Total Conversion of Tissue Inhibitor of Metalloproteinase (TIMP) for Specific Metalloproteinase Targeting

FINE-TUNING TIMP-4 FOR OPTIMAL INHIBITION OF TUMOR NECROSIS FACTOR-α-CONVERTING ENZYME*

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Tissue inhibitors of metalloproteinases (TIMPs) are the endogenous inhibitors of the matrix metalloproteinases, the ADAMS (a disintegrin and metalloproteinase) and the ADAM-TS (ADAM with thrombospondin repeats) proteinases. There are four mammalian TIMPs (TIMP-1 to -4), each TIMP has its own profile of metalloproteinase inhibition. TIMP-4 is the latest member of the TIMPs to be cloned, and it has never been reported to be active against the tumor necrosis factor-α-converting enzyme (TACE, ADAM-17). Here we examined the inhibited properties of the full-length and the N-terminal domain form of TIMP-4 (N-TIMP-4) with TACE and showed that N-TIMP-4 is a far superior inhibitor than its full-length counterpart. Although full-length TIMP-4 displayed negligible activity against TACE, N-TIMP-4 is a slow tight-binding inhibitor with low nanomolar binding affinity. Our findings suggested that the C-terminal subdomains of the TIMPs have a significant impact over their activities with the ADAMS. To elucidate further the molecular basis that underpins TIMP/TACE interactions, we sculpted N-TIMP-4 with the surface residues of TIMP-3, the only native TIMP inhibitor of the enzyme. Transplantation of only three residues, Pro-Phe-Gly, onto the AB-loop of N-TIMP-4 resulted in a 10-fold enhancement in binding affinity; the $K_i$ values of the resultant mutant were almost comparable with that of TIMP-3. Further mutation at the EF-loop supported our earlier findings on the preference of TACE for leucine at this locus. Drawing together our previous experience in TACE-targeted mutagenesis by using TIMP-1 and -2 scaffolds, we have finally resolved the mystery of the selective sensitivity of TACE to TIMP-3.

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Tissue inhibitor of metalloproteinases (TIMP(s))1 are the endogenous regulators of the zinc-dependent metalloproteinases (MPs) of the matrix metalloproteinase (MMP), the ADAM (a disintegrin and metalloproteinase), and the ADAM-TS (ADAMs with thrombospondin repeats) families. There are four mammalian TIMPs identified to date (TIMP-1 to -4), and they are all small molecules of ~24 kDa in molecular mass. The TIMPs are 40–50% identical in amino acid sequence (reviewed in Ref. 1), and structural analysis reveals that the molecules are composed of two very distinct N- and C-terminal domains. The N-terminal domain (N-TIMP) encompasses nearly the first two-thirds of the polypeptide, and the domain is made up of five-stranded pleated sheets in the shape of a conical β-barrel. The tertiary configuration of the domain is typical of that of an oligonucleotide/oligosaccharide-binding motif (2–4). It is within the N-terminal domain that the inhibitory activity of a TIMP resides. The C-terminal domain is ~8 kDa in mass. In contrast to the N terminus, this domain is less well defined (3, 5). There are three disulfide bonds in each domain, but only the full-length and the N-terminal forms of TIMPs have been expressed so far and refolded from Escherichia coli inclusion bodies (6–10). No success has yet been achieved in expressing the C terminus as an independent entity either in mammalian or in prokaryotic cells.

TIMP inhibits the enzymatic function of an MP by inserting the wedge-shaped edge of its N-terminal domain, the so-called “MMP-binding ridge,” into the catalytic groove of the target MP to form a tight but essentially noncovalent 1:1 stoichiometric complex (3, 5). The MMP-binding ridge, by its very definition, is composed of the N terminus, the AB-loop, CD-loop, and the EF-loop of the molecule. Despite the high degree of similarity in their tertiary structures, each TIMP has its own range of metalloproteinase (MP) targets, and the profiles of MP inhibition among the TIMPs are acutely variable between the species. For instance, with the exception of several cell surface-bound membrane-type MMPs and MMP-19, all secreted MMPs are sensitive to TIMP-1 inhibition (11, 12). TIMP-2, on the contrary, inhibits membrane-type MMPs as well as MMP-19 at subnanomolar affinities (11, 12). The ADAMS have slightly different sensitivity profiles in comparison to the MMPs. ADAM-10, for example, is inhibited by TIMP-1 and -3 and not TIMP-2 (13). TACE, in contrast, is selectively inhibited by TIMP-3 (14). Notwithstanding the successful delineation of two co-crystal structures of TIMP-MMP complexes, namely TIMP-1/stromelysin-1 (PDB code 1UEA) and TIMP-2/MT1-MMP (PDB code 1BUV), the molecular basis that underpins TIMP/MP selectivity is still not fully understood.

Unlike its counterparts TIMP-1, -2, and -3, the inhibitory characteristics of TIMP-4 with the MP have so far not been investigated in detail. Because it is the last TIMP to be isolated, our knowledge of its functions, biochemical or cellular, is admittedly still fairly limited. Our laboratory is interested in elucidating the molecular basis that governs MP/TIMP interactions, above all members of the cell surface-associated MPs.
that are of clinical importance, such as the tumor necrosis factor-α-converting enzyme (TACE). TACE is unique among the MPs because of its versatility in the shedding of a broad range of membrane-bound bioactive molecules (reviewed in Ref. 15). Furthermore, it is selectively inhibited by TIMP-3 and not the other TIMPs (14). Biochemically, it is both interesting as well as challenging to unlock the molecular basis that underpins TACE/TIMP selectivity, and no TIMP is more challenging than TIMP-4. Hence, we chose TACE as the subject of our investigation in this study and TIMP-4 as the prototype for mutagenesis.

