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The C-terminal domains of TACE weaken the inhibitory action of N-TIMP-3

Meng-Huee Lee[a,*], Vandana Verma[a], Klaus Maskos[b], J. David Becherer[c], Vera Knäuper[a,1], Philippa Dodds[a], Augustin Amour[d], Gillian Murphy[a]

[a] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[b] *Max-Planck Institut für Biochemie, D-82152 Martinsried, Germany*
[c] *Glaxo Smith Kline Research and Development Inc., 5 Moore Drive, Research Triangle Park, NC 27709, USA*
[d] *Glaxo Smith Kline, Stevenage, Herts SG1 2NY, UK*

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Abstract Tumor necrosis factor-α converting enzyme (TACE) is an ADAM (a disintegrin and metalloproteinases) that comprises an active catalytic domain and several C-terminal domains. We compare the binding affinity and association rate constants of the N-terminal domain form of wild-type tissue inhibitor of metalloproteinase (TIMP-3; N-TIMP-3) and its mutants against full-length recombinant TACE and the truncated form of its catalytic domain. We show that the C-terminal domains of TACE substantially weaken the inhibitory action of N-TIMP-3. Further probing with hydroxamate inhibitors indicates that both forms of TACE have similar active site configurations. Our findings highlight the potential role of the C-terminal domains of ADAM proteinases in influencing TIMP interactions. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: N-TIMP-3; TACE-cat; TACE-long; Binding affinity; Association rate constants

1. Introduction

Tumor necrosis factor-α (TNF-α) is a key pro-inflammatory cytokine produced by activated monocytes and macrophages as part of the self-defence machinery. Excessive levels of TNF-α are known to be responsible for the inflammatory features of many debilitating diseases such as rheumatoid arthritis, Alzheimer's disease, Crohn's disease as well as various types of autoimmune disorders [1–5]. The precursor of the cytokine, pro-TNF-α, is a membrane-bound trimeric molecule of 26 kDa monomers. Upon cleavage by TNF-α converting enzyme (TACE), the active, 17 kDa form of mature TNF-α is released into the surrounding medium. TACE is a zinc-dependent metalloproteinase belonging to the ADAM proteinase (a disintegrin and metalloproteinases) family (ADAM-17). Apart from the enzymatically active catalytic domain, TACE also contains a pro-domain at its N-terminus and several domains at its C-terminus. These C-terminal domains are commonly referred to as the disintegrin, epidermal growth factor (EGF)-like, crambin-like, transmembrane and cytoplasmic domains. Although there is plenty of evidence confirming the important roles of these domains in inter-cellular adhesion and fertilization [6–9], to date, no investigation has yet studied their effects on the inhibitory action of tissue inhibitor of metalloproteinases (TIMP).

Amongst the four species of TIMP discovered so far, only TIMP-3 has been shown to be capable of inhibiting TACE [10–12]. The TIMP-3 molecule is composed of 178 amino acids, and the N-terminal two-thirds of the polypeptide is believed to be folded into a very distinct domain highly reminiscent of the oligosaccharide/oligonucleotide-binding protein motif [13–14]. The truncated N-terminal domain of the TIMPs has been known to form a stable, autonomous unit capable of inhibiting many matrix metalloproteinases (MMPs), ADAMs and ADAM proteinases with thrombospondin domains at the C-termini (ADAM-TS) [11–12,15–17]. Previously we reported that full-length recombinant TIMP-3 and its N-terminal domain truncate (N-TIMP-3) displayed comparable tightness of binding against TACE [11]. We have subsequently demonstrated that N-TIMP-3 could be further engineered into a powerful and selective inhibitor against the catalytic domain of TACE [12]. In this report, we provide the first evidence that the C-terminal domains of TACE could exert a significant impact on the inhibitory activity of N-TIMP-3 molecule.

2. Materials and methods

2.1. TACE-cat and TACE-long

TACE-cat (also known as TACE-473) is the recombinant form of TACE enzyme that encompasses only the catalytic domain (i.e. residues 215–473) of the enzyme polypeptide. TACE-long (TACE-651), on the other hand, is the soluble extracellular form of TACE that incorporates the catalytic domain as well as the disintegrin, EGF-like and crambin-like domains at the C-terminus (i.e. residues 215–651) [18]. Both TACE-cat and TACE-long are kind gifts from Glaxo Smith Kline Research and Development Inc., NC, USA.

