Tailoring tissue inhibitor of metalloproteinases-3 to overcome the weakening effects of the cysteine-rich domains of tumour necrosis factor- α converting enzyme

Meng-Huee LEE*, Philippa DODDS*, Vandana VERMA*, Klaus MASKOS†, Vera KNÄUPER*¹ and Gillian MURPHY*²

*School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K., and †Max-Planck Institut für Biochemie, D-82152 Martinsried, Germany

Tumour necrosis factor- α (TNF- α) converting enzyme (TACE) is a membrane-anchored, multiple-domain zinc metalloproteinase responsible for the release of the potent pro-inflammatory cytokine, TNF- α . The extracellular part of the active enzyme is composed of a catalytic domain and several cysteine-rich domains. Previously, we reported that these cysteine-rich domains significantly weakened the inhibitory potency of the N-terminal-domain form of tissue inhibitor of metalloproteinases-3 (N-TIMP-3). In the present paper, we describe a novel strategy developed to overcome this weakening effect. We have engineered a new generation of N-TIMP-3 mutants that are capable of withstanding the repulsion of the cysteine-rich domains by the formation of electrostatic bonds with the catalytic domain of the enzyme. These N-TIMP-3 mutants displayed markedly improved binding affinity with the

soluble extracellular domain form of recombinant TACE. With K_i (app) values of < 0.1 nM, these mutants were dramatically better than the wild-type N-TIMP-3 [K_i (app) 1.7 nM]. We accounted for this by proposing that Glu³¹, an acidic residue situated at the base of the AB-loop of N-TIMP-3, is drawn into contact with Lys³¹⁵, a prominent basic residue adjacent to the TACE catalytic site. The mutagenesis strategy involved re-orientation of the edge of N-TIMP-3; in particular, the β -strand A where Glu³¹ was located. Further expression of one of the mutants, Lys^{26/27/30/76} → Glu, in a mammalian expression system confirmed that TIMP-3 associates with the extracellular matrix via its C-terminal domain.

Key words: binding affinity, conformation tuning, protein engineering.

INTRODUCTION

Tumour necrosis factor- α (TNF- α) converting enzyme (TACE; also known as ADAM-17) is a member of the zinc-dependent ADAM (a disintegrin and metalloproteinase) metalloproteinase family. The enzyme has been implicated in the ectodomain shedding of many important growth factors and bioactive cell-surface molecules, one of the most studied being the pro-inflammatory cytokine, TNF- α [1–4]. TACE is a cell-surface-anchored enzyme consisting of several distinctive domains. Preceding the catalytic domain is a pro-domain of approx. 20 kDa that includes a consensus cysteine switch motif (¹⁸¹PKVC¹⁸⁶GY¹⁸⁶) that serves to co-ordinate the catalytic zinc in the active site of the enzyme. The catalytic domain extends from residue 215 to 473, and contains a typical zinc-binding motif sequence (HEXXHXXGXXH) common to the majority of metzincins. The crystal structure of the soluble truncated catalytic domain revealed a polypeptide fold and an active-site environment reminiscent of that of the snake venom metalloproteinases [5]. Following the catalytic domain are the disintegrin, epidermal growth factor (EGF)-like and the Crambin-like domains. These domains are frequently referred to as the ‘cysteine-rich’ domains. To date, the structures and functions of these extracellular domains have not been fully delineated. At the C-terminus of the enzyme are the transmembrane and cyto-

plasmic domains that exceed 150 amino acids in length. Given the pivotal role of TACE in many clinical complications, intense efforts have been made in the pharmaceutical industry to search for an inhibitor that could ultimately be used to modulate the activities of the enzyme.

Tissue inhibitor of metalloproteinases (TIMP)-3 is the only member of the mammalian TIMP family that has been demonstrated to be capable of inhibiting TACE [6]. TIMP-3 is composed of 178 amino acids, and the molecule is believed to be folded into two well-defined N- and C-terminal domains. The N-terminal domain encompasses almost the entire first two-thirds of the molecule. The overall configuration of the domain is considered to be similar to the oligosaccharide/oligonucleotide binding (OB) moiety, as found in TIMP-1 and TIMP-2 [7,8]. The C-terminal domain, on the other hand, is structurally less well defined. Both the N- and C-terminal domains of TIMPs contain three disulphide bonds.

Previously, we discovered that the N-terminal domain of TIMP-3 (N-TIMP-3) retained full inhibitory activity against TACE [9]. Subsequently, we showed that the binding affinity of N-TIMP-3 against the catalytic domain of TACE (TACE-cat, or TACE-473) could be improved significantly by the introduction of bulkier and more hydrophobic amino acids at the Ser⁴ position [10]. Indeed, the K_i (app) values of these mutants with TACE-cat were substantially lower than 60 pM. However, ensuing kinetic

Abbreviations used: ADAM, a disintegrin and metalloproteinase; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; gel-A, gelatinase-A (also known as MMP-2); K26E, etc., mutant bearing an amino acid substitution of Lys²⁶ → Glu, etc.; MMP, matrix metalloproteinase; N-TIMP-3, N-terminal-domain form of TIMP-3; OB, oligosaccharide/oligonucleotide binding; PDB, Protein Data Bank; TNF- α , tumour necrosis factor- α ; TACE, TNF- α converting enzyme; TACE-cat, the catalytic domain form of TACE; TACE-long, TACE incorporating the catalytic and the cysteine-rich domains; TIMP, tissue inhibitor of metalloproteinases.

