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The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs

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Abstract The ADAM family of proteases are type I transmembrane proteins with both metalloproteinase and disintegrin containing extracellular domains. ADAMs are implicated in the proteolytic processing of membrane-bound precursors and involved in modulating cell–cell and cell–matrix interactions. ADAM8 (MS2, CD156) has been identified in myeloid and B cells. In this report we demonstrate that soluble ADAM8 is an active metalloprotease in vitro and is able to hydrolyse myelin basic protein and a variety of peptide substrates based on the cleavage sites of membrane-bound cytokines, growth factors and receptors which are known to be processed by metalloproteinases. Interestingly, although ADAM8 was inhibited by a number of peptide analogue hydroxamate inhibitors, it was not inhibited by the tissue inhibitors of metalloproteinases (TIMPs). We also demonstrate that the activity of recombinant soluble ADAM9 (meltrin-γ, MDC9) lacks inhibition by the TIMPs, but can be inhibited by hydroxamate inhibitors. The lack of TIMP inhibition of ADAM8 and 9 contrasts with other membrane-associated metalloproteinases characterised to date in this respect (ADAM10, 12, 17, and the membrane-type metalloproteinases) which have been implicated in protein processing at the cell surface.

Key words: Metalloproteinase; Disintegrin metalloproteinase; ADAM; Tissue inhibitor of metalloproteinases; Membrane-type metalloproteinase

1. Introduction

The ADAMs are a recently discovered family of membrane-anchored metalloproteinases with a complex domain structure including a metalloproteinase, disintegrin, cysteine-rich, transmembrane and cytoplasmic domains [1]. About half of the 33 ADAMs cloned to date are predicted to be active metalloproteinases based on the presence of the HEXXH zinc binding motif. Of these, ADAM17, or tumour necrosis factor-α (TNFα) converting enzyme (TACE), is the most thoroughly characterised member. In addition to processing precursor membrane-bound TNFα to its soluble form, TACE also cleaves other membrane proteins [2]. Some of the ADAMs predicted to be active metalloproteinases have also subsequently been demonstrated to be able to participate in similar proteolytic activities as TACE in vitro in cell-based systems [1]. The catalytic activities of purified recombinant ADAMs have been studied using the in vitro proteins α2-macroglobulin and myelin basic protein (MBP) as well as various peptides [3–6]. These assays have allowed their susceptibility towards the tissue inhibitors matrix metalloproteinases (TIMPs), potential physiological regulators of ADAM proteolytic activity in vivo, to be evaluated, as well as many low molecular weight synthetic inhibitors. Four different TIMPs have been identified so far, but only TIMP-3 was found to inhibit TACE and ADAM12 [7,8], whilst both TIMP-1 and TIMP-3 could inhibit ADAM10 [3]. Furthermore, TIMP-3 also inhibited the aggrecanases ADAMTS-4 and ADAMTS-5, which are members of the related family of disintegrin metalloproteinases with thrombospondin domains [9,10]. This contrasts with the membrane-type metalloproteinases (MT-MMPs) which are inhibited by TIMP-2, 3 and 4 (e.g. MT1-MMP) or TIMP-1, 2, 3 and 4 (e.g. MT4-MMP) [11,12], W. English and V. Knäuper, unpublished observations). There is some evidence that the MT-MMPs may also participate in proteolytic processing of membrane-anchored proteins [11,13,14]. Characterising the TIMP inhibition profile of the remaining proteolytically active ADAMs will increase their usefulness as tools in the identification of metalloproteinases involved in cellular events, aid in determining their potential as therapeutic agents and explain consequences of TIMP activity such as TIMP-3's ability to promote apoptosis in certain cell types [15,16].

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

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Here, we have extended the study of TIMP susceptibility to ADAM8 and 9. ADAM8 was further characterised to evaluate its catalytic properties in comparison to those of other ADAMs and the MT-MMPs.