2.2. Construction, purification and refolding of wild-type and mutant N-TIMP-3

In our previous report, we described the creation of a series of 'tailor made' N-TIMP-3 mutants that displayed extreme binding tightness against TACE-cat [12]. In this study, we extend these mutants to

*Corresponding author. Fax: (44)-1603-592250.
E-mail address: meng.lee@uea.ac.uk (M.-H. Lee).

[1] Present address: Department of Biology, University of York, York YO10 5DD, UK.

Abbreviations: TIMP-3, tissue inhibitor of metalloproteinases-3; TNF-α, tumor necrosis factor-α; TACE, tumor necrosis factor-α converting enzyme; ADAM-TS, ADAM proteinase with thrombospondin domains; Mca, (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenyl

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characterize the TACE-long enzyme. Hence, the construction, refolding and purification details of the mutants have been described elsewhere [11–12]. In essence, N-TIMP-3 inclusion bodies expressed in *Escherichia coli* were dissolved in 50 ml 6 M guanidinium-Cl (GdmCl) in 50 mM Tris–HCl, pH 7.8, in the presence of 10 mM dithiothreitol. Solubilized proteins were pumped slowly (0.5 ml/min) into 2 l refolding buffer containing 1.5 M GdmCl, 50 mM Tris–HCl, pH 10, containing reduced and oxidized glutathione (GSH 0.10 mM and GSSG 0.40 mM). Refolding was allowed for at least 16 h at room temperature. The following day, the refolding solutions were dialyzed twice into 30 l of 50 mM Tris–HCl, pH 7.8. Insoluble N-TIMP-3 proteins were removed by centrifugation at 12 000 g for 30 min. Soluble active N-TIMP-3 were extracted with 50 ml Ni-NTA agarose, eluted with 0.2 M imidazole before final dialysis in 50 mM Tris–HCl, pH 7.8, containing 25% glycerol.

2.3. Enzyme assays

The quenched fluorescent substrate, QF-45 (Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys-Dnp-NH_2) was used for TACE-cat and TACE-long assays as described in our previous paper [11,12]. A Perkin-Elmer LS-50B spectrofluorometer with thermostatic cuvette holders was used for both k_{on} and K_i experiments. All enzymatic assays were carried out at 27°C unless otherwise stated. All wild-type and mutant N-TIMP-3 were titrated against both TACE-cat and gelatinase-A (MMP-2) for accurate estimation of concentration. Fluorescence assay buffer (10 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5, 0.05% Brij-35, 1% DMSO, 0.02% NaN_3) buffer was used as diluent throughout the experiments.

2.4. Association rate constant (k_{on}) measurement

Association rate constant measurements were started by adding various concentrations of N-TIMP-3 proteins (5–150 nM) to TACE-cat or TACE-long (0.1 nM), depending on the potency of the mutants. For TACE-cat, less than 5 nM of Ser-4Met/Lys/Arg mutants were needed for near total inhibition in less than 2000 s. TACE-long, comparatively, required more N-TIMP-3 for inhibition. The time course of inhibition was monitored using a continuous fluorometric assay until steady state was reached, usually after 12 000 s. The progress curve was analyzed using the Enzfitter program (Biosoft, Cambridge, UK) and the equation:

$$P = V_s t + (V_0 - V_s)(1 - e^{-kt})/k$$

where P = product concentration; V_0 = initial velocity; V_s = steady state velocity; k = 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.

All k_{on} measurements were performed at least twice to confirm the reproducibility of the results.

2.5. Inhibition constant (K_i^{app}) measurement

In all K_i^{app} studies, TACE-cat and TACE-long (0.2 nM) were preincubated with different concentrations of N-TIMP-3 or its mutants in a final volume of 2.5 ml. Due to the diverse potency of various N-TIMP-3 mutants against TACE-cat and TACE-long, the concentrations of N-TIMP-3 proteins required differed among the mutants. TACE-cat required less inhibitors, the concentrations of some tight-binding mutants (e.g. Ser-4Met, Ser-4Lys and Ser-4Arg) typically ranged from 0.1 to less than 5 nM. For the less potent mutants (i.e. all Thr-2 mutants), up to 50 nM of proteins were required. On the contrary, substantially more inhibitors were needed for all TACE-long assays, the concentrations of some N-TIMP-3 mutants could be as