¹ Present address: Department of Biology, Biomedical Tissue Research Group, University of York, Heslington, York YO10 5YW, U.K.

² To whom correspondence should be addressed, at the present address: Room 6.9/11, Department of Oncology, University of Cambridge, Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, U.K. (e-mail gm290@cam.ac.uk).

analysis with the extracellular form of TACE that incorporated the catalytic and the cysteine-rich domains (TACE-long, or TACE-651) showed no real improvement in affinity, the K_i (app) values of these mutants being identical with that of the wild-type N-TIMP-3 (≈ 1.5 nM) [11]. In our opinion, the cysteine-rich domains pose a steric hindrance towards an incoming N-TIMP-3, preventing the inhibitor from docking comfortably with the catalytic domain of the enzyme. In order to overcome this hindrance, we decided to modify our previous mutagenesis strategy and adopt a novel approach that would entail reorientating part of the N-TIMP-3 molecule. In the present paper, we report our findings on the development of a series of N-TIMP-3 mutants that exhibited vastly improved potency against the TACE-long enzyme.

EXPERIMENTAL

Sources of TACE-cat and TACE-long

TACE-cat (also known as TACE-473) is the catalytic domain of the enzyme that spans across residues 215 to 473 of the TACE polypeptide. TACE-long (also known as TACE-651), on the other hand, is the extracellular form that encompasses not only the catalytic domain, but also the disintegrin, EGF-like as well as the Crambin-like domains (extending from residues 215 to 651) [12]. Gelatinase-A (gel-A) was obtained from mouse myeloma cells and activated with organomercurials, in accordance with our previous paper [13].

N-TIMP-3 model

The N-TIMP-3 model in this work was constructed with the help of the protein modelling server Swiss-Model version 3.5 (Glaxo-SmithKline, Geneva, Switzerland) by superimposing N-TIMP-3 sequence on the templates of TIMP-2 [Protein Data Bank (PDB) accession number 1BR9 or 2TMP] and TIMP-1 (PDB accession number 1UEA). The structure of TACE was retrieved from the

PDB (PDB accession number 1BKC), with the inhibitor removed. The details on N-TIMP-3/TACE docking and interactions have been explained in one of our earlier papers [14]. The inter-atomic distance calculation was performed with Rasmol (created by Roger Sayle). Figures 1 and 2 were prepared with the molecular simulation programme WebLab Viewer (Accelrys).

N-TIMP-3 and full-length TIMP-3 mutagenesis

All mutants in this study were constructed by means of PCR using *Pwo* polymerase (Boehringer Mannheim, Lewes, East Sussex, U.K.). To introduce the desired mutations into wild-type N-TIMP-3 cDNA in pRSET-c vector (Invitrogen, Inchinnan Business Park, Paisley, Scotland, U.K.), we designed several PCR forward primers bearing Lys²⁶ → Glu (K26E) and/or K27E and/or K30E and/or E31K single or multiple point mutations. K71E and K76E mutations were incorporated by PCR reverse primers. PCR amplicons generated with these primers were used to replace the corresponding restriction fragment from the wild-type N-TIMP-3 cDNA.

To express TIMP-3 in HeLa cells, the *Bst*XI–*Bsp*MI fragment bearing the K26/27/30/76E mutations was subcloned into full-length and N-TIMP-3 cDNA in pcDNA 3.1(+) expression vector (Invitrogen). All mutants used in this study have been sequenced to confirm the incorporation of the correct mutations.

Protein expression in *Escherichia coli* and *in vitro* refolding

N-TIMP-3 mutants were expressed in *E. coli* 'super-competent' cells [strain BL21(DE3) pLys] following the protocols outlined in our earlier publications [9,10]. The conditions used for refolding were identical with those reported earlier [10]. The final concentrations of refolded N-TIMP-3 mutants were measured by titration against gel-A, collagenase-3 and TACE to ensure that accurate concentrations had been obtained [9–11]. The amount of protein was measured using a BioRad protein assay kit.