2. Material and methods

2.1. Materials and general procedures

Protein A and soybean 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 (non-secreter zero) mouse myeloma cells and purified as previously described [17]. Full-length mouse TIMP-4 was expressed and refolded from *Escherichia coli* (V. Knäuper, unpublished). Recombinant human MT1-MMP ectodomain and the catalytic domain of mouse MT4-MMP were prepared as described previously [11,12]. ADAM9 and ADAM17 were prepared as described previously [6,7]. The peptides Ac-LPPVAASSLR-NH_2 (KL1), Ac-KENSFEMQKGAQ-NH_2 (CD40L), Ac-TVKEASSTFSWG-NH_2 (IL1R) and Ac-GLSLPVENRLYTYD-NH_2 (HB-EGF) are based on the respective cleavage sites for the TNF-related cytokine c-kit ligand-1 (KL1), CD40 ligand, the interleukin-1 receptor (IL-1R), and heparin binding epidermal growth factor (HB-EGF). These peptides were a kind gift from R. Black, Immunex (Seattle, WA, USA). The APP (H-YEVHHQKLVFF-OH) and MBP (H-YGSLPQKAQRPQDEN-OH) peptide fragments were from Bachem (Bubendorf, Switzerland). The TNF-based peptide was as previously published [7].

High performance liquid chromatography (HPLC) analyses were performed 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. Electrospray mass analysis of the peptide fragments was conducted as previously described [7].

N-terminal sequencing was done by electrophoretically transferring 5 μg of purified protein onto a polyvinylidene difluoride membrane (Immobilon P, Millipore), which was then stained with Ponceau red and washed extensively with distilled water. The band corresponding to the protein was excised for sequencing using an Applied Biosystems 470 microsequencer [3].

2.2. Expression and purification of recombinant ADAM8

A full-length cDNA for murine ADAM8 was isolated essentially as previously described for ADAM17 [7]. The DNA fragment encoding the prepro-catalytic domain was amplified by PCR and ligated into the vector pEE12 such that it was joined at a *Sal*I site to a sequence encoding an enterokinase cleavage site ([$V_{405\ in\ ADAM8}$V]DDDDK$^{\downarrow}$) followed by the human IgG_1 heavy chain constant region, hinge, CH2 and CH3 domains. The vector was transfected into mouse NS0 myeloma cells and clones expressing soluble recombinant protein were used as a source of conditioned media for purification of the ADAM8 catalytic domain as described for ADAM17 [7]. Briefly, 100 ml of conditioned medium was treated with 0.5 ml of protein A-Sepharose. The gel was washed and ADAM8 was eluted by specific cleavage at the enterokinase cleavage site using 20 U of recombinant enterokinase. The enterokinase was removed with soybean trypsin inhibitor-Sepharose and the eluted protein was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (10% polyacrylamide) under reducing conditions and by N-terminal sequencing.

2.3. Enzyme assays

ADAM8 (5 μg/ml) was incubated at 37°C with MBP (500 μg/ml) and peptides (50 μM) in 10 mM HEPES, 0.05% Brij-35 (v/v), pH 7.5. MBP and enzyme incubation mixtures were analysed by 14% 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. Comparative analysis of the cleavage patterns produced by MT-MMPs was conducted at 25°C. The fragment peaks were collected and identified by mass spectrometry. The percentage of peptide cleavage was calculated on the basis of the peak areas of start and end products from absorbance monitored at 220 nm. Activity of ADAM9 in 20 mM HEPES, 0.01% Triton X-100 (v/v), pH 7.5 was monitored by following hydrolysis of the quenched fluorescent TNFα-based peptide essentially as described in [7]. The concentration of ADAM9 was estimated by active site titration with the hydroxamate inhibitor CGS27023 (GlaxoSmithKline, Stevenage, UK) or BB94 (British Biotech, Oxford, UK). Inhibition by TIMP-1, 2 and 3 was followed as described in [7] by pre-incubation of 8 nM ADAM9 with TIMP at concentrations from 1 nM to 0.2 μM for 16 h prior to the addition of the TNFα peptide substrate.