In line with the design and progress of the project, the results are divided into three sections. In the first section, we compare the inhibitory characteristics of full-length and the N-terminal domain forms of TIMP-4 (N-TIMP-4) with TACE. In particular, the discrepancy between their affinities and association rates will be examined. In the second part of this paper, we describe the mutagenesis process leading to the creation of a potent, slow, and tight-binding TIMP-4 inhibitor. The relevance of the TIMP-3 AB-loop to the current project will be discussed in detail. The last section under “Results” deals specifically with residue Leu101, the pivotal amino acid on the EF-loop that is underpinned by TACE/TIMP selectivity, and no TIMP is more challenging than TIMP-4. Hence, we chose TACE as the subject of our investigation in this study and TIMP-4 as the prototype for mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—TACE (ADAM-17) enzyme (the catalytic domain of TACE or TACE-473) was the kind gift from Dr. J. David Becherer, Glaxo-SmithKline, Research Triangle Park, NC (16). Human TIMP-4 cDNA was given by Dr. Rob Nuttall and Dr. Dylan Edwards, University of East Anglia, Norwich, UK. Native full-length TIMP-4 was purchased from R&D Systems, Minneapolis, MN. All chemicals and reagents in this study were purchased from Sigma unless otherwise stated. Vent DNA polymerase for mutagenesis and the restriction enzymes for subcloning were obtained from New England Biolabs (Hitchin, Hertfordshire, UK). The fluorescent substrate for TACE assay ([7-methoxycaumarin-4-yl]-acetyl-Ser-Pro-Leu-Ala-Glu-Ala-Val-Arg-Ser-Ser-Arg-Lys-2,4-di-nitrophenyl-NH2) was synthesized and purified by Dr. Graham Knight, Department of Biochemistry, University of Cambridge, as reported in our early papers (17, 18).

**Construction and Site-directed Mutagenesis of Full-length and N-TIMP-4 Mutants**—Full-length and the N-terminal domain forms of TIMP-4 (N-TIMP-4; corresponding to residue Cys1 to Gly129) were amplified from the human cDNA template by PCR before being subcloned into the E. coli expression plasmid pRSET-c (Invitrogen). A hexahistidine tag was added to the C termini of the TIMP-4 constructs during PCR amplification for the ease of downstream purification. Mutagenesis was achieved by either forward or reverse oligonucleotides, depending on the MP being amplified from the human cDNA template by PCR before being subcloned into the E. coli expression plasmid pRSET-c (Invitrogen). A hexahistidine tag was added to the C termini of the TIMP-4 constructs during PCR amplification for the ease of downstream purification.

**Production, Refolding, and Activity Assessment of Full-length and N-TIMP-4**—The protocols for the production, refolding, and titration of full-length and N-TIMP-4 were almost identical to those of N-TIMP-2 elaborated in our previous papers with the exception that 0.6 M l-arginine was included in the refolding solution (17, 18). The concentration of active TIMPs in each preparation was determined by titration against a known amount of gelatinase-A (MMP-2) and/or collagenase-3 (MMP-13) as described before (19).

Inhibition Constant Measurement (Ki)—Inhibition (Ki) and association (k_on) assays were performed at 27 °C constant temperature in fluorescence assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl2 supplemented with 0.05% Brij-35, 1% Me2SO, 0.02% NaN3) with a PerkinElmer Life Sciences LS-50B spectrofluorimeter equipped with thermostatic cuvette holders (17–19). TACE (0.22 nm) was preincubated at room temperature with TIMP-4 or its mutants, ranging from 0.1 to 1000 nm, for a minimum of 2 h prior to steady state (V_S) measurement at 27 °C. Reactions were initiated by adding quenched fluorescent peptides to a final concentration of 1 μM. All data were fitted into competitive tight binding equations with the computer program Grafit to obtain an estimation of the Ki values with Equation 1 (20),

$$V_S = (V_S / 2E_r) 	imes (K_e - E_r - K_i + (K_e + E_r - E_i)^2 + 4E_r K_i)^{1/2}$$

(Eq. 1)

Where V_S is the rate in the absence of inhibitor; E_r is the total enzyme concentration, and I_t is the total inhibitor concentration.

Association Rate Constant Measurement (k_on)—k_on measurements were performed by adding TIMP-4 or its mutants (up to 2000 nM) to 80 pm TACE. The rate of inhibition was monitored using a continuous fluorimetric assay at 27 °C until steady state was reached. The progress curve was analyzed by using Equation 2 (20),

$$P = V_{St} + (V_S - V_{St})(1 - e^{-kt})$$

(Eq. 2)

where P is the product concentration; V_S is the initial velocity; V_st 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 ln k on TIMP concentrations.