Figure 1 Hypothetical TACE–N-TIMP-3 complex, highlighting the strategic positions of Glu³¹ (N-TIMP-3) and Lys³¹⁵ (TACE)

(a) Lateral view of the TACE–N-TIMP-3 association complex; (b) top view of the same complex. Glu³¹ is located at the junction between the β -strand A and the AB-loop of N-TIMP-3. The residue is estimated to be 13 Å from the side chain of Lys³¹⁵, a prominent amino acid on the TACE surface. Note the gap between the two amino acids and the open space surrounding Glu³¹ (b). The aim of this project is to bring the two residues together to form electrostatic bonding by 'tilting' Glu³¹ towards Lys³¹⁵. In (a) and (b), the Thr² and Ser⁴ residues of N-TIMP-3 that interact with the TACE S1' and S3' catalytic pockets are highlighted in red. The β -strand A and β -strand B of N-TIMP-3 are labelled 'A' and 'B' respectively (a).

Figure 2 Surface charges of N-TIMP-3 model, with Lys²⁶ being the tactical 'epicentre' for (first) reorientating the β -strand A and (secondly) ECM-binding mutagenesis

Lys²⁶ is strategically positioned at the epicentre of our mutagenesis study. We predicted that substitution of Lys²⁶ and one or more of the surrounding lysine residues with glutamate would bring about slight reorientation of the β -strand A.

Apparent inhibition constant [K_i (app)] and association rate (k_{on}) kinetic characterization of N-TIMP-3 mutants

Kinetic assays for K_i (app) and k_{on} have been described comprehensively in our previous publications [9–11]. K_i (app) measurements were performed with TACE-cat (0.25 nM) and TACE-long (0.30 nM) pre-incubated with increasing concentrations of N-TIMP-3 mutants, ranging from 0.05 to 50 nM. The activity of remaining TACE in each sample was measured at 3 h post-incubation with the quenched fluorescent peptide, QF-45 [Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys-Dnp-NH₂], where 'Mca' and 'Dnp' represent (7-methoxycoumarin-4-yl)acetyl and 2,4-dinitrophenyl respectively, at the final concentration of 1 μ M. The K_i (app) values were calculated by least-squares fitting to an equation developed for competitive tight-binding inhibition by Morrison and Walsh [15].

k_{on} measurements were performed by following the rate of inhibition using a continuous fluorimetric activity assay, as explained in our previous papers [10,14].

Extracellular matrix (ECM) binding study

An ECM binding study was performed using HeLa cells cultured in 1.5 ml of 10% (v/v) fetal-calf serum ('FCS')-enriched Dulbecco's modified Eagle's medium (DMEM) in 12-well tissue-culture dishes. For the purpose of TIMP-3 transfection, cells were seeded at a density of 1×10^4 /well. Transfections were performed with FuGene 6 Transfection Reagent® (Roche, Lewes, East Sussex, U.K.) following the manufacturer's instructions.

Typically, we transfected 0.4 μ g of full-length or N-TIMP-3 plasmid DNA into each well. At 24 h post-transfection, cells were washed twice with serum-free DMEM medium, before a further incubation of 48 h in the same medium. ECM preparation was carried out essentially using the methods developed by Leco et al. [16]. The conditioned media of N-TIMP-3 samples were concentrated 10-fold with a 10 kDa MWCO Centricon® centrifugal filter unit (Millipore, Bedford, MA, U.S.A.), before final analysis by reverse zymography. This latter technique was performed in non-reducing SDS/12.5% (w/v) polyacrylamide gels supplemented with gel-A and 2 mg/ml gelatin, as described elsewhere [17].

Reducing and non-reducing SDS/PAGE

Reducing and non-reducing SDS/PAGE were performed in 4% stacking/12.5% resolving gels. In non-reducing samples, 2-mercaptoethanol was excluded from the loading buffer. The gels were stained in Coomassie Brilliant Blue R250.

RESULTS

Reorientating the β -strand A of N-TIMP-3: 'drawing' residue Glu³¹ to form electrostatic bonding with Lys³¹⁵ of TACE-long

Comparison of free and complexed forms of TIMP-2 by NMR and crystallography studies showed that the AB-loop of TIMP-

2 was reorientated by as much as 8 Å (1 Å $\equiv$ 0.1 nm) to establish contacts with the MT-1 loop of MT-1 matrix metalloproteinase (MMP) [18]. Structural superimposition of free and MMP-bound forms of TIMP-1 also revealed a comparable pattern of bending in and around the AB-loop region [19]. In the case of N-TIMP-3, there are two glycine residues (indicated subsequently in bold-face) within the constituency of the AB-loop (-Glu³¹-**Gly**³²-Pro³³-Phe³⁴-**Gly**³⁵-Thr³⁶-). For this reason, we expected the AB-loop of N-TIMP-3 to enjoy a similar, if not a higher, degree of flexibility in conformational changes. In our N-TIMP-3-TACE complex model, the first amino acid of the loop, Glu³¹, is approximately 13 Å away from the side chain of Lys³¹⁵, a prominent residue protruding from the surface of TACE (Figures 1a and 1b). The strategic positions of these two residues are best illustrated with an aerial view of the complex, as shown in Figure 1(b). According to our calculation, if Glu³¹ could be drawn by 6–8 Å towards the contact perimeter of Lys³¹⁵, the residue might be able to establish electrostatic bonding with the side chain of Lys³¹⁵. The result, in our opinion, would be an extremely tight enzyme–inhibitor binary complex. A hypothetical way of achieving this is by ‘inclining’ the section of the β -strand A that lies before residue Glu³¹ ‘downwards’ and towards the surface of TACE.