3. Results and discussion

3.1. Purification of ADAM8

Recombinant ADAM8 catalytic domain was purified by affinity chromatography on protein A-Sepharose and enterokinase treatment to remove the C-terminal Fc tag (Fig. 1A). The MW of the resulting protein was 33 kDa by SDS–PAGE and Coomassie staining (Fig. 1B). N-terminal sequencing of the purified material indicated that ADAM8 was processed after Arg187, which is situated between the predicted

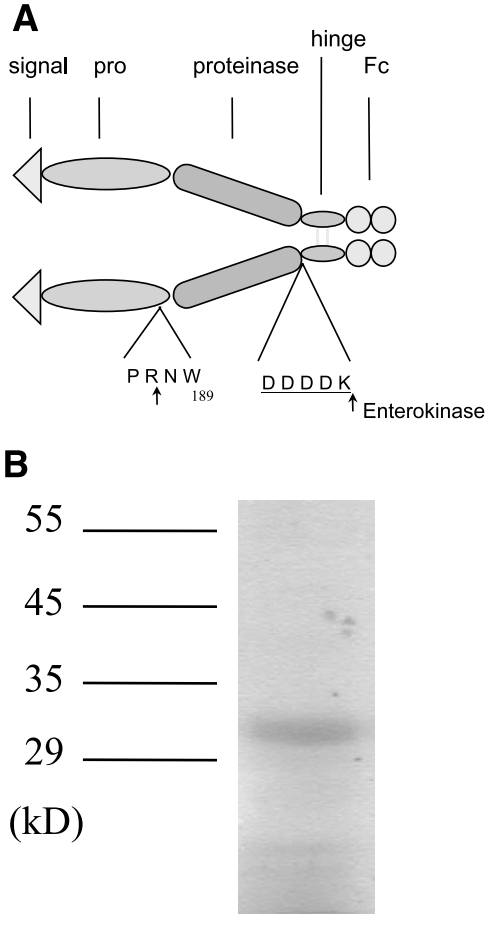

Fig. 1. SDS–PAGE analysis of purified ADAM8. A: Schematic representation of the ADAM8 prepro-catalytic domain expressed as a Fc fusion. The arrowheads to the left indicate the propeptide processing sites as determined by N-terminal sequencing. The arrowhead to the right indicates the anticipated cleavage that occurs after treatment with enterokinase. B: Coomassie-stained SDS–PAGE of purified ADAM8. The fusion protein could be bound through the Fc portion to protein A-Sepharose and free ADAM8 eluted by enterokinase cleavage, yielding a protein band at an apparent molecular weight of 33 kDa. N-terminal sequencing of this protein band showed that it corresponded to the catalytic domain of ADAM8 lacking the pro-domain.