**RESULTS**

**N-TIMP-4 Is a Far Superior TACE Inhibitor Than Full-length TIMP-4**—TIMP-4 is the latest TIMP variant to be cloned, and our understanding of its inhibitory functions with the MMPs, the ADAMs, and the ADAM-TS proteinases in general is therefore very limited. To date, there have been several reports on the inhibitory activity of TIMP-4 with a number of MMPs and ADAM-TSs (21–23) but none has so far looked at the ADAMs. As a first step toward delineating ADAM/TIMP-4 interactions, we compared the binding affinity and the association rate of full-length TIMP-4 and its N-terminal form, N-TIMP-4, with the ADAM proteinase, TACE. The results are listed in Table I. As shown, N-TIMP-4 is functionally much more active than full-length TIMP-4; its affinity (Ki = 180 nM) was at least an order of magnitude better than that of the full-length species (Ki = 120 nm). To rule out the caveat that the poor activity of refolded full-length TIMP-4 is caused by misfolding in the C-terminal domain, we repeated the measurement using native TIMP-4 expressed and purified from the eukaryotic expression system (Ki > 180 nM). The findings are unequivocal, i.e. full-length TIMP-4 is a much poorer inhibitor than N-TIMP-4. Further study on the association profile revealed that N-TIMP-4 is clearly a slow, tight binding inhibitor, whereas full-length TIMP-4 is not. The contrast between the two forms of TIMP-4 is best illustrated by their k_on profiles, as shown in Fig. 1 (k_on of N-TIMP-4 = 6.4 × 10^{-3} M^{-1} s^{-1}; whereas no k_on could be determined for full-length TIMP-4 because of its low inhibitory activity).

**Strategy for Creating a Fully Active, Potent N-TIMP-4 TACE Inhibitor**—In contrast to its counterparts the N-TIMP-1 (Ki = 356 nm) and the N-TIMP-2 (Ki = 893 nm), N-TIMP-4 is a compara-measurements (Table I) and the association rate of full-length TIMP-4 and its N-terminal form, N-TIMP-4, with the ADAM proteinase, TACE. The results are listed in Table I. As shown, N-TIMP-4 is functionally much more active than full-length TIMP-4; its affinity (Ki = 180 nM) was at least an order of magnitude better than that of the full-length species (Ki = 120 nm). To rule out the caveat that the poor activity of refolded full-length TIMP-4 is caused by misfolding in the C-terminal domain, we repeated the measurement using native TIMP-4 expressed and purified from the eukaryotic expression system (Ki > 180 nM). The findings are unequivocal, i.e. full-length TIMP-4 is a much poorer inhibitor than N-TIMP-4. Further study on the association profile revealed that N-TIMP-4 is clearly a slow, tight binding inhibitor, whereas full-length TIMP-4 is not. The contrast between the two forms of TIMP-4 is best illustrated by their k_on profiles, as shown in Fig. 1 (k_on of N-TIMP-4 = 6.4 × 10^{-3} M^{-1} s^{-1}; whereas no k_on could be determined for full-length TIMP-4 because of its low inhibitory activity).

**Strategy for Creating a Fully Active, Potent N-TIMP-4 TACE Inhibitor**—In contrast to its counterparts the N-TIMP-1 (Ki = 356 nm) and the N-TIMP-2 (Ki = 893 nm), N-TIMP-4 is a compara-
tively better inhibitor in terms of TACE inhibition. On the other hand, its potency against TACE is still lagging behind that of the N-TIMP-3 \( (\text{Ki} \ 0.22 \ \text{nM}) \) (Table II). As part of our effort in the elucidation of the molecular basis that governs TACE/TIMP selectivity, we wondered if N-TIMP-4 could ever be engineered to be as potent as N-TIMP-3, and if so, which part(s) of the molecule should be targeted for engineering? The most likely candidates, in our conjecture, are the amino acids located at the MMP-binding ridge as these are the parts of the molecule that would be in direct physical contact with the target enzyme upon the formation of TACE/H18528 TIMP complex.

Furthermore, we have demonstrated previously (17–19) that the selectivity profile of a TIMP variant could be re-modeled by adjusting the amino acid composition of its MMP-binding ridge. Fig. 2 compares the primary sequences of N-TIMP-1, -2, -3, and -4. Clearly, the most conspicuous difference between the four TIMPs is the AB-loop region, and to a lesser extent residues 2 (Ser2) and 4 (Ala4) at the N terminus and residue 100 (His100) on the EF-loop. If our hypothesis on TIMP/MP selectivity is correct, these would be the residues worthy of investigation if the activity profile of N-TIMP-4 is to be tuned against TACE.

In this work, our strategy was to divide our mutagenesis targets into two categories. The first category consisted of mutants at the N terminus (i.e. residue Ser2 and Ala4) and the EF-loop (i.e. residue His100). The second category, on the other hand, was exclusively AB-loop-related. The residues that were deemed to be important for TACE selectivity were either retained or replaced by amino acids that are, according to our modeling prediction, capable of enhancing the affinity with TACE. The goals of the current project are 2-fold. In the short term, we intend to enhance the activity of N-TIMP-4 specifically against TACE for the purpose of finding out the part(s) of the TIMP molecule that is/are involved in TACE recognition and selectivity. If the objective can be attained, our long term goal would be to create a potent, TACE-inhibiting “N-TIMP-4 probe” to answer the many contentious issues surrounding the biological and cellular functions of TIMPs.

**First Category N-TIMP-4 Mutants, the N Terminus and the EF-loop—**Three mutants were created in this category, namely Ser2 to threonine (S2T) mutant, Ala4 to serine (A4S) mutant, and Ala4 to methionine (A4M) mutant (Table II). S2T and A4X mutants, regardless of the biochemical nature of the amino acid substitutes, are poorer inhibitors than the wild-type protein \( (\text{Ki} \ 9.5–19 \ \text{nM}) \).

The reason His100 was chosen for mutagenesis in this work was because of its close proximity to Leu101, a residue that, according to our hypothesis, could be of critical importance in TACE recognition (details will be elaborated below). Being adjacent to Leu101, His100 could in theory exert significant influence upon the movement of the Leu101 side chain (such as methionine) at position 4 enhanced the affinity of N-TIMP-3 with TACE (24). The findings are shown in Table II. Among the three, only S2T displayed possible signs of improvement in affinity \( (\text{Ki} \ 7.6 \ \text{nM}) \), whereas H100T mutation severely compromised the activity of N-TIMP-4 with TACE \( (\text{Ki} \ 170 \ \text{nM}) \).