As illustrated in Figure 2, the section of β -strand A immediately preceding the Glu³¹ residue, namely -Lys²⁷-Leu²⁸-Val²⁹-Lys³⁰-, emerges from the OB core of N-TIMP-3 in the shape of a ‘blade’, overhanging the edge of the molecule. Incidentally, at the root of this ‘blade’ is a group of adjoining lysine residues: the ‘Lys²⁶/Lys²⁷/Lys⁷⁶ triplet’ (Figure 2). We believe that the overall angle of the β -strand A ‘blade’ is critically dependent on the strategic alignment of these lysine residues, as well as on the precise orientation of their individual side chains. Our modelling study predicts that if one or more of the lysine residues within this Lys²⁶/Lys²⁷/Lys⁷⁶ triplet, and in particular its ‘epicentre’ Lys²⁶, is replaced by an oppositely charged amino acid, such as glutamate, the ‘blade’ could be inclined slightly towards the TACE surface. Hence we created three mutants, K26E, K26/27E and K26/27/76E, to investigate this proposition.

The ‘Lys³⁰/Lys⁷¹/Lys⁷⁶ lysine triangle’: a target for validating the ECM-binding domain of TIMP-3

Full-length TIMP-3 is notorious for being tightly ECM-associated, and therefore problematical in terms of purification from overexpressing cells. In particular, the area surrounding Lys²⁶ has been proposed to be the most likely binding site for glycosaminoglycans [20]. Apart from being a prime site for the design of tight-binding N-TIMP-3 mutants, Lys²⁶ and its vicinity also present an attractive opportunity for us to investigate the underlying cause for the unique ECM-binding characteristics of the molecule. In the present study, we decided against mutating these lysine residues individually; instead, we again nominated Lys²⁶ as the ‘epicentre’ of the investigation and mutated the encircling lysine residues progressively. Since Lys²², Lys⁴² and Lys⁴⁵ are conserved among all four members of mammalian TIMPs, on their own, they are unlikely to confer ECM-binding capability to TIMP-3. Therefore we selected only the non-conserved residues, namely Lys²⁶, Lys²⁷, Lys³⁰, Lys⁷¹ and Lys⁷⁶, in an imaginary ‘Lys³⁰/Lys⁷¹/Lys⁷⁶ lysine triangle’, to be the target for our analysis (Figure 2).

In addition to the aforementioned K26E, K26/27E and K26/27/76E mutants, four more mutants were created to cover all the chosen targets: the K26/30E double mutant, K26/27/30/76E and K26/27/71/76E quadruple mutants and the K26/27/30/71/76E multiple mutant.

Figure 3 Analysis of refolded wild-type N-TIMP-3 and its lysine-to-glutamate mutants in (a) non-reducing and (b) reducing SDS/12.5% PAGE

In the non-reducing samples, the lysine-to-glutamate mutants migrate slightly faster than the wild-type N-TIMP-3.

In vitro refolding and SDS/PAGE analysis of N-TIMP-3 mutants

The majority of the mutants in this study refolded without too much difficulty, with the exception being those bearing the Lys⁷¹ → Glu mutations (i.e. K26/27/71/76E and K26/27/30/71/76E mutants). The final yields of the mutants were comparable with that of the wild-type N-TIMP-3 [10]. Typically, we generated approx. 20 ml of active N-TIMP-3 of approx. 2–5 μ M in concentration from 500 ml of *E. coli* culture. Among the five refoldable mutants (i.e. K26E, K26/27E, K26/30E, K26/27/76E and K26/27/30/76E), K26/27/76E displayed clear signs of heterogeneity under non-denaturing SDS/PAGE analysis, an indication of the presence of misfolded proteins (Figure 3a). Therefore we titrated all the mutants against two MMPs (gel-A and collagenase-3, for instance) as well as an ADAM (TACE), in order to calculate the accurate concentrations of active N-TIMP-3 in each sample. In this study, the proportion of active N-TIMP-3 mutants ranged from 50 % to > 85 % (by comparing the titration concentrations with the amounts of protein measured by BioRad bicinchoninic acid assays). Fortunately, the misfolded N-TIMP-3 proteins did not interfere with our kinetic assays, as we have previously compared the inhibitory profiles of high (95 % active)- and low (40 % active)-quality N-TIMP-3 against various MMPs, and the results were identical (J. R. Worley, P. B. Thompkins, M. H. Lee, M. Hutton, P. Soloway, D. R. Edwards, G. Murphy and V. Knäuper, unpublished work).

