The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3

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Received 3 March 2000; received in revised form 15 April 2000

Edited by Pierre Jolles

Abstract A recombinant soluble form of the catalytic domain of human ADAM-10 was expressed as an Fc fusion protein from myeloma cells. The ADAM-10 was catalytically active, cleaving myelin basic protein and peptides based on the previously described 'metallosheddase' cleavage sites of tumour necrosis factor α, CD40 ligand and amyloid precursor protein. The myelin basic protein degradation assay was used to demonstrate that hydroxamate inhibitors of matrix metalloproteinases (MMPs) were also inhibitors of ADAM-10. The natural MMP inhibitors, TIMP-2 and TIMP-4 were unable to inhibit ADAM-10, but TIMP-1 and TIMP-3 were inhibitory. Using a quenched fluorescent substrate assay and ADAM-10 we obtained approximate apparent inhibition constants of 0.1 nM (TIMP-1) and 0.9 nM (TIMP-3). The TIMP-1 inhibition of ADAM-10 could therefore prove useful in distinguishing its activity from that of TACE, which is only inhibited by TIMP-3, in cell based assays.

Key words: Metalloproteinase; Disintegrin metalloproteinase; Tissue inhibitor of metalloproteinase

1. Introduction

The ADAMs are type I membrane proteins containing a disintegrin and a metalloproteinase domain [1]. ADAMs are predicted to be involved in important cellular events, including cellular adhesion and membrane protein shedding [2,3]. The latter role concerns the ADAMs that are likely to be active proteases based on the integrity of their active site [4]. Approximately half of the ADAMs identified fall into this category, thereby forming a branch of the metzincin superfamily of metalloproteinases which also includes the matrix metalloproteinases (MMPs) [5]. In particular, ADAM-17, also known as the tumour necrosis factor-α (TNF-α) converting enzyme (TACE), sheds membrane-anchored TNF-α generating its soluble form [6,7]. More recently, TACE has been suggested to be involved in the shedding of other membrane proteins. In common with as yet unidentified 'sheddases', TACE is inhibited by synthetic inhibitors (MMP inhibitors; MMPIs) originally designed to target MMPs and is upregulated by activators of protein kinase C, such as phorbol myristate acetate [8].

The X-ray crystal structure of the catalytic domain of human TACE suggested that the structure of the active site not only allowed MMPIs to bind efficiently, but also, theoretically, the tissue inhibitors of MMPs (TIMPs) [9]. In practice, only TIMP-3 out of the four TIMPs tested could efficiently inhibit TACE [10]. In parallel, other studies showed that TIMP-3 was able to block the cellular shedding of proTNF-α, L-selectin and the interleukin-6 (IL-6) receptor [11,12]. Recently, other TIMPs have been shown to share the ability to inhibit membrane protein proteolysis. In particular, TIMP-2 also inhibited the shedding of the TNF receptors [13], whereas TIMP-1 was found to prevent the shedding of the heparin-binding epidermal growth factor (HB-EGF) and the tyrosine kinase receptor HER-2 [14,15]. These observations implicate the involvement of metallosheddases that can be distinguished from TACE through their differing sensitivity to TIMPs.

In this study, we examine the proteolytic activity and inhibition characteristics of ADAM-10. ADAM-10 was initially cloned from bovine brain and was found to be expressed in a wide variety of tissues [16]. ADAM-10 not only shares a closer sequence similarity and potential structural features with TACE than with any other mammalian ADAM [3] but it is also able to cleave TNF-α based TACE substrates [17]. Both enzymes have also been shown to cleave peptides based on the α-secretase-sensitive sequence of amyloid precursor protein [18,19]. Purified ADAM-10 is able to degrade myelin basic protein (MBP) and type IV collagen, although it remains to be determined whether these activities are relevant in vivo [16,20,21]. The *Drosophila* ADAM-10 orthologue KUZ has been linked to the processing of the Notch receptor ligand Delta and is thus implicated in cell fate determination [22]. However, it is not known whether ADAM-10 shares the ability of KUZ to cleave Notch receptor ligands.

In this report, the catalytic activity of recombinant human ADAM-10 has been assayed to characterise its sensitivity towards MMPIs and TIMPs. Such data are relevant to the analysis of the inhibitory profile of cell surface shedding events mediated by metalloproteinases. The substrate specificity of ADAM-10 towards peptides mimicking the membrane-proximal cleavage site of proteins shed by metalloproteinases was also investigated.