The reason His100 was chosen for mutagenesis in this work was because of its close proximity to Leu101, a residue that, according to our hypothesis, could be of critical importance in TACE recognition. The closest residue to Leu101, His100, could in theory exert significant influence upon the movement of the Leu101 side chain or even the mobility of the EF-loop as a whole; hence the creation of H100T and H100G mutants to mimic the EF-loops of TIMP-1, -2, and -3, respectively (highlighted in Fig. 2). Of the two, H100G exhibited possible signs of improvement in affinity \( (\text{Ki} \ 7.7 \ \text{nM}) \) versus wild-type N-TIMP-4 \( (\text{Ki} \ 8.7 \ \text{nM}) \).

The reason His100 was chosen for mutagenesis in this work was because of its close proximity to Leu101, a residue that, according to our hypothesis, could be of critical importance in TACE recognition (details will be elaborated below). Being adjacent to Leu101, His100 could in theory exert significant influence upon the movement of the Leu101 side chain or even the mobility of the EF-loop as a whole; hence the creation of H100T and H100G mutants to mimic the EF-loops of TIMP-1, -2, and -3, respectively (highlighted in Fig. 2). Of the two, H100G exhibited possible signs of improvement in affinity \( (\text{Ki} \ 7.6 \ \text{nM}) \), whereas H100T mutation severely compromised the activity of N-TIMP-4 with TACE \( (\text{Ki} \ 170 \ \text{nM}) \).

**Second Category N-TIMP-4 Mutants, Fine-tuning the AB-loop—**None of the mutations carried out at the N terminus and
the EF-loop significantly improved the affinity of N-TIMP-4 with TACE. If the affinity of N-TIMP-4 for TACE is to be enhanced to a level on a par with that of the N-TIMP-3 ($K_i$ 0.22 nM), it is very unlikely that the mutations so far offer any realistic chance of success. In other words, the answer to the successful creation of a tight-binding N-TIMP-4 variant must lie elsewhere in the molecule. The next promising locus on the MMP-binding ridge was no doubt the AB-loop (Figs. 2 and 3). It is worth noting that the AB-loops of TIMP-2 and -4 are longer than those of the TIMP-1 and -3 by five to six residues (Fig. 3). Furthermore, the amino acid composition of the AB-loop is highly variable among the four TIMPs (Figs. 2 and 3). If the AB-loop is indeed the key that confers TACE-inhibiting activity upon TIMP-3, transplantation of the fragment onto TIMP-4 should generate a TIMP-4 inhibitor with improved affinity against the enzyme. Our modeling exercise on the TIMP-3 AB-loop suggests that the residues most likely to come into contact with TACE in a hypothetical TACE-N-TIMP-3 complex are Pro$^{33}$, Phe$^{34}$, and Gly$^{35}$ (Fig. 3). The equivalent residues in TIMP-4 Thr$^{38}$, Glu$^{39}$, Lys$^{40}$, respectively, as highlighted in Fig. 3 (upper panel). Could these residues be the key to TACE recognition? As the first step toward delineating the molecular significance of the loop, we created an N-TIMP-4 triple mutant with three amino acid substitutions: T38P, E39F, and K40G. Indeed, the $K_i$ value of the resultant mutant (T38P/E39F/K40G) was an order of magnitude better than that of the wild-type N-TIMP-4 ($K_i$, T38P/E39F/K40G of 0.86 nM versus wild-type of 8.7 nM). Its association rate was almost 2.5-fold that of the wild-type inhibitor (T38P/E39F/K40G, $k_{on}$ of $10^5$ s$^{-1}$ versus wild-type of $10^3$ s$^{-1}$) (Table III).

The findings, although impressive, immediately prompted two further questions. 1) Which residue(s) within the three is/are responsible for the improvement in activity? 2) Given that TIMP-4 AB-loop is five amino acids longer than that of the TIMP-3, what would the effects be for shortening the length of the AB-loop of N-TIMP-4 mutant T38P/E39F/K40G to that of the TIMP-3 AB-loop? To address the first question, we dissected the triple T38P/E39F/K40G mutation into six individual single and double mutants as follows: T38P, E39F, K40G, T38P/E39F, T38P/K40G, and E39F/K40G (Table III).
tively, these six mutants should reveal the key residue(s) that is/are critical for TACE recognition. Among the three single mutants, only T38P demonstrated marginal signs of improvement in binding affinity (T38P, $K_i$ of 3.91 nM versus wild-type N-TIMP-4 of 8.68 nM). The affinities of the other two were either impaired (E39F, $K_i$ 9.86 nM) or not altered (K40G, $K_i$ 8.80 nM).

In contrast, all the double mutants displayed subtle enhancement in activities ($K_i$ T38P/E39F, T38P/K40G, and E39F/K40G between 3.7 and 5.0 nM) (Table III). The affinities of the other two were either impaired (E39F, $K_i$ 9.86 nM) or not altered (K40G, $K_i$ 8.80 nM).

In contrast, all the double mutants displayed subtle enhancement in activities ($K_i$ T38P/E39F, T38P/K40G, and E39F/K40G between 3.7 and 5.0 nM) (Table III). Most interestingly, the enhancement was noted even in the E39F/K40G double mutant ($K_i$ 4.90 nM) that was made up of two single mutations (E39F and K40G) that on their own have either negligible or deteriorating effects on TACE association.