Interestingly, we noted a slight variation in the mobility of some proteins under non-reducing conditions (Figure 3). As shown in Figure 3, N-TIMP-3 mutants migrate faster than the wild-type protein in non-reducing SDS/PAGE. It appeared that K26E, the mutant with only one lysine-to-glutamate substitution, migrated at a slightly slower pace than its counterparts with two or more mutations. Subsequent analysis of recombinant full-length K26/27/30/76E mutant with reverse zymography also revealed a similar pattern of migration.

Figure 4 Inhibition profiles of K26/27/30/76E and one of our previous N-TIMP-3 mutant, Ser-4Arg (S4R), against TACE-long

(a) Inhibition profiles of K26/27/30/76E [K_i (app) < 0.1 nM] and S4R [K_i (app) ≈ 0.5 nM] mutants against TACE-long. S4R is a high-affinity mutant against TACE-cat [K_i (app) < 60 pM], but not against TACE-long [11]. (b) Time courses showing the association profiles of the same mutants against TACE-long. Note that much less K26/27/30/76E enzyme was needed to inactivate TACE-long than S4R. At equilibrium, TACE-long enzyme was significantly less active in the K26/27/30/76E samples than those of the S4R mutant.

Inhibitory constants [K_i (app)] against TACE-cat and TACE-long

We subjected the refoldable mutants (K26E, K26/27E, K26/30E, K26/27/76E and K26/27/30/76E) to kinetic analysis with the two different forms of TACE, TACE-cat and TACE-long. Without exception, the inhibitory constants of all the mutants against both forms of TACE were below 0.1 nM: beyond the sensitivity limits of our fluorimetric assays. Figure 4 highlights the inhibition profiles of the K26/27/30/76E mutant against TACE-long. For comparison, mutant Ser⁴ $\rightarrow$ Arg (S4R), one of the most effective N-TIMP-3 mutants described in our previous paper, is also included in the same Figure [10,11]. Whereas S4R was created exclusively to target the S3' pocket of the TACE catalytic site, Figure 4(a) vividly shows that the mutant is unquestionably much less potent than its counterpart, the K26/27/30/76E mutant as a TACE-long inhibitor.

Association rates (k_{on}) against TACE-cat and TACE-long

An interesting trend emerged from the study on the association rates with TACE-cat and TACE-long. Table 1 indicates that the two mutants harbouring the K30E mutation (i.e. K26/30E and

K26/27/30/76E mutants), originally designed for ECM-association studies, display 2- to 3-fold-higher association rates against TACE than those without (k_{on} K26/30E and K26/27/30/76E $\approx 9.5 \times 10^5$ M⁻¹ · s⁻¹, others $\approx 3.9 \times 10^5$ M⁻¹ · s⁻¹ with TACE-cat; k_{on} K26/30E and K26/27/30/76E $\approx 1.2 \times 10^5$ M⁻¹ · s⁻¹, others $\approx 0.6 \times 10^5$ M⁻¹ · s⁻¹ with TACE-long). The trend is paralleled in both TACE-cat and TACE-long. The findings support our proposal that introduction of another negative residue near the vicinity of the AB-loop could result in better association with our intended TACE target, i.e. the Lys³¹⁵ residue. The relative potency of the S4R and K26/27/30/76E mutants is best illustrated by their association-rate profiles, as shown in Figure 4(b).

K26E/E31K 'revert mutant': verification of the strategic role of Glu³¹ in forming a tight binary complex with Lys³¹⁵ of TACE

The strategy of this project is based on the proposition that Glu³¹ could be reorientated to forge electrostatic bonding with the Lys³¹⁵ side chain. Hitherto, all the lysine-to-glutamate mutants are tight-binding inhibitors against TACE-long. If the improvements in binding affinity displayed by these mutants are genuinely

Table 1 Association rate constants (k_{on}) of N-TIMP-3 and its Lys → Glu mutants with TACE-cat and TACE-long

Note that the two mutants bearing K30E mutations (i.e. K26/30E and K26/27/30/76E) displayed 2- to 3-fold-higher association rates against TACE-cat and TACE-long than those without. The K_i (app) constants of the mutants (K26E, K26/27E, K26/30E, K26/27/76E and K26/27/30/76E) with both TACE-cat and TACE-long were all below 0.1 nM, beyond the sensitivity limits of our fluorimetric assays. 'u' represents mutants that are unable to refold; asterisks (*) denote data published in our previous papers [9,11]; WT, wild-type.