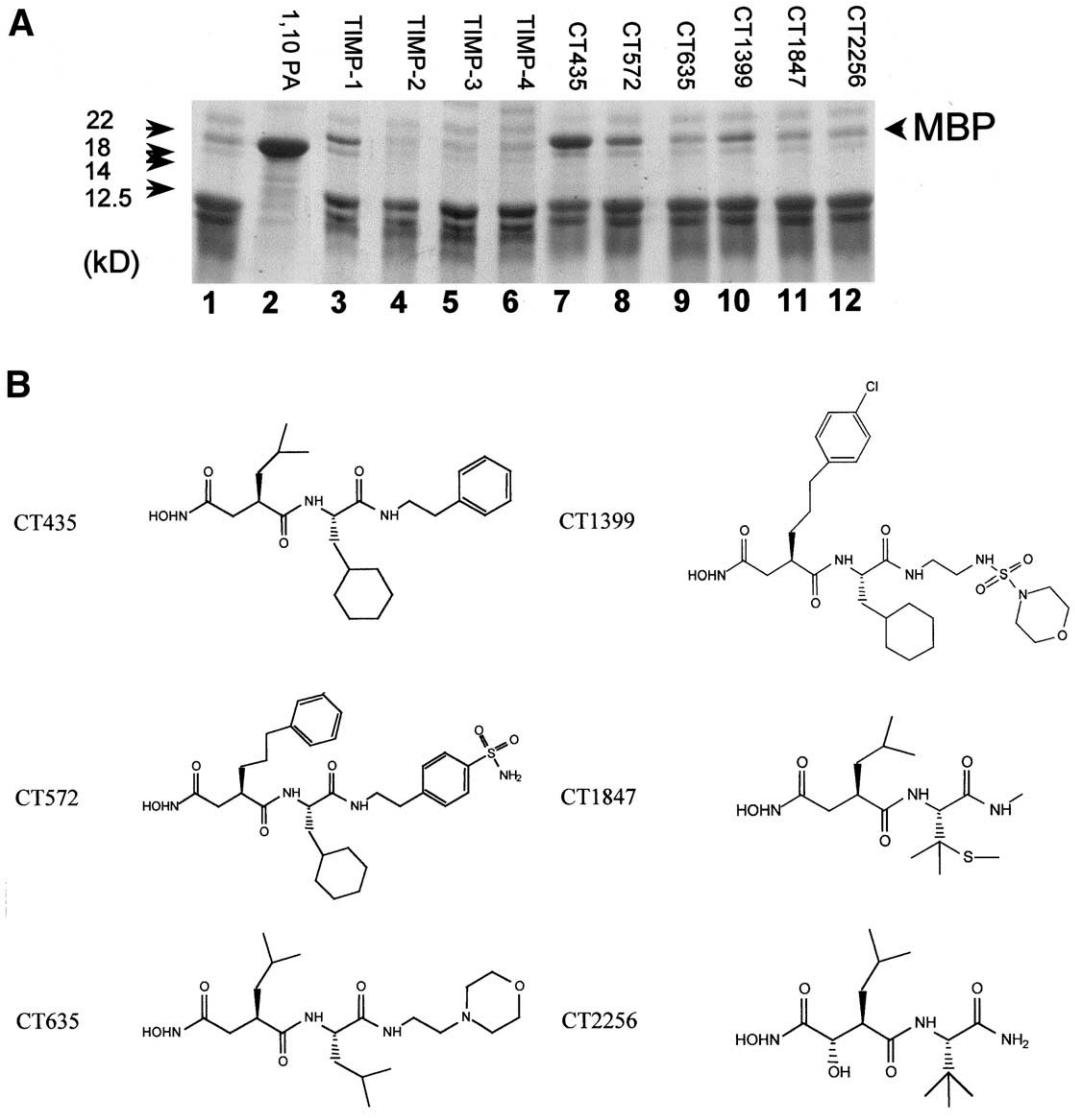

Fig. 2. MBP degradation by ADAM8. A: Bovine MBP (500 μg/ml) was incubated at 37°C for 4 h with ADAM8 (5 μg/ml). Reactions were stopped by the addition of loading buffer followed by heating. The protein (5 μg) was analysed by 14% SDS–PAGE under reduced conditions. The gel was stained with Coomassie brilliant blue R-250. Lane 1, dimethylsulphoxide. Lane 2, 1 mM 1,10-phenanthroline. Lane 3, 1 μM TIMP-1. Lane 4, 1 μM TIMP-2. Lane 5, 1 μM TIMP-3. Lane 6, 1 μM TIMP-4. Lane 7, 5 μM CT435. Lane 8, 5 μM CT572. Lane 9, 5 μM CT635. Lane 10, 5 μM CT1399. Lane 11, 5 μM CT1847. Lane 12, 5 μM CT2256. B: Structures of inhibitors tested on ADAM8.

propeptide and catalytic domains (Fig. 1A). Thus, the MW and N-terminal sequence of the purified material are consistent with it being the ADAM8 catalytic domain from which the propeptide has been removed.