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Abbreviations: DMSO, dimethylsulfoxide; HB-EGF, heparin-binding epidermal growth factor; HPLC, high performance liquid chromatography; K_i, inhibition constant; MBP, myelin basic protein; MMP, matrix metalloproteinase; MMP-1, collagenase-1; MMP-2, gelatinase A; MMP-3, stromelysin-1; MMPI, MMP inhibitor; NS0, non-secreter zero; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TACE, tumour necrosis factor-α converting enzyme; TFA, trifluoroacetic acid; TIMP, tissue inhibitor of metalloproteinases

PII: S0014-5793(00)01528-3

2. Materials and methods

2.1. Materials and general procedures

Protein A and soya bean trypsin inhibitor-conjugated Sepharose and all other non-specified reagents were from Sigma (Poole, UK). Recombinant enterokinase was from Novagen (Cambridge Bioscience, Cambridge, UK). Recombinant human TIMPs-1, -2 and -3 were expressed in NS0 mouse myeloma cells and purified as previously described [23]. Full-length mouse TIMP-4 was expressed from *Escherichia coli* and refolded (V. Knäuper, unpublished). The broad-spectrum MMPI BB94 was a kind gift from British Biotech Pharmaceuticals Ltd. (Oxford, UK). The peptides Ac-KENSFEMQKGAQ-NH_2 (CD40L), Ac-LPPVAASSLR-NH_2 (KL1) and Ac-GLSLPV-ENRLYTYD-NH_2 (HB-EGF) were respectively based on the cleavage site of the juxtamembrane region of the cytokines CD40-ligand and c-kit ligand-1 and the growth factor HB-EGF. These peptides were a kind gift from R. Black (Immunex Corp. Ltd., Seattle, WA, USA). The peptides H-YEVHHQKLVFF-OH, based on the α-secretase cleavage site of APP, and H-YGSLPQKAQRPQDEN-OH, based on the ADAM-10 cleavage site of MBP, were from Bachem (Bubendorf, Switzerland).

Fluorimetric measurements were made with a Perkin-Elmer LS50B spectrofluorimeter ($\lambda_{ex} = 328$ nm, $\lambda_{em} = 393$ nm) at 37°C in 2.5 ml of buffer A (10 mM HEPES, pH 7.5, 0.02% NaN_3, 0.05% Brij 35) containing a constant amount of dimethylsulfoxide (DMSO) (1% v/v). The quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(2,4-dinitrophenyl)-NH_2 (QF45), based on the TACE-sensitive sequence in human TNF-α, were as previously described [10].

High performance liquid chromatography (HPLC) analyses were done on a Perkin-Elmer Integral 4000 instrument equipped with a Vydac 218TP54 column maintained at 40°C. Solvents were: A, 0.1% trifluoroacetic acid (TFA) in water; B, 0.08% TFA in acetonitrile. A linear gradient from 5 to 95% B was run over 20 min at 1.25 ml/min and the eluate was monitored at 230 nm.

2.2. Expression and purification of recombinant ADAM-10

A full-length cDNA for ADAM-10 was isolated from the human T cell line Jurkat (ECACC Ref. No.: 88042803), essentially as previously described [10]. The DNA fragment encoding the pre pro catalytic domain was amplified by PCR and a 5′ *Hin*dIII and Kozak sequence upstream of the initiating methionine codon and a 3′ *Sal*I site were introduced. The coding sequence was then ligated into the vector pEE12 [10] such that it was joined at the *Sal*I site to a sequence encoding the human IgG1 heavy chain constant region, hinge, CH2 and CH3 domains. A DNA adapter encoding an enterokinase cleavage site ([V]DDDDK) was further introduced at the *Sal*I site. The vector was transfected into mouse NS0 myeloma cells and clones expressing recombinant protein were identified and grown as previously described [10].

For purification of recombinant ADAM-10, the transfected NS0 cells were grown in conditioned medium containing the MMPI BB94 (1 μM) to prevent proteolytic degradation of ADAM-10 catalytic domain Fc fusion, as previously reported [17]. After binding of the fusion protein (rADAM-10) to protein A-Sepharose, the gel was washed with 50 volumes of buffer A. rADAM-10 was eluted with five volumes of 100 mM glycine, pH 3.0, and immediately neutralised with 1 M Tris, pH 7.5. The absence of BB94 after purification was checked using an MMP-2 fluorimetric activity assay [24].