Enzyme	$k_{on} \times 10^5$ ($M^{-1} \cdot s^{-1}$)	
	TACE-cat	TACE-long
WT N-TIMP-3*	$3.65 \pm 0.33^*$	$0.49 \pm 0.24^*$
Ser-4Arg*	$1.84 \pm 0.19^*$	$0.20 \pm 0.02^*$
K26E	3.78 ± 1.02	0.49 ± 0.19
K26/27E	3.95 ± 0.18	0.52 ± 0.12
K26/30E	9.18 ± 1.59	1.08 ± 0.06
K26/27/76E	4.35 ± 0.26	0.69 ± 0.08
K26/27/30/76E	9.70 ± 0.83	1.41 ± 0.59
K26/27/71/76E	u	u
K26/27/30/71/76E	u	u
K26E/E31K	1.14 ± 0.14	0.28 ± 0.02

due to electrostatic bonding between Glu³¹ and Lys³¹⁵, replacement of Glu³¹ with a neutral or positively charged residue must bring about complete abolition of the improved affinity displayed so far. Consequently, we created a K26E/E31K double mutant for the purpose of validating this hypothesis. Indeed, the affinity [K_i (app) 0.15 nM with TACE-cat; 1.70 nM with TACE-long] as well as the association rates (k_{on} $1.14 \times 10^5 M^{-1} \cdot s^{-1}$ with TACE-cat; $0.28 \times 10^5 M^{-1} \cdot s^{-1}$ with TACE-long) of this mutant was comparable with that of the wild-type N-TIMP-3, as shown in Table 1.

ECM-association profiles of full-length and N-TIMP-3 K26/27/30/76E mutants

Figure 5 depicts the distribution of full-length and N-terminal-domain forms of wild-type TIMP-3 and its K26/27/30/76E mutant after expression in HeLa cells for 48 h. Evidently, full-length TIMP-3 bearing the K26/27/30/76E mutations is still ECM-associated. No significant amount of the protein could be detected in the conditioned media (Figure 5a). On the other hand, the N-terminal-domain forms of TIMP-3 are localized exclusively in the conditioned media, irrespective of being wild-type or mutant (Figure 5b).

DISCUSSION

Novel strategy in TIMP-3 mutagenesis

One of the most interesting findings revealed by the crystal structure of TACE is the large number of 'insertion loops' that dominate the surface of the enzyme. Indeed, Maskos et al. [5] have envisaged the possibility of designing a new generation of more potent and specific TACE inhibitors by making use of the structural peculiarity 'in the forms of surface charge and shape'. In our previous paper on TIMP-1/TIMP-3 chimaera mutagenesis analysis, we presented our findings on some of the so-called 'alternative' pockets that could be targeted for the design of a better TIMP inhibitor [14]. In the present study, we demonstrate that the design of a good TACE inhibitor does not necessarily have to be focused exclusively on the conventional S1' and S3' catalytic pockets.

In comparison with our previous series of N-TIMP-3 mutants aimed at investigating the S1' and S3' pockets, all the lysine-to-

Figure 5 Reverse zymography: expression and distribution of full-length (FL) and N-TIMP-3 in HeLa cells

(a) Reverse zymography, showing the distribution of wild-type (WT) full-length (FL) TIMP-3 and its K26/27/30/76E mutant in HeLa cells. Despite conversion of four lysine residues into glutamate, the K26/27/30/76E mutant is still ECM-associated. (b) N-TIMP-3, regardless of whether it is in the WT or mutant form, is exclusively confined to the conditioned media, and is not in the matrix. The K26/27/30/76E mutant displays a weak signal in the reverse zymography gel because the K_i (app) value of the mutant against gel-A was 10-fold higher (> 2.5 nM) than that of the WT N-TIMP-3 (0.25 nM; J. R. Worley, P. B. Thompson, M. H. Lee, M. Hutton, P. Soloway, D. R. Edwards, G. Murphy and V. Knäuper, unpublished work). The positions of recombinant TIMP-3 and its mutant are highlighted by an asterisk (*). Note that in both N- and FL TIMP-3, K26/27/30/76E mutants migrate slightly faster than the WT proteins.

glutamate mutants described in this study are significantly better at inhibiting the TACE-long form of the enzyme. Interestingly, one single mutation (i.e. K26E) is sufficient to bring about such improvement in binding affinity, although another lysine-to-glutamate substitution (K30E) is required to increase the association rate. Owing to the extreme sensitivity of the enzyme to salts, we were not able to perform binding assays in high-salt concentrations [12]. As an alternative, we relied on the K26E/E31K mutant to verify our hypothesis. So far, all the evidence gathered does suggest that the β -strand A has indeed been 'fine-tuned' and that the Glu³¹ residue is 'drawn' to form electrostatic bonding with the Lys³¹⁵ side chain of TACE. The definitive answer, however, would undoubtedly require information from structural elucidation of a TACE–N-TIMP-3 mutant complex.

Throughout this study, we were not able to measure the K_i (app) values of N-TIMP-3 mutants with both TACE-cat and TACE-long, in part due to the poor k_{cat}/K_m value of the synthetic

QF-45 peptide with TACE enzyme ($\leq 10^5$ /s). We are currently investigating the possibility of increasing the sensitivity limit of our fluorimetric assays in the fM to pM range, and the results will be reported in the future.

The findings in this work are significant, not least because highly potent N-TIMP-3 mutants have been created to inhibit TACE-long, but also because of the unconventional approach taken to achieve such an objective. In addition, these tight-binding inhibitors are ideal candidates for co-crystallization with the form of TACE bearing the cysteine-rich domains. At present, we are evaluating the potential of these mutants as a therapeutic agent against TACE-related diseases. The findings of the study will be presented in our next paper.