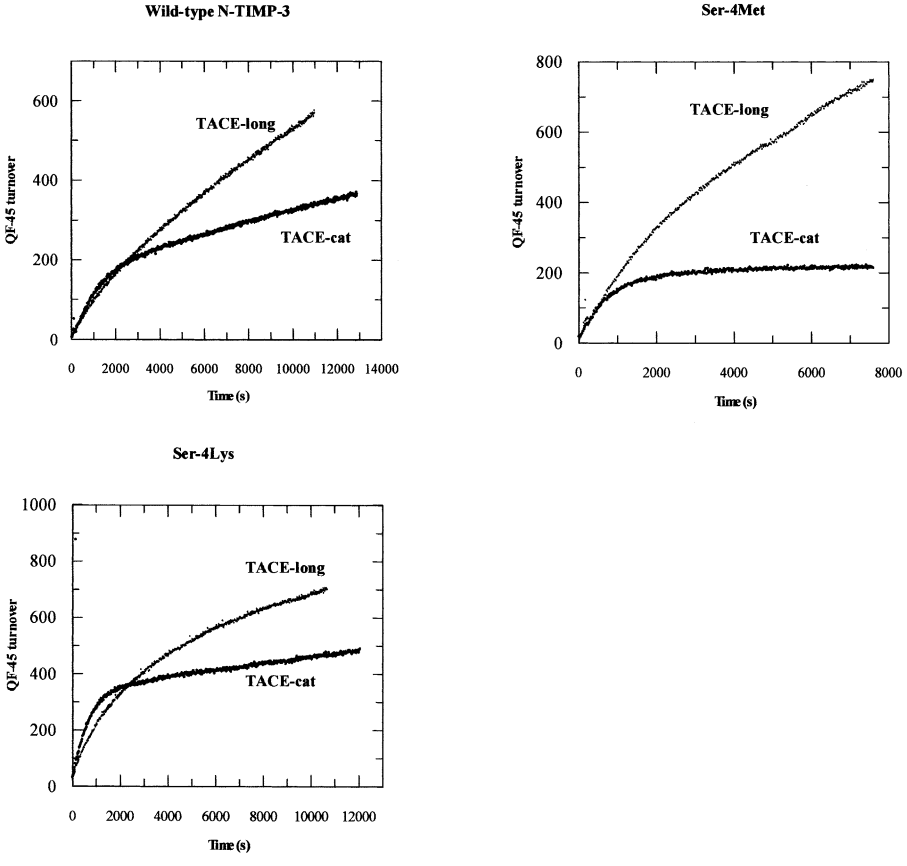

Fig. 1. Time courses of the inhibition profiles (k_{on}) of wild-type N-TIMP-3, Ser-4Met and Ser-4Lys mutants against TACE-cat and TACE-long. Wild-type N-TIMP-3 or the Ser-4 mutants were added to 0.1 nM TACE-cat or TACE-long to a final concentration of 2.5 nM and the turnover of QF-45 substrate monitored over a period of 15 000 s. At equilibrium, Ser-4Met completely inactivated TACE-cat, but not TACE-long. TACE-cat enzyme in the Ser-4Lys sample was still slightly active, although the K_i^{app} value of Ser-4Lys could not be determined accurately. The time scale of the experiments varied, due to the difference in potency among the mutants. *Y*-axis: fluorescence in arbitrary units; *X*-axis: time in seconds.

much as 150 nM. Incubation was allowed for 3 h at room temperature prior to steady state (V_s) measurement. Measurements of enzyme activities were initiated by adding fluorescent substrate QF-45 to a final concentration of 1 μM. The time courses were fitted into competitive tight binding equations with the computer program Grafit to obtain an estimation of K_i^{app} values:

$$V_s = (V_0/2E_t)\times\{(E_t - I_t - K_i^{app}) + [(K_i^{app} + I_t - E_t)^2 + 4E_t\ K_i^{app}]^{1/2}\}$$

where V_0 = rate in the absence of inhibitor; E_t = total enzyme concentration; I_t = total inhibitor concentration.