2.3. Enzyme assays

The active site concentration and inhibition of ADAM-10 estimated with TIMP-1 and TIMP-3 as well as the activity assay with the quenched fluorescent peptide QF45 were as previously described for TACE [10]. rADAM-10 (150 nM) was incubated at 37°C with MBP (500 μg/ml) or peptides (50 μM) in buffer A containing a constant amount of DMSO (1% v/v). MBP and enzyme mixtures were incubated for 4 h and then analysed by 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions. Peptide cleavage reactions were stopped at different times by dropping the pH to 4. The samples (100 μl) were then analysed by HPLC. The fragment peaks were collected and identified by mass spectrometry. The catalytic specificities (k_{cat}/K_M) for the cleavage of peptides were obtained by measuring the HPLC peak areas of start and end products at early time points so that less than 10% of the peptide substrate was turned over.

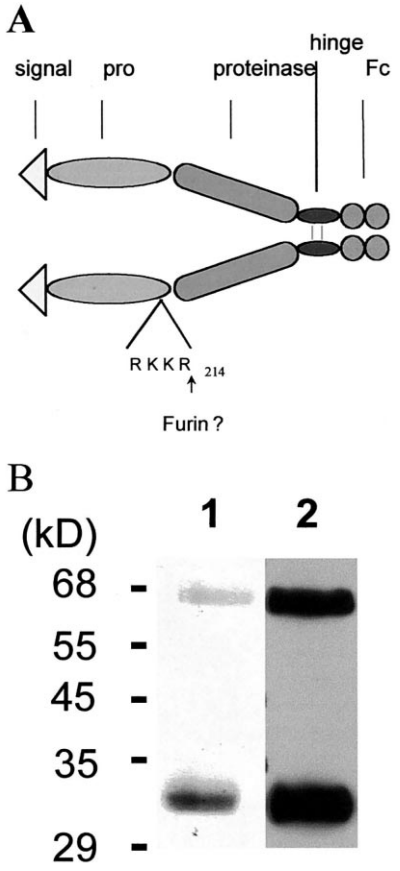

Fig. 1. SDS–PAGE analysis of purified rADAM-10. (A) Schematic representation of the ADAM-10 pre pro catalytic domain expressed as a Fc fusion, the arrow indicates where a furin-like proteinase is likely to activate ADAM-10. (B) SDS–PAGE of purified rADAM-10. The fusion protein was bound to protein A-Sepharose and eluted under acid conditions: lane 1, Coomassie blue-stained; lane 2, Western blot using an anti-human Fc antibody.

3. Results and discussion

3.1. Purification of rADAM-10

The material eluted from protein A-Sepharose yielded two bands of M_r 65 and 31 kDa on SDS–PAGE (Fig. 1B, lane 1). These two protein species contained the Fc fusion domain as they were both detected with an anti-human Fc antibody by Western blotting (Fig. 1B, lane 2). This observation combined with predicted molecular weights for the domains of rADAM-10 suggested that the 65 and 31 kDa proteins correspond to the Fc with and without the catalytic domain of rADAM-10. Thus, as previously observed by Rosendahl et al., rADAM-10 was partially cleaved, most likely at the proteolytically sensitive Fc hinge region [17]. The 65 kDa band was no longer observed on SDS–PAGE and Western blots if the MMPI BB94 was omitted from the conditioned medium, indicating that the proteolytic cleavage of the Fc hinge region is at least partially metalloproteinase-mediated. We propose that this process might be autocatalytic, as BB94 efficiently inhibits ADAM-10 catalytic activity (J.D. Becherer, personal communication).