To address the second question, we selected four N-TIMP-4 variants (i.e. wild-type N-TIMP-4, E39F single mutant, E39F/K40G double mutant, and T38P/E39F/K40G triple mutant) and truncated five residues from their AB-loops to generate a group of “AB-loop-less” versions of the equivalent mutants. The five AB-loop residues selected for removal are Ala$^{33}$, Asp$^{34}$, Pro$^{35}$, Ala$^{36}$, and Asp$^{37}$, chosen purely on the basis of sequence alignment (Fig. 3, upper panel). These AB-loop-less mutants are henceforth designated as follows: (i) $\Delta$AB, (ii) $\Delta$AB + E39F, (iii) $\Delta$AB + E39F/K40G, and (iv) $\Delta$AB + T38P/E39F/K40G (Table III). The kinetic profiles of these $\Delta$AB mutants are listed in Table III. In short, all AB-loop-less mutants were considerably weaker than their AB-loop-intact counterparts not only in affinities but also in association rates ($K_i$, $\Delta$AB mutants varied from 14 to 60 nM; $k_{on}$, $\Delta$AB mutants in the range of $2\times10^{-3}$ to $10^{-3}$ M$^{-1}$ s$^{-1}$ versus wild-type N-TIMP-4 of $6.4\times10^{-3}$ M$^{-1}$ s$^{-1}$). Notably, the $K_i$ values of these $\Delta$AB mutants were 3–15 times higher than those with intact loops, whereas the drop in $k_{on}$
conserved in TIMP-2 (Leu100) and TIMP-3 (Leu94) but not in N-TIMP-4 Mutant—
against TACE simply by replacing its Thr98 residue with (17, 18). As a matter of fact, TIMP-1 could be rendered active impact on the ability of a TIMP to recognize and inhibit TACE choice of amino acid occupant at this locus has a profound interest to us is because previous mutagenesis investigation of the AB-loop.

slight enhancement in affinity, the shown in Table IV. Indeed, incorporation of S2T resulted in “good” mutations at the N terminus and EF-loop, singularly as nM). In an attempt to further increase its potency, we combined the activities of the compounded H100G conclusion that could be clearly derived from the affinity values was only 2–3-fold, an indication that dissociation rate values ranged from 4 to 25 nM (Table V). The worst residues were no doubt proline (K_i 105 nM), tryptophan (K_i 185 nM), and aspartate (K_i 144 nM); replacement of Leu^{191} with these residues resulted in near complete elimination of the activities of the prototype with TACE. A conspicuous aspect worth noting is the discrepancy between the aspartate (K_i 144 nM) and the glutamate (K_i 13.3 nM) mutants. Despite the most modest difference in their biophysical properties, the residues have vastly dissimilar impact on the activities of the N-TIMP-4 prototype with TACE.

DISCUSSION

Table VI summarizes the latest progress of our TIMP engineering projects aimed specifically at two of the clinically most important membrane-associated MP, i.e. MT1-MMP and TACE. To date, we have engineered two variants of TIMP-1 mutants that are superb inhibitors for MT1-MMP (V4A/P6V/ T98L, K_i 1.80 nM) and S2T + H100G + T38P/E39F/K40G (K_i 2.65 nM) mutants. L101X Mutations—Leucine 101 is located at the turn of the EF-loop, right before the second disulfide bond (Cys^{396}-Cys^{180}) that bridges the loop to the N terminus of TIMP (Fig. 4, disulfide bonds are shown in yellow stick format). The amino acid is conserved in TIMP-2 (Leu^{180}) and TIMP-3 (Leu^{289}) but not in TIMP-1 (Fig. 2). Its equivalent in TIMP-1, as shown in Fig. 2, is threonine (Thr^{289}). The reason the residue is of immense interest to us is because previous mutagenesis investigation using TIMP-1 and TIMP-2 scaffolds demonstrated that the choice of amino acid occupant at this locus has a profound impact on the ability of a TIMP to recognize and inhibit TACE (17, 18). As a matter of fact, TIMP-1 could be rendered active against TACE simply by replacing its Thr^{289} residue with leucine (K_i 170 nM). Further mutagenesis analysis with TIMP-1 and -2 scaffolds showed that the presence of a leucine residue at the position was indispensable for the generation of tight-binding, TACE-active TIMP mutants (17, 18). Would the same rule apply to TIMP-4?

Using S2T + T38P/E39F/K40G as the prototype, we mutated Leu^{101} to all the existing amino acids, except cysteine. The kinetic properties of these L101X mutants are summarized in Table V. Indeed, leucine again fared best (K_i S2T + T38P/E39F/K40G/L101I 3.44 nM), and tyrosine (K_i S2T + T38P/ E39F/K40G/L101Y 3.55 nM) (L101X mutations highlighted in boldface for easy recognition). Apart from these three, the majority of the other amino acids was reasonably well tolerated; their K_i values ranged from 4 to 25 nM (Table V). The worst residues were no doubt proline (K_i 105 nM), tryptophan (K_i 185 nM), and aspartate (K_i 144 nM); replacement of Leu^{191} with these residues resulted in near complete elimination of the activities of the prototype with TACE. A conspicuous aspect worth noting is the discrepancy between the aspartate (K_i 144 nM) and the glutamate (K_i 13.3 nM) mutants. Despite the most modest difference in their biophysical properties, the residues have vastly dissimilar impact on the activities of the N-TIMP-4 prototype with TACE.