2.6. Inhibition constant (K_i^{app}) with hydroxamate acids

As with N-TIMP-3 mutants in Section 2.5, similar concentrations of TACE-cat and TACE-long were used in determining the K_i^{app} values of hydroxamate inhibitors. Enzymes were pre-incubated with up to 10 nM of hydroxamate acids for 3 h at room temperature before steady state velocities (Vs) were measured.

3. Results

3.1. K_i^{app} profiles of Thr-2, Ser-4 and Leu-67 mutants against TACE-cat and TACE-long

Most Ser-4 mutants displayed extremely high binding affinities against TACE-cat ($K_i^{app} < 60$ pM) (Table 1 and Fig. 1). In fact, the accurate K_i^{app} values of Ser-4Met, Ser-4Lys and Ser-4Arg mutants were beyond the sensitivity limit of our fluorometric assays. As shown in Fig. 2, TACE-cat was totally inactivated by the Ser-4Met mutant at the inhibitor:enzyme (I:E) ratio of 5:1 when steady state was finally achieved. TACE-long, on the contrary, could not be completely inactivated by the same mutant even when the I:E ratio was raised to 20:1. The contrast between the inhibitory potency of N-TIMP-3 mutants against the two variants of TACE is best reflected by the time courses of their action. Fig. 1 summarizes the k_{on} profiles of wild-type N-TIMP-3, Ser-4Met and Ser-4Lys mutants against TACE-cat and TACE-long. At equilibrium, TACE-cat and TACE-long enzymes in the wild-type N-TIMP-3 samples still showed detectable activity against QF-45. On the other hand, the Ser-4Met mutant was capable of completely inactivating TACE-cat, but not TACE-long. In any event, regardless of the mutants, TACE-long was always more resilient to N-TIMP-3 inhibition than TACE-cat.

Table 1
K_i^{app} of wild-type N-TIMP-3, Thr-2, Ser-4 and Leu-67 mutants against TACE-cat and TACE-long

	K_i^{app} (nM)	
	TACE-cat[a]	TACE-long
FL-TIMP-3	0.20 ± 0.03	0.74 ± 0.04[a]
N-TIMP-3[a]	0.22 ± 0.07	1.75 ± 0.20[a]
Thr-2Leu	0.49 ± 0.07	2.78 ± 0.20
Thr-2Met	0.77 ± 0.06	4.20 ± 0.10
Thr-2Tyr	1.30 ± 0.16	8.03 ± 0.03
Ser-4Met	< 0.060	1.01 ± 0.03
Ser-4Lys	< 0.060	0.96 ± 0.13
Ser-4Arg	< 0.060	0.49 ± 0.07
Ser-4Leu	0.066 ± 0.016	0.53 ± 0.10
Ser-4Tyr	0.063 ± 0.012	0.96 ± 0.05
Leu-67Met	0.073 ± 0.014	1.67 ± 0.33

All Ser-4 and Leu-67 mutants have higher affinities against TACE-cat in comparison with the wild-type N-TIMP-3. The K_i^{app} values of Ser-4Met/Lys/Arg mutants against TACE-cat could not be measured accurately due to the tight binding nature of the inhibitors. Note that the K_i^{app} values of Thr-2 mutants increase with the length of the residue. Also, all Ser-4 mutants displayed similar affinities against TACE-long. FL-TIMP-3: full-length TIMP-3.
[a]Results previously published [11,12].

Table 2
Association rate constants (k_{on}) of wild-type N-TIMP-3, Thr-2, Ser-4 and Leu-67 mutants against TACE-cat and TACE-long

	k_{on} ($\times 10^5$ M^{-1} s^{-1})	
	TACE-cat[a]	TACE-long
FL-TIMP-3	9.94 ± 0.46	0.98 ± 0.13[a]
N-TIMP-3[a]	3.65 ± 0.33	0.46 ± 0.14[a]
Thr-2Leu	1.12 ± 0.05	0.43 ± 0.07
Thr-2Met	3.34 ± 1.01	2.32 ± 0.11
Thr-2Tyr	0.48 ± 0.05	0.23 ± 0.04
Ser-4Met	3.00 ± 0.42	2.27 ± 0.69
Ser-4Lys	3.12 ± 0.22	0.29 ± 0.04
Ser-4Arg	1.84 ± 0.19	0.20 ± 0.02
Ser-4Leu	2.66 ± 0.04	0.47 ± 0.07
Ser-4Tyr	2.71 ± 0.02	4.47 ± 1.16
Leu-67Met	2.96 ± 1.44	0.59 ± 0.14

Wild-type N-TIMP-3 has lower association rate with TACE-long than with TACE-cat. However, Thr-2Met, Ser-4Met and Ser-4Tyr mutants show improved association rates against TACE-long in comparison with the wild-type N-TIMP-3. FL-TIMP-3: full-length TIMP-3.
[a]Results previously published [11,12].