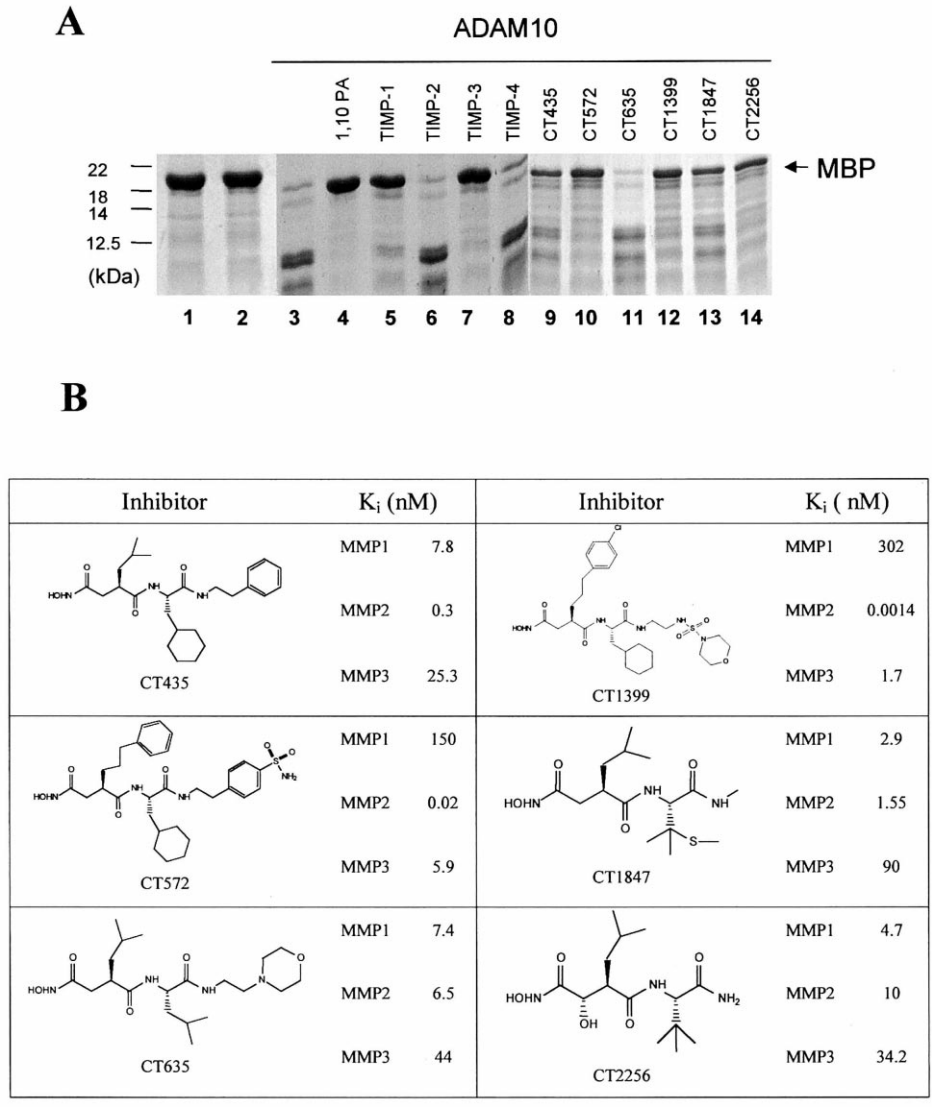

Fig. 2. A: MBP degradation by rADAM-10. Bovine MBP was incubated at 37°C for 4 h with rADAM-10. Reactions were stopped by the addition of sample buffer followed by heating and analysed by 14% SDS–PAGE under reduced conditions. Lanes 1 and 2: 10 μl of MBP alone before (1) and after incubation (2). Lanes 3–18: 10 μl of incubation mixture containing rADAM-10 (0.15 μM) and MBP (0.5 mg/ml) with 1% (v/v) DMSO (3), 10 mM 1,10-phenanthroline (4), 0.25 μM TIMP-1, -2, -3 or -4 (5–8), 5 μM CT435, CT572, CT635, CT1399, CT1847 or CT2256 (9–14). B: MMPIs tested on ADAM-10 with inhibition constants versus selected MMPs.

3.2. MBP degrading activity of rADAM-10

To determine whether rADAM-10 was catalytically active, the purified material was incubated with MBP and its cleavage monitored by SDS–PAGE, as described by [20]. After a 4 h incubation, the 20 kDa MBP was completely degraded with the appearance of two prominent protein bands of about 9 and 11 kDa (Fig. 2A) consistent with previous reports [16,21]. Glynn and collaborators also determined the site for the initial cleavage of MBP by purified bovine ADAM-10 which occurred at the Pro-73–Gln-74 bond [25]. A MBP-based peptide substrate was cleaved by rADAM-10 at the Pro–Gln bond, confirming that the substrate specificity of rADAM-10 mimicked that of purified bovine ADAM-10 (Table 1).