Delineating TACE/TIMP Selectivity

| N-TIMP-4 mutants bearing T38P, E39F, and K40G mutations | K_i (nM) | k_on (× 10^9 M^{-1} s^{-1}) | k_off (× 10^4 s^{-1}) |
|--------------------------------------------------------|----------|----------------------------|---------------------|
| AB-loop mutant carrying triple mutationsT38P/E39F/K40G | 0.86 ± 0.20 | 15.91 ± 0.02 | 1.37 |
| Mutants carrying single or double mutations at T38P/E39F/K40G<sup>b</sup> | | | |
| T38P | 3.91 ± 0.81 | 6.47 ± 0.13 | 2.53 |
| E39F | 9.64 ± 2.13 | 3.51 ± 0.22 | 3.38 |
| K40G | 8.80 ± 1.36 | 5.84 ± 0.40 | 5.14 |
| T38P/E39F | 5.03 ± 0.71 | 8.44 ± 0.44 | 4.24 |
| T38P/K40G | 3.67 ± 0.85 | 7.39 ± 0.54 | 2.71 |
| E39F/K40G | 4.90 ± 0.38 | 6.74 ± 0.06 | 3.30 |
| Mutants with truncated AB-loop (∆AB)<sup>d</sup> | | | |
| ∆AB | 24.29 ± 2.45 | 4.17 ± 0.21 | 10.13 |
| ∆AB + E39F | 60.68 ± 0.34 | 1.99 ± 0.21 | 12.07 |
| ∆AB + E39F/K40G | 14.66 ± 2.84 | 3.04 ± 0.15 | 4.46 |
| ∆AB + S2T/E39F/K40G | 13.81 ± 0.91 | 5.18 ± 0.26 | 7.15 |

<sup>a</sup> k_off is calculated by K_i × k_on.
<sup>b</sup> Mutants that carry the triple mutations T38P/E39F/K40G.
<sup>c</sup> Mutants with single or double mutations at the T38P/E39F/K40G site.
<sup>d</sup> Mutants with truncated AB-loop.
against this background that our current series of TIMP engineering projects was conceived and initiated. As mentioned before, TACE was chosen as the target of our investigation not only because of its prominent roles in inflammatory diseases such as arthritis, but more so on the basis of its discriminate sensitivity to TIMP-3. Hence, the immediate objective of this project was to delineate this selectivity by identifying the critical features of the TIMP crystal structure that differentiate TACE from TIMP-3. As shown in FIG. 4, this formula was successfully generated for TACE-inhibiting TIMPs. To convert an otherwise inactive TIMP into a fully active one against TACE, there are four rules to be observed as follows: 1) a leucine residue on the EF-loop immediately before the second disulfide bond; 2) a leucine residue on the CD-loop immediately before the first disulfide bond; 3) a threonine residue at the P1 position; and 4) a proline-phenylalanine-glycine triad at the AB-loop.

**TABLE IV**
The combining effects of the "good" mutations at the N terminus (S2T), the AB-loop (T38P/E39F/K40G), and the EF-loop (H100G)

|        | $K_i$ (nm) | $k_{on}$ ($\times 10^{-3}$ M$^{-1}$ s$^{-1}$) | $k_{off}$ ($\times 10^5$ s$^{-1}$) |
|--------|-----------|---------------------------------|-------------------------------|
| T38P/E39F/K40G | 0.86 ± 0.20 | 15.91 ± 0.02 | 1.37 |
| S2T + T38P/E39F/K40G | 0.73 ± 0.15 | 11.40 ± 0.11 | 0.83 |
| H100G + T38P/E39F/K40G | 1.80 ± 0.43 | 20.32 ± 1.22 | 3.66 |
| S2T + H100G + T38P/E39F/K40G | 2.65 ± 0.46 | 11.91 ± 1.60 | 3.16 |

$^a k_{off}$ is calculated by $K_i \times k_{on}$.

**TABLE V**
N-TIMP-4 prototype S2T + T38P/E39F/K40G with Leu-101X mutations

|        | $K_i$ (nm) | $k_{on}$ ($\times 10^{-3}$ M$^{-1}$ s$^{-1}$) | $k_{off}$ ($\times 10^5$ s$^{-1}$) |
|--------|-----------|---------------------------------|-------------------------------|
| Prototype S2T + T38P/E39F/K40G | 0.73 ± 0.15 | 11.40 ± 0.11 | 0.83 |
| Leu-101X mutants | | | |
| Hydrophobic | | | |
| Ile | 3.44 ± 0.85 | 10.24 ± 0.43 | 3.52 |
| Val | 7.20 ± 0.95 | 11.30 ± 0.41 | 8.14 |
| Met | 3.30 ± 0.52 | 7.28 ± 0.46 | 2.40 |
| Phe | 5.99 ± 0.88 | 6.44 ± 0.55 | 3.86 |
| Pro | 105 ± 42 | NA | NA |
| Small | | | |
| Gly | 24.02 ± 2.48 | 5.82 ± 1.27 | 13.98 |
| Ala | 10.19 ± 2.07 | 8.01 ± 0.18 | 8.16 |
| Nucleophilic | | | |
| Ser | 11.74 ± 1.52 | 11.30 ± 0.70 | 13.27 |
| Thr | 7.88 ± 0.95 | 10.71 ± 0.52 | 8.44 |
| Aromatic | | | |
| Tyr | 3.55 ± 0.69 | 12.11 ± 0.20 | 4.30 |
| Trp | 185 ± 3 | NA | NA |
| Acidic | | | |
| Asp | 144 ± 21 | NA | NA |
| Glu | 13.31 ± 2.75 | 4.56 ± 0.79 | 6.07 |
| Amide | | | |
| Asn | 5.41 ± 1.27 | 11.92 ± 0.51 | 6.45 |
| Gln | 4.56 ± 1.38 | 6.88 ± 0.17 | 3.14 |
| Basic | | | |
| His | 6.23 ± 0.61 | 8.43 ± 0.30 | 5.25 |
| Lys | 12.90 ± 1.44 | 8.09 ± 0.45 | 10.44 |
| Arg | 6.97 ± 0.57 | 11.61 ± 0.04 | 8.09 |

$^a k_{off}$ is calculated by $K_i \times k_{on}$.

