Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10

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INTRODUCTION

ADAM (a disintegrin and metalloproteinase) proteases are a family of type I transmembrane proteins with a modular structure consisting of a prodomain and a metzincin-type metalloproteinsere catalytic domain followed by disintegrin and cysteine-rich domains, a transmembrane region and a short cytoplasmic tail [1]. A major function of ADAM proteases is the extracellular juxtamembrane cleavage of cell surface proteins, or ectodomain shedding. TACE [TNFα (tumour necrosis factor α) converting enzyme, also known as ADAM17] and ADAM10 are the best characterized members of the ADAM family, and are the most closely related members in mammals, having approx. 50% sequence similarity within the catalytic domain [2]. TACE was originally identified as the enzyme responsible for processing the inflammatory cytokine TNFα from its membrane-bound precursor to its soluble circulating form [3,4]. However, genetic studies in mice have established that TACE plays an essential role in vivo in processing ligands for the EGF (epidermal growth factor) receptor family, including TGF-α (transforming growth factor α) and HB-EGF (heparin-binding EGF) [5–7]. Although ADAM10 is also the dedicated processing enzyme for particular EGFR (EGF receptor) ligands [8], its critical function in development appears to be regulation of Notch signalling, potentially through cleavage of Notch and its cognate ligand Dll-1 (delta-like ligand-1) [9,10]. Numerous protein substrates have subsequently been identified for both TACE and ADAM10, including cytokine receptors, chemokines and adhesion molecