that is effected by MMP-2 cannot proceed [16,22]. We hypothesize that MMP-2 is only weakly attached to the cell in the absence of Glu\(^{192}\) and Asp\(^{193}\), and therefore autocleavage reactions would be less efficient.

**Membrane protein ectodomain proteolysis by metalloproteinases**

Metalloproteinases have been linked to the proteolysis of a very large number of structurally and functionally diverse membrane proteins, a process known as ectodomain shedding, which is often essentially a solubilization of the whole ectodomain of the protein. Roles in the shedding of epidermal growth factor receptor ligands, cytokines, their receptors and adhesion molecules have been attributed to both MMPs and the ADAM family of metalloproteinases [23]. The regulation of cell surface proteolytic activities is a key issue, and could occur at a number of levels. Induction of shedding by physiological stimuli leading to intracellular signalling cascades is of importance. The interactions of the enzyme and substrate within the plasma membrane, or at focused locations determined by interactions with other extracellular or intracellular proteins, are thought to be key levels of regulation.
MMPs and ADAMs all require activation by proteolysis of the propeptide that resides in the active-site cleft (cysteine switch mechanism [24]).

Regulation of ADAM and ADAMTS activity by TIMPs

We have investigated the potential role of TIMPs in the regulation of ectodomain shedding events mediated by metalloproteinases. About half of the 33 ADAMs cloned to date are predicted to be active metalloproteinases, based on the presence of the HEXXHXXGXXH zinc-binding motif. Of these, ADAM17, also known as tumour necrosis factor α (TNFα)-converting enzyme (TACE), is the most thoroughly characterized member. In addition to processing precursor membrane-bound TNFα to its soluble form, TACE also cleaves other membrane proteins [25]. Some of the ADAMs predicted to be active metalloproteinases have also subsequently been demonstrated to be able to participate in proteolytic activities similar to those of TACE in cell-based systems [26]. The catalytic activities of purified recombinant ADAMs have been studied using α2-macroglobulin and myelin basic protein, as well as various peptides [27,28]. These assays have allowed their susceptibility towards the TIMPs, potential physiological regulators of ADAM proteolytic activity in vivo, to be evaluated, as well as the effects of many low-molecular-mass synthetic inhibitors. Of the TIMPs, only TIMP-3 was found to inhibit TACE, ADAM12 and ADAM19 [29–31], while both TIMP-1 and TIMP-3 could inhibit ADAM10. Furthermore, TIMP-3 also inhibited the aggrecanases ADAMTS-4 and ADAMTS-5 as well as ADAMTS-1, which are members of the related family of disintegrin metalloproteinases with thrombospondin domains [32,33]. In contrast, ADAM8 and ADAM9 were not inhibited by any of the TIMPs [34]. In the few cases where TIMP-2 has been found to be an inhibitor of a proteolytic shedding event, it seems likely that an MMP is involved [35]. In cell-based studies of ectodomain shedding, TIMP-3 has frequently been shown to be an effective inhibitor of the processing of TNFα, L-selectin, the interleukin-6 receptor, CD30 and the p55 TNF receptor 1. In many cases this may be due to the inhibition of TACE, but substantial further study is necessary. Since TIMP-3 is associated primarily with the extracellular matrix, it is effectively localized to the pericellular environment of cells and may represent a significant physiological regulator of membrane metalloproteinases, including those involved in ectodomain shedding. This has not been definitively established, however. Studies of the TIMP-3−/− mouse have indicated the importance of TIMP-3 in the regulation of extracellular matrix turnover in the lung [36] and the involuting mammary gland [37].

Overexpression of TIMP-3 in vivo significantly inhibited MMP-driven neointima formation by smooth muscle cells in a pig vein graft model [38]. Cell-based studies showed that this was due to apoptosis of smooth muscle cells [39,40]. Indeed, high levels of TIMP-3 promote apoptosis in many cell types, and this effect has been associated with TNF receptor modulation [41]. TIMP-3 may also modulate the levels of other death receptors [42,43].
We have started to investigate some of the structural features of TIMP-3 that determine its ability to inhibit cell surface proteinases such as the ADAMs. Initially we showed that full-length TIMP-3 and the construct Δ122–188-TIMP-3, comprising the three N-terminal disulphide-bonded loops, had similar $k_{\text{on}}$ and $K_i^{\text{app}}$ values for interaction with a soluble form of the TACE catalytic domain (Δ474–824-TACE; TACE-473) or the whole ectodomain of TACE (Δ652–824-TACE; TACE-651) [44]. This is a comparison with its interaction with MMP-2, where the loss of the C-terminal three loops of TIMP-3 markedly abrogates binding (Table 2) [45]. It was noted that TIMP-3 associated rather more efficiently with the TACE catalytic domain than with the full ectodomain, suggesting that the disintegrin and cysteine-rich domains either modify the catalytic domain or interfere with TIMP-3 interactions.

By site-directed mutagenesis, we showed that it was possible to specifically enhance TIMP-3 binding to the TACE active-site cleft by modifying Ser⁴ to Met, Tyr, Lys or Arg [46], leading to a $>3$-fold drop in $K_i^{\text{app}}$. Binding to MMP-2 was concomitantly increased approx. 10-fold; hence an element of selectivity can be introduced into TIMP-3 by the modification of a single residue. This suggests that further mutagenesis studies should allow further specificity to be engineered. However, the problem of interference by the non-catalytic domains of TACE needs to be addressed. Since TIMP-3 is largely a matrix-associated protein, the role of the extracellular matrix in its activity and the effects on TACE function need to be considered. We have shown previously that the rate of association between TIMP-3 and MMP-2 was increased by various glycosaminoglycans, including heparan sulphate [47]. These charged molecules could therefore play a significant role in both focusing and

### Table 2 Association rate constants (a) and apparent binding constants (b) for TIMP-3, TACE and MMP-2.

<table>
<thead>
<tr>
<th></th>
<th>TACE-651</th>
<th>TACE-473</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>$10^{-5} \times k_{\text{on}} \left( \text{M}^{-1} \text{s}^{-1} \right)$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.98</td>
<td>9.94</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>Δ122–188-TIMP-3</td>
<td>0.45</td>
<td>3.65</td>
<td>0.25</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_i^{\text{app}} \left( \text{nM} \right)$</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.74</td>
<td>0.20</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Δ122–188-TIMP-3</td>
<td>1.75</td>
<td>0.22</td>
<td>0.25</td>
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potentiating TIMP-3 activity at the cell surface. Further studies of the precise nature of the extracellular matrix binding sites and the structural features of TIMP-3 involved in binding are needed in order to address the significance of TIMP-3 sequestration on the extracellular matrix.

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References

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