ECM-binding properties of N-TIMP-3

Purification of TIMP-3 from natural sources is technically difficult, mainly because of the propensity of the protein to adhere tightly to the ECM [21]. Furthermore, TIMP-3 is known to induce apoptosis in mammalian cells overexpressing the recombinant protein [22–24]. The exact rationale that enables TIMP-3, but not TIMP-1, -2 or -4, to bind to the ECM is a subject of great interest in our laboratory. So far, there have been two independent reports on the ECM-binding mechanism of TIMP-3. The conclusions of these studies seem to be contradictory, because Langton et al. [25] showed that TIMP-3 associated to the ECM predominantly through its C-terminal domain. Yu et al. [20], on the other hand, suggested that the N-terminal domain was more likely to fulfil the requirements for a glycosaminoglycan-binding site.

Here, our cell-based assays confirmed that N-TIMP-3 was soluble and exclusively confined to the conditioned medium. Full-length TIMP-3, on the other hand, was tightly associated with the ECM. Furthermore, despite extensive conversion of lysine residues into glutamate, full-length TIMP-3 carrying the K26/27/30/76E mutations was still ECM-associated. In short, our data are in agreement with the findings by Langton et al. [25] in that the C-terminal domain is the key determinant that confers ECM-binding characteristics to the TIMP-3 molecule. In the paper by Yu et al. [20], although the authors did express full-length TIMP-3 in mammalian BHK cells, no attempt was made to express N-TIMP-3 in the same cell line. Instead, the authors postulated the putative ECM-binding potential of TIMP-3 by: (i) measuring the binding/elution profiles of full-length and N-TIMP-3 on heparin-agarose; and (ii) DNase II-based peptide competition assays [20]. As pointed out explicitly in our recent publication on TACE-long, it is difficult to hypothesize on actual cellular events using biochemical data derived solely from *in vitro* observations [11]. Furthermore, it is quite possible that TIMP-3 binds to other ECM proteins besides those with heparan sulphate or chondroitin sulphate side chains.

As demonstrated in the present study, recombinant full-length TIMP-3 is much less soluble than N-TIMP-3 in mammalian cell ECM. Earlier, we reported that full-length TIMP-3 was significantly more potent than N-TIMP-3 in inhibiting c-Met, as well as TNF- α shedding [10]. Hence our data suggest that the solubility of TIMP-3 does affect the potency of the inhibitor *in vivo*. At present, we are very interested in the solubility and bioavailability profiles of these lysine-to-glutamate mutants *in vivo*. No doubt, these factors will be of the utmost importance if the mutants are to be engineered further to become therapeutic agents against TACE-related diseases. We are currently investigating these factors with our cell-based study.

In conclusion, we have developed a new generation of high-affinity N-TIMP-3 mutants against the extracellular domain

form of TACE. Our results also show that TIMP-3 binds to the ECM via its C-terminal domain. We believe our findings here will be of great consequence to future development of TIMP-3 as a prospective therapeutic agent.

We thank Dr J. D. Becherer (GlaxoSmithKline, Research Triangle Park, NC, U.S.A.) for generous provision of TACE-cat and TACE-long enzymes. This work was funded by the Arthritis Research Campaign (grant no. M0602), the Medical Research Council, the Wellcome Trust and the Biotechnology and Biological Sciences Research Council, U.K.