3.3. Catalytic activity of rADAM-10 towards peptide substrates

Several different types of intrinsic proteins are released from the plasma membrane by metalloproteases. To evaluate fur-

Table 1
Peptides mimicking the membrane-proximal cleavage site of proteins shed by metalloproteases were evaluated for cleavage by rADAM-10

Substrate	Peptide	ADAM-10
CD40L	KENSFE*MQKGAQ	KENSFEM↓QKGAQ 0.5×10^3
APP	YEVHHQK*LVFF	YEVHHQK↓LVFF 0.7×10^3
TNF-α	SPLAQA*VRSSSR	SPLAQA↓VRSSSR 2.0×10^3
MBP	YGSLP*QKAQRPQDEN	YGSLP↓QKAQRPQDEN 0.9×10^3

The predicted cleavage site is between the two residues represented by an asterisk and the observed cleavage sites are indicated with an arrow (↓). The rates of peptide turnover were determined by HPLC and are reported as k_{cat}/K_M (M^{-1} s^{-1}) with S.E.M. below 30%.

ther the cleavage specificity of ADAM-10, the recombinant proteins were incubated with peptides corresponding to cleavage sites of specific membrane proteins that are known to be released by metalloproteases [8]. rADAM-10 was able to cleave both the TNF-α and APP peptides at the correct cleavage site, as previously reported [17,18]. The TNF-α-based peptide QF45 was two orders of magnitude less sensitive for ADAM-10 than for TACE [10]. The peptides based on the cleavage site of the juxtamembrane region of the cytokine c-kit ligand-1 and the growth factor HB-EGF were not cleaved by rADAM-10. The CD40L (also known as TRAP or CD154)-based peptide [26] was hydrolysed by ADAM-10, but at a different site than the one expected for the CD40L sheddase (Table 1).

3.4. Inhibition of rADAM-10 activity

The MBP degradation assay was also used to evaluate the inhibitory potential of MMPIs as well as TIMPs towards rADAM-10. Of the six hydroxamate-based MMPIs tested (Fig. 2B), only CT635 did not inhibit rADAM-10. From this, it appears that ADAM-10 inhibition is not affected by the length of the side-chain in position P_1' and this contrasts with the observation that sheddases are usually only inhibited by hydroxamate inhibitors with an isobutyl group in that position [8]. In that respect, ADAM-10 resembles more MMP-2 or MMP-3 than MMP-1 or TACE (Fig. 2B, [10]).

Importantly, the rADAM-10-catalysed degradation of MBP was abolished with low submicromolar concentrations of both TIMP-1 and -3. This was confirmed using the quenched fluorescent TACE substrate, QF45. TIMP-1 and -3 behaved as active site titrants, confirming that they are both potent inhibitors of rADAM-10. However, for rADAM-10, the QF45 assay was not sensitive enough ($k_{cat}/K_M = 2\times10^3$ M^{-1} s^{-1}) to allow the accurate estimation of apparent inhibition constants (K_i'). The lowest enzyme concentration used to monitor activity (4 nM) still exceeded the K_i' values estimated for TIMP-1 (0.1 nM) and TIMP-3 (0.9 nM). Thus, although both TACE and ADAM-10 are prone to TIMP-3 inhibition, these enzymes are distinguished by a different susceptibility to TIMP-1. TIMP-1 was efficient in inhibiting rADAM-10-induced MBP degradation at concentrations similar to those used to inhibit aggrecan degradation by the soluble disintegrin metalloproteinase ADAM-TS4 [27]. As TIMP-2 was inactive on ADAM-TS4, it would be interesting to determine the effect of TIMP-3 on this enzyme and how it compares to ADAM-10 and TACE.

The TIMP-1 inhibition of ADAM-10 could prove useful in distinguishing its activity from that of TACE in cell-based assays. The lack of TIMP-1 inhibition for the shedding of proTNF-α, L-selectin and the receptors to the cytokines TNF-α and IL-6 [11,13] precludes the participation of ADAM-10 in the solubilisation of these membrane proteins. Controversially, TIMP-1 can inhibit the shedding of the tyrosine kinase receptor HER-2 [15], as well as the growth factor HB-EGF [15], but our peptide cleavage data suggest that the latter may not be processed by ADAM-10. ADAM-10 and TACE share some structural similarities which distinguish them from the other ADAMs and, to date, appear to have a more general proteolytic capacity [3]. It could be hypothesised that these two ADAMs are more susceptible to TIMP inhibition because of their special structure, a topic which is under detailed investigation.

Acknowledgements: This work was supported by the Arthritis Research Campaign, the Wellcome Trust and the Medical Research Council, UK. We would like to thank Dr Roy Black (Immunex Corp. Ltd, Seattle, WA, USA) for providing us with the peptides CD40L, HB-EGF and KL1. We also thank Dr J. O'Connell for access to his data on the inhibition of MMPs by CT hydroxamates.

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