3.2. MBP degrading activity of ADAM8

To determine whether ADAM8 was catalytically active, the purified materials were incubated with MBP and its cleavage monitored by SDS–PAGE, as previously described [18]. After a 2-h incubation, the 20-kDa MBP is completely degraded with the appearance of two prominent protein bands of about 9 and 11 kDa (Fig. 2A). N-terminal sequencing of the 11-kDa fragment was consistent with cleavage occurring between Pro73 and Gln74 as previously observed for ADAM10 and 28 [5,18]. MBP was also a good TACE substrate, although cleavage occurred at Phe42–Phe43 and Phe88–Phe89 [19]. Various proteases have been implicated in MBP degradation an important feature of demyelinating diseases such as multiple sclerosis (MS) [20]. Although the direct involvement of ADAMs in demyelination remains to be reported, TACE expression is increased during experimental allergic encephalomyelitis (EAE), an animal model for MS [21], whilst the disintegrin domain of ADAM8 had a protective effect on EAE [22].

3.3. Inhibition of ADAM8 activity

The zinc chelator 1,10-phenanthroline inhibited the MBP degradation by ADAM8, confirming the zinc dependence of the ADAM8 activity (Fig. 2). Of the four TIMPs, only TIMP-1 (1 μM) showed slight inhibitory activity after 2 h incubation time. In contrast, ADAM-10 was fully inhibited by 0.25 μM of TIMP-1 and 3 under similar conditions [3]. Furthermore, no additional inhibitory effect was observed at shorter intermediate incubation time points. In a separate assay system, we

Table 1
Susceptibility profile of putative metallosheddases by TIMP inhibition

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
ADAM8	–	–	–	–
ADAM9	–	–	–	nd
ADAM10[a]	++	–	++	nd
ADAM12[b]	–	–	+	nd
ADAM17[c]	(+)	–	++	–
MT1-MMP[d]	–	++	++	++
MT4-MMP[e]	++	++	++	++

Data in a–c are from [3,7,8] respectively. Data in d and e are from [11,12] and W. English and V. Knäuper, unpublished observations. – indicates no inhibition observed at $>10^{-6}$ M; (+), $10^{-6} \leq K_i > 10^{-7}$ M; +, $10^{-7} < K_i > 10^{-8}$ M; ++, 10^{-8} M $< K_i$ (approximations from experimental data).

also evaluated the ability of the TIMPs to inhibit the soluble ectodomain of ADAM9 using a quenched fluorescent peptide based on TNFα. Although ADAM9 was inhibited by hydroxamic acid inhibitors CGS27023 and BB94, it was not inhibited by TIMP-1, 2 or 3 at concentrations up to 200 nM. TIMP-1 was a weak inhibitor of the aggrecanases ADAMTS-4 and 5 [10], whilst it inhibited ADAM10 well [3]. This is in contrast to ADAM17 (TACE), which is only inhibited by TIMP-3 [7], and the MT-MMPs (MT1 by TIMP-2, 3 and 4, MT4 by TIMP-1, 2, 3 and 4 [11,12]; W. English and V. Knäuper, unpublished observations). In each case where TIMP inhibition was observed K_i has typically been estimated to be in the low nM to pM range. We conclude therefore that the poor potency of the TIMPs towards ADAM8 and 9 indicates that they do not regulate their proteolytic activity. The varying specificity of membrane bound metalloproteinases towards TIMPs should provide a powerful tool in the identification of the type of protease activity observed in cell-based systems (summarised in Table 1).

The MBP degradation assay was also used to carry out an initial evaluation of the inhibitory potential of various hydroxamate-based metalloproteinase inhibitors towards ADAM8. Of the six inhibitors tested, CT435 showed the most potential as an inhibitor of ADAM8, followed by CT572 and CT1399 whereas CT635 and CT2256 were poor ADAM8 inhibitors (Fig. 2A). CT572 and CT1399 have an extended side chain in P_1' (Fig. 2B) indicating that ADAM8 may resemble MMPs such as gelatinase A or collagenase-3 which have a deep S_1' specificity pocket. At P_2', the cyclohexylmethyl group of CT435, CT572 and CT1399 was preferred to the smaller isobutyl and *tert*-butyl groups of CT635 and CT2256, respectively. Taken together these data suggest that ADAM8 inhibitor potency can be obtained by optimisation of the P_2' and P_1' residues.