REFERENCES

- Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L. et al. (1994) Matrix metalloproteinases and processing of pro-TNF- α . *Nature (London)* **370**, 555–557
- McGeehan, G. M., Becherer, J. D., Bast, R. C., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S. et al. (1994) Regulation of tumour necrosis factor- α processing by a metalloproteinase inhibitor. *Nature (London)* **370**, 558–561
- Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997) A metalloproteinase disintegrin that releases tumor-necrosis factor- α from cells. *Nature (London)* **385**, 729–732
- Moss, M. L., Jin, S. L. C., Milla, M. E., Burkhardt, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R. et al. (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumor-necrosis factor- α . *Nature (London)* **385**, 733–736
- Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G. P., Bartunik, H., Ellestad, G. A., Reddy, P., Wolfson, M. F., Rauch, C. T., Castner, B. J. et al. (1998) Crystal structure of the catalytic domain of human tumour necrosis factor- α -converting enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3408–3412
- Amour, A., Slocombe, P. M., Webster, A., Butler, M., Knight, C. G., Smith, B. J., Stephens, P. E., Shelly, C., Hutton, M., Knäuper, V., Docherty, A. J. P. and Murphy, G. (1998) TNF- α converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* **435**, 39–44
- Gomis-Rüth, F., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G. P. et al. (1997) Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. *Nature (London)* **389**, 77–81
- Williamson, R. A., Martorell, G., Carr, M. D., Murphy, G., Docherty, A. J., Freedman, R. B. and Feeney, J. (1994) Solution structure of the active domain of tissue inhibitor of metalloproteinases-2. A new member of the OB fold protein family. *Biochemistry* **33**, 11745–11759
- Lee, M. H., Knäuper, V., Becherer, J. D. and Murphy, G. (2001) Full-length and N-TIMP-3 display equal inhibitory activities towards TNF- α convertase. *Biochem. Biophys. Res. Commun.* **280**, 945–950
- Lee, M. H., Verma, V., Maskos, K., Nath, D., Knäuper, V., Dodds, P., Amour, A. and Murphy, G. (2002) Engineering N-terminal domain of tissue inhibitor of metalloproteinase (TIMP)-3 to be a better inhibitor against tumour necrosis factor- α converting enzyme. *Biochem. J.* **364**, 227–234
- Lee, M. H., Verma, V., Maskos, K., Becherer, J. D., Knäuper, V., Dodds, P., Amour, A. and Murphy, G. (2002) The C-terminal domains of TACE weaken the inhibitory action of N-TIMP-3. *FEBS Lett.* **520**, 102–106
- Milla, M. E., Leesnitzer, M. A., Moss, M. L., Clay, W. C., Carter, H. L., Miller, A. B., Su, J. L., Lambert, M. H., Willard, D. H., Sheeley, D. M. et al. (1999) Specific sequence elements are required for the expression of functional tumor necrosis factor α -converting enzyme (TACE). *J. Biol. Chem.* **274**, 30563–30570
- Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D. and Docherty, A. J. P. (1992) The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. *Biochem. J.* **283**, 637–641
- Lee, M. H., Maskos, K., Knäuper, V., Dodds, P. and Murphy, G. (2002) Mapping and characterization of the functional epitopes of tissue inhibitor of metalloproteinases (TIMP)-3 using TIMP-1 as the scaffold: a new frontier in TIMP engineering. *Protein Sci.* **11**, 2493–2503
- Morrison, J. F. and Walsh, C. T. (1995) The behavior and significance of slow binding enzyme inhibitors. *Methods Enzymol.* **248**, 201–301
- Leco, K. J., Khokha, R., Pavloff, N., Hawkes, S. P. and Edwards, D. R. (1994) Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J. Biol. Chem.* **269**, 9352–9360

- 17 Janowska-Wieczorek, A., Marquez, L. A., Matsuzaki, A., Hashmi, H. R., Larratt, L. M., Boshkov, L. M., Turner, A. R., Zhang, M. C. and Edwards, D. R. (1999) Expression of matrix metalloproteinases (MMP-2 and -9) and tissue inhibitors of metalloproteinases (TIMP-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. *Br. J. Haematol.* **105**, 402–411
- 18 Tuuttila, A., Morgunova, E., Bergmann, U., Lindqvist, Y., Maskos, K., Fernandez-Catalan, C., Bode, W., Tryggvason, K. and Schneider, G. (1998) Three-dimensional structure of human tissue inhibitor of metalloproteinases-2 at 2.1 Å resolution. *J. Mol. Biol.* **284**, 1133–1140
- 19 Wu, B., Arumugam, S., Gao, G., Lee, G., Semchenko, V., Huang, W., Brew, K. and Van Doren, S. R. (2000) NMR structure of tissue inhibitor of metalloproteinases-1 implicates localized induced fit recognition of matrix metalloproteinases. *J. Mol. Biol.* **295**, 257–268
- 20 Yu, W. S., Yu, S. C., Meng, Q., Brew, K. and Woessner, J. F. (2000) TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. *J. Biol. Chem.* **275**, 31226–31232
- 21 Blenis, J. and Hawkes, S. P. (1984) Characterization of a transformation-sensitive protein in the extracellular matrix of chicken embryo fibroblasts. *J. Biol. Chem.* **259**, 11563–11570
- 22 Ahonen, M., Baker, A. H. and Kahari, V. M. (1998) Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer Res.* **11**, 2310–2315
- 23 Bond, M., Murphy, G., Bennett, M. R., Amour, A., Knäuper, V., Newby, A. C. and Baker, A. H. (2000) Localisation of the death domain of TIMP-3 to the N-terminus: metalloproteinase inhibition is associated with pro-apoptotic activity. *J. Biol. Chem.* **275**, 41358–41363
- 24 Bond, M., Murphy, G., Bennett, M. R., Newby, A. C. and Baker, A. H. (2002) Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent type II apoptotic pathway. *J. Biol. Chem.* **277**, 13787–13795
- 25 Langton, K. P., Barker, M. D. and McKie, N. (1998) Localization of the functional domains of human tissue inhibitor of metalloproteinase-3 and the effects of a Sorby's fundus dystrophy mutation. *J. Biol. Chem.* **273**, 16778–16781

Received 2 October 2002/23 January 2003; accepted 30 January 2003

Published as BJ Immediate Publication 30 January 2003, DOI 10.1042/BJ20021538