$^b$ NA, not able to determine due to low activity.
Throughout this series of TIMP engineering projects, we have designed and created a whole new generation of TIMP mutants that are capable of inhibiting MPs otherwise insensitive to the wild-type inhibitors. We have engineered a TIMP-1 mutant that inhibits MT1-MMP, as well as TIMP-1, TIMP-2, and TIMP-4 mutants that are highly active against TACE. The binding affinities (Ki) of the TIMPs before (wild type) and after (mutants) are listed in 4th and 5th columns of the table. We were interested in making use of these mutants to address the many previously unanswered questions surrounding the biological functions of TIMPs that might not be related to their inhibitory functions; a good example is the ability of the TACE-active TIMP-1, TIMP-2, and TIMP-4 mutants to induce apoptosis in mammalian cells. A summary of the cellular functions of TIMPs can be found in the review by Baker et al. (32).

### Table VI

| Scaffold | Target MP | Mutation | Ki, wild-type | Ki, mutant | Ref. | New cellular function? (Ref. 32) |
|---------|-----------|----------|---------------|------------|------|---------------------------------|
| TIMP-1  | MT1-MMP   | V4A/P6V/T98L | 178           | 1.66       | 19   | Growth factor still? |
| TIMP-1  | TACE      | V4S/TIMP-3 AB-loop/V69L/T98L | 356           | 0.14       | 17   | Induces apoptosis? |
| TIMP-2  | TACE      | S2T/TIMP-3 AB-loop/A705V/T71L | 883           | 1.49       | 18   | Growth factor still? |
| TIMP-4  | TACE      | S2T/T3SP/E39F/K40G | 8.68          | 0.73       | This work | Inhibits angiogenesis? |

The findings of this investigation are, to a large extent, in agreement with the conclusions from our earlier TACE/TIMP mutagenesis studies that employed TIMP-1 and -2 scaffolds (17, 18). The principles leading to the creation of slow, tight-binding inhibitors may be varied subtly among the TIMPs; however, the essence is identical. In fact, we are now able to explain the molecular basis of the selective sensitivity of TACE to TIMP-3. The rules that underpin TACE/TIMP recognition are herein summarized. First and foremost, in order to forge tight binding with TACE, the amino acid immediately preceding the second disulfide bond on the EF-loop (i.e., residue Thr-98 in TIMP-1, residue Leu100 in TIMP-2, residue Leu84 in TIMP-3) demonstrated that the C terminus of TIMP-3 has negligible impact on TACE inhibition (Ki of N- and full-length TIMP-3 being equal at 0.2 nM) (9). Thus, our results support the notion that the C termini of TIMPs are also crucial in the determination of their MP inhibition profiles.

The findings of this investigation are, to a large extent, in agreement with the conclusions from our earlier TACE/TIMP mutagenesis studies that employed TIMP-1 and -2 scaffolds (17, 18). The principles leading to the creation of slow, tight-binding inhibitors may be varied subtly among the TIMPs; however, the essence is identical. In fact, we are now able to explain the molecular basis of the selective sensitivity of TACE to TIMP-3. The rules that underpin TACE/TIMP recognition are herein summarized. First and foremost, in order to forge tight binding with TACE, the amino acid immediately preceding the second disulfide bond on the EF-loop (i.e., residue Thr-98 in TIMP-1, residue Leu100 in TIMP-2, residue Leu84 in TIMP-3)
TIMP-3, and residue Leu$^{101}$ in TIMP-4 of a TIMP must be none other than a leucine. For reasons not yet clear, leucine is capable of bridging the MMP-binding ridge of TIMP to the surface of TACE and bringing about the establishment of a tight enzyme-inhibitor binary complex. Isoleucine and methionine are tolerated at the locus, but the affinities of the resultant TIMP mutants are always somewhat poorer than those with leucine (17, 18). On the other hand, occupation of the position by proline, tryptophan, and aspartate completely abrogates the activities of a TIMP toward TACE. Second, an identical stringency of amino acid requirement also occurred at the CD-loop, the site where TIMPs interact with the S2 pocket at the nonprimed side of the catalytic zinc (Fig. 5). The residue immediately before the first disulfide bond (i.e. the equivalent of valine 69 in TIMP-1, valine 71 in TIMP-2, leucine 67 in TIMP-3, and leucine 72 in TIMP-4) must again be leucine. Third, this rule relates to the preferred choice of amino acid at the P1 position (i.e. residue 2 at the very N terminus of the TIMPs). Occupation of the locus by threonine is always unfavorable to serine, the native residues of TIMP-2 and -4. The last and perhaps the most important rule that enables TIMP/TACE recognition is the absolute requirement of three residues, namely Pro-Phe-Gly, in concert, at the AB-loop. For the successful generation of a potent, TACE-active TIMP variant, the substitution of any therapeutic use, however, has yet to be determined, but the very fact that there are stiff challenges to be overcome adds excitement, puts a premium upon sound judgment, and renders the results still more interesting.