3.4. Catalytic activity of ADAM8 and towards peptide substrates

The catalytic activity of ADAM8 towards peptide substrates based on the cleavage region within the juxta-membrane stalk of amyloid precursor protein (APP), TNFα, CD40 ligand (CD40L), HB-EGF, IL-1R and KL, were compared with two different members of the MT-MMP family, MT1 and MT4-MMP (Table 2). These proteins have also been shown to be able to function as metallosheddases [11,13], hence it is of interest to compare the specificity of these proteins with those of the ADAMs. This has also allowed us to compare our data with those previously reported for ADAM9 and 10 ectodomains. ADAM8 was found to cleave the APP peptide at H↓Q, like ADAM9 [6], rather than at the α-secretase site, K↓L, cleaved by ADAM10 [3]. MT4-MMP was found to cleave at both K↓L and H↓Q, whereas MT1-MMP was only found to cleave at K↓L. ADAM8 cleavage of the MBP peptide confirmed the cleavage site, P↓Q, found within the protein itself. ADAM10 has also been shown to cleave MBP at this site [3] and although we found that MT1 and MT4-MMP were able to cleave this peptide at a single site, we were unable to determine the site of hydrolysis. On comparison of the cleavage sites found within the TNFα peptide, ADAM8 was found to hydrolyse at two sites both A↓V, as ADAM10 [3] and ADAM17 (TACE, [7]) and A↓Q, rather than at Q↓A like MT4-MMP, or at the two sites A↓Q/S↓S (ADAM9, MMP-1, MMP-9 [6]). MT1-MMP was found to be unable to cleave this peptide. ADAM8 was able to cleave KL and although MT1 and MT4-MMP were also able to cleave this peptide, we were unable to identify the site. This differs from ADAM9, which has been reported to cleave this peptide at multiple sites [6]. Although we were able to demonstrate that ADAMs and MT-MMPs were all able to cleave some peptides in common, albeit with differing specificity in most cases, we found some instances where more marked abilities in peptide specificity were apparent. For example, neither ADAM8 nor ADAM10 were able to cleave the peptide corresponding to the stalk region of HB-EGF, yet both MT1-MMP and MT4-MMP were able to hydrolyse this peptide at P↓V/E↓N and E↓N/R↓L respectively. ADAM8 was able to process the peptide of IL-1R, yet ADAM10, MT1 and MT4 were unable to do so. We previously reported that ADAM10 was able to cleave a peptide based on CD40L [3], yet ADAM8, MT1 and MT4 showed no activity towards this peptide. It has been proposed that specificity of metallosheddases is governed, in part, by the amino acid sequence of the peptide in the stalk region of the shed molecule [23]. Providing these peptides are suitable mimics of the shed region of the molecule and they are not in a region containing significant secondary or tertiary structure, our comparisons of different metallosheddase specificity indi-

Table 2
Metalloproteinase-mediated cleavage of peptides based on the proteolytically sensitive sequences of shed proteins

	Predicted cleavage site	ADAM8	ADAM9	ADAM10	MT1-MMP	MT4-MMP
CD40L	KENSFE↓MQKGAQ	NC	ND	M↓Q	NC	NC
HB-EGF	GLSLPVE↓NRLYTYD	NC	ND	NC	P↓V+E↓N	E↓N+R↓L
IL-1Rc	TVKEAS↓STFSWG	S↓S	ND	NC	NC	NC
KL	LPPVA↓A↓SSLR	A↓S	S↓S+A↓S+A↓A	NC	?↓?	?↓?
TNFα	SPLAQA↓VRSSSRK	A↓Q+A↓V	A↓Q+S↓S	A↓V	NC	Q↓A
APP	YEVHHQK↓LVFF	H↓Q	H↓Q	K↓L	K↓L	K↓L+H↓Q

MT1 and MT4-MMP cleaved the KL peptide at a single site that could not be identified, ADAM9 results are from [6].

cates this could indeed be partly how substrate specificity is regulated. However our study also suggests that it is not merely the P_1 and P_1' which influence this but also the surrounding amino acids, suggesting these enzymes recognise a wider 'footprint'.

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