The K_i^{app} profiles of the same mutants against TACE-cat and TACE-long are shown in Fig. 2.

In comparison to wild-type N-TIMP-3, all Thr-2 mutants have impaired affinities against both TACE-cat and TACE-long. Despite the general reduction in binding tightness, yet again, all Thr-2 mutants demonstrated a similar pattern of inhibition as their Ser-4 counterparts, i.e. their affinities with TACE-long were consistently lower than with TACE-cat (Table 1). As a matter of fact, without exception, all wild-type as well as mutant N-TIMP-3 exhibited nearly 10-fold lower affinity against TACE-long than TACE-cat.

3.2. Association rates (k_{on}) of N-TIMP-3 mutants with TACE-cat and TACE-long: increase efficiency of Thr-2Met, Ser-4Met and Ser-4Tyr

Association rate constants (k_{on}) are the reflection of the swiftness of TACE and N-TIMP-3 molecules in forming a stable binary complex. In comparison with TACE-cat, wild-type N-TIMP-3 was nearly an order of magnitude less efficient in forming a binary complex with TACE-long (TACE-long $k_{on} \sim 0.5\times10^5$ M^{-1} s^{-1}; TACE-cat $k_{on} \sim 4\times10^5$ M^{-1} s^{-1}); so were most of the N-TIMP-3 mutants (Table 2). When all the Thr-2, Ser-4 and Leu-67 mutants were aligned against wild-type N-TIMP-3, a clear pattern emerged suggesting that Thr-2Met, Ser-4Met and Ser-4Tyr mutants were in fact more efficient than wild-type N-TIMP-3 in associating with TACE-long (wild-type $k_{on} \sim 0.5\times10^5$ M^{-1} s^{-1}; Thr-2Met, Ser-4Met, Ser-4Tyr $k_{on} \sim 2$–5×10^5 M^{-1} s^{-1}). The gain in efficiency was no doubt the direct result of enhancing the length and hydrophobicity of residue-2 and -4 side chains.

3.3. Inhibition constants (K_i^{app}) of TACE-cat and TACE-long with hydroxamate inhibitors

Four different species of hydroxamate compounds were chosen for this study, with the criteria for their selection based primarily on the discrepancy in the bulkiness of their P1′ and P3′ sub-units (Fig. 3). CT2256 has relatively small P1′ and P3′ groups that should fit relatively well into the S1′ and S3′ pockets of TACE-cat, although we suspect there might still be space for expansion in the P3′ sub-unit. CT1399 and CT435, on the other hand, were chosen for their large P1′