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REFERENCES

1. Douglas, D. A., Shi, Y. E., and Sang, Q. A. (1997) Protein Chem. 16, 237–255
2. Williamson, R. A., Martorell, G., Carr, M. D., Murphy, G., Docherty, A. J., Freedman, R. B., and Feneely, J. (1994) Biochemistry 33, 11745–11759
3. Gomis-Ruth, F., Maskos, K., Bergmann, D. H., Huber, R., Yashida, N., Nagase, H., Brew, K., Bourenkov, G. P., Bartunik, H., and Bode, W. (1997) Nature 389, 77–81
4. Tsuttila, A., Morganova, E., Bergmann, U., Lindqvist, Y., Maskos, K., Fernandez-Catalan, C., Bode, W., Tryggvason, K., and Schneider, G. (1998) J. Mol. Biol. 284, 1133–1140
5. Fernandez-Catalan, C., Bode, W., Huber, R., Turk, D., Calvete, J. J., Lichte, A., Tarchesche, H., and Maskos, K. (1998) EMBO J. 17, 5238–5248
6. Williamson, R. A., Bartels, H., Murphy, G., and Freedman, R. B. (1994) Protein Eng. 7, 1035–1040
7. Negro, A., Onisto, M., Massiero, L., and Garbisa, S. (1995) FEBS Lett. 360, 52–56
8. Lee, M. H., Knauper, V., Becherer, J. D., and Murphy, G. (2001) Biochem. Biophys. Res. Commun. 290, 945–950
9. Williamson, R. A. (2001) Methods Mol. Biol. 151, 257–265
10. Davies, D. (2001) Methods Mol. Biol. 151, 267–273
11. Stracke, J. O., Hutton, M., Stewart, M., Pendás, A. M., Smith, B., López-Otin, C., Murphy, G., and Knauper, V. (2000) J. Biol. Chem. 275, 14809–14816
12. Fernandez-Barrantes, S., Bernardo, M., Toth, M., and Fridman, R. (2000) Semin. Cancer Biol. 12, 131–138
13. Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Docherty, A. J., and Murphy, G. (2000) FEBS Lett. 473, 275–279
14. Amour, A., Slocombe, P. M., Webster, A., Butler, M., Knight, C. G., Smith, B. J., Stephens, P. E., Shelley, C., Hutton, M., Knauper, V., Docherty, A. J., and Murphy, G. (1998) FEBS Lett. 435, 39–44
15. Kheradmand, F., and Werb, Z. (2002) Bioessays 24, 8–12
16. Mills, M. A., Leesnitzer, M. A., Moss, M. L., Clay, W. C., Carter, H. L., Miller, A. R. S., Ju, J. L., Lambem, G., Willard, D. H., Shelley, D. M., Koel, T. A., Burkhart, W., Moyer, M., Blackburn, R. K., Pahel, G. L., Mitchell, J. L., Hoffman, C. R., and Becherer, J. D. (1999) J. Biol. Chem. 274, 30583–30579
17. Lee, M. H., Bapti, M., Knauper, V., and Murphy, G. (2004) J. Biol. Chem. 279, 17562–17569
18. Lee, M. H., Bapti, M., and Murphy, G. (2004) J. Biol. Chem. 279, 45121–45129
19. Lee, M. H., Bapti, M., and Murphy, G. (2003) J. Biol. Chem. 278, 40324–40330
20. Morrison, J. F., and Walsh, C. T. (1995) Methods Enzymol. 248, 201–301
21. Stratmann, B., Farr, M., and Tarchesche, H. (2001) Biol. Chem. 382, 987–991
22. Troeberg, L., Tanaka, M., Waki, R., Shi, Y. E., Brew, K., and Nagase, H. (2002) Biochemistry 41, 10525–10535
23. Hashimoto, G., Aoki, T., Nakamura, H., Tanzawa, K., and Okada, Y. (2001) FEBS Lett. 494, 192–195
24. Lee, M. H., Verma, V., Maskos, K., Nath, D., Knauper, V., Dodds, P., Amour, A., and Murphy, G. (2002) Biochem. J. 364, 227–234
25. Leco, K. J., Waterhouse, P., Sanchez, O. H., Gowling, K. L., Poole, A. R., Wakeham, A., Mak, T. W., and Knoch, R. (2001) J. Clin. Invest. 108, 817–829
26. Fata, J. E., Leco, K. J., Voura, E. B., Yu, H. Y., Waterhouse, P., Murphy, G., Moorehead, R. A., and Knoch, R. (2001) J. Clin. Investig. 108, 831–841
27. Abraham, M., Baker, A. H., and Kahari, V. M. (1998) Cancer Res. 58, 2310–2315
28. Baker, A. H., George, S. J., Zaltsman, A. B., Murphy, G., and Newby, A. C. (1999) Br J Cancer 79, 1347–1355
29. Ahonen, M., Poukkula, M., Baker, A. H., Kashiwagi, M., Nagase, H., Eriksson, T. E., and Kahari, V. M. (2001) Oncogene 22, 2121–2128
30. Docherty, A. J., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J., Murphy, G., and Reynolds, J. J. (1985) Nature 318, 66–69
31. Williamson, G., Bapti, M., Hutton, M., Knauper, V., Docherty, A. J., Carr, M. D., and Murphy, G. (2001) J. Biol. Chem. 276, 32966–32970
32. Baker, A. H., Edwards, D. R., and Murphy, G. (2002) J. Cell Sci. 115, 3719–3727