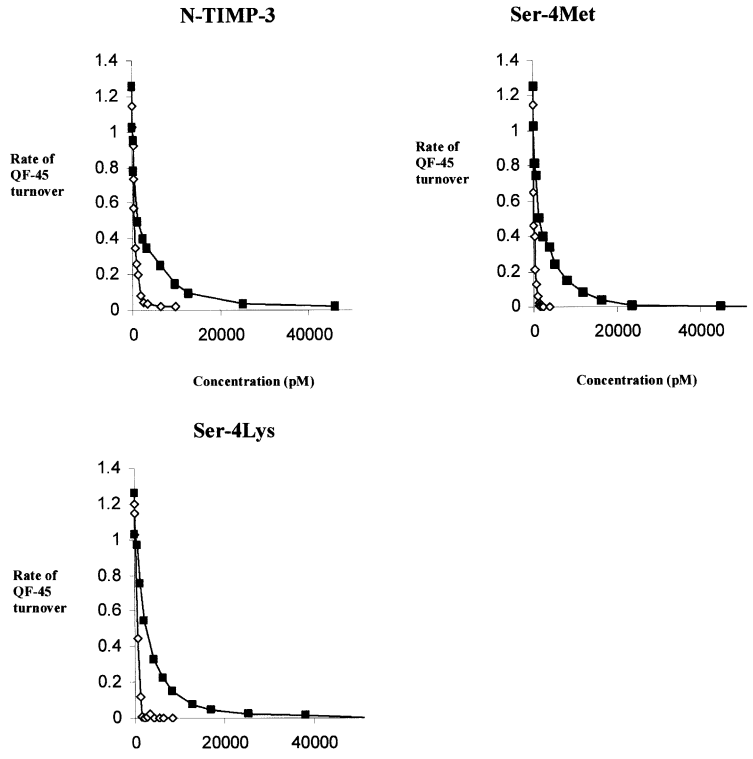

Fig. 2. K_i^{app} inhibition profiles of wild-type N-TIMP-3, Ser-4Met and Ser-4Lys mutants against TACE-cat and TACE-long. The accurate values for Ser-4Met and Ser-4Lys against TACE-cat could not be determined accurately due to total inhibition of the enzyme at higher concentrations of inhibitors. On the contrary, TACE-long was much more resilient towards N-TIMP-3 inhibition. *Y*-axis: rate of QF-45 catalysis; *X*-axis: concentration of N-TIMP-3 and mutants in pM; ◇: TACE-cat; ■: TACE-long.

and/or P3′ sub-units. In our opinion, if TACE-cat and TACE-long do differ in their active site configuration, these two compounds should be able to reflect the divergence by yielding different K_i^{app} values with the two species of TACE. The last compound, AG3340, belongs to the novel class of inhibitor without the conventional P1′ and P3′ sub-units. Evidently, all CT2256, CT1399, AG3340 and CT435 exhibited near identical K_i^{app} values with both TACE-cat and TACE-long. Thus, our results effectively rule out the possibility that TACE-cat and TACE-long have major differences in their overall active site configuration.

4. Discussion

In our previous report, we described the creation of a novel generation of 'super' N-TIMP-3 mutants that bind to the catalytic domain truncate of TACE with extremely tight affinity ($K_i^{app} < 60$ pM) [12]. The design of these 'super-mutants' was based solely on the published crystal structure of the catalytic domain truncate of the TACE enzyme [19]. In spite of the remarkable improvement in binding tightness in vitro, our cell-based TNF-α shedding analysis showed that, among others, the super-mutant Ser-4Met ($K_i^{app} < 60$ pM against TACE-cat) was only marginally better than the wild-type N-TIMP-3 in inhibiting native TACE activity [12]. One of the most likely causes for such discrepancy in potency, in our opinion, was the interference posed by the C-terminal domains of the native TACE enzyme. Therefore, we decided to extend our studies to TACE-long, the variant of recombinant TACE that bore more resemblance to the native enzyme as it retained the disintegrin, EGF-like and crambin-like domains at its C-terminus. Here, we demonstrate that these C-terminal domains do significantly weaken the inhibitory activity of N-TIMP-3.

At this stage, we ruled out the likelihood that the C-terminal domains have somehow altered the overall configuration of the S1′ and S3′ pockets. TACE-cat and TACE-long showed indistinguishable K_i^{app} values with all the hydroxamate inhibitors, indicating no extensive dissimilarity in their active site configuration. Moreover, in the original paper describing the expression and purification of the enzymes by Milla et al., TACE-cat and TACE-long were also demonstrated to have similar k_{cat}/K_m values in synthetic peptide turnover (k_{cat}/K_m TACE-cat 1.3×10^5 M^{-1} s^{-1}; TACE-long 1.8×10^5 M^{-1} s^{-1}) [18].

Instead, our findings point to steric hindrance being the more likely explanation for the lower potency of N-TIMP-3 with TACE-long. In particular, the k_{on} data of Thr-2Met, Ser-4Met and Ser-4Tyr mutants suggest that the association rate against TACE-long could be improved by increasing the length and hydrophobicity of residue-4. In other words, our results seem to hint at the presence of an 'obstacle' in TACE-long that hinders N-TIMP-3 from docking snuggly onto the surface of the enzyme. This 'obstacle', in our view, originates from the physical obstruction posed by the C-terminal domains upon an approaching N-TIMP-3 molecule. Besides, our previous kinetic studies with full-length TIMP-3 also revealed similar patterns of inhibition, i.e. the binding affinities and the association rate constants of full-length TIMP-3 with TACE-long were consistently lower than those with TACE-cat (Tables 1 and 2) [11]. Most amazingly, this 'obstructive effect' was also noted by the workers describing the expression

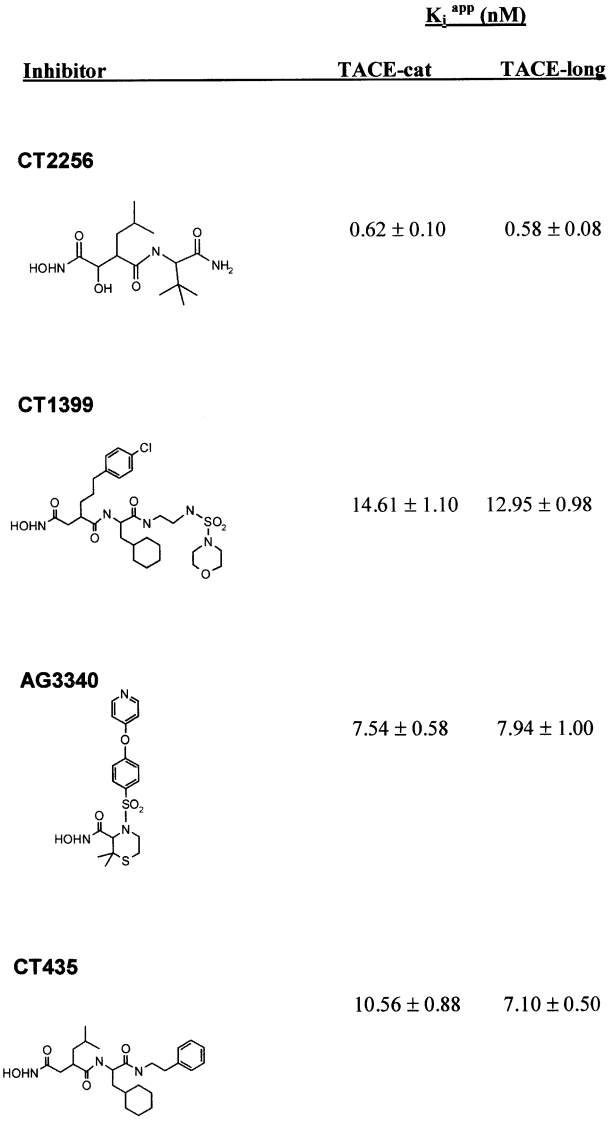

Fig. 3. K_i^{app} equilibrium constants of CT2256, CT1399, AG3340 and CT435 against TACE-cat and TACE-long. A panel of hydroxamate inhibitors of different P1′ and P3′ sub-units were chosen to probe the active site S1′ and S3′ pockets of TACE-cat and TACE-long. The data support the view that TACE-cat and TACE-long have similar active site configuration.

and purification of the TACE-cat and TACE-long enzymes. In the paper by Milla et al., TACE-cat was observed to be capable of forming a tight, inactive complex with the pro-domain of TACE during protein expression. In order to release the enzyme, up to 4 M of urea was required to denature one or both members of the complex. TACE-long, on the contrary, was incapable of forming such a complex with the pro-domain [18].

An interesting yet demanding challenge prompted by the current study is the question of whether the soluble form of TACE-long enzyme in our solution assay system genuinely resembles native TACE that exist on the cell surface. Given the fact that TIMP-3 appears to be a significant regulator of TACE in vivo, it is important to recognize its role in relation to the development of many TACE-related clinical disorders. Currently, a number of other ADAM and ADAM-TS proteinases are known to be inhibited by TIMP-1 and/or -3 [10–12,17,20]. Many biochemical and kinetic data derived from these ADAM proteinases could only be performed with soluble or truncated forms of enzyme frequently lacking various domains. Thus, our findings highlight the difficulty of relating in vitro biochemical data to actual cellular events and the importance of conducting the latter type of assays. Understanding the effects of these C-terminal domains on TIMP action would no doubt be of utmost importance in drug design and targeting in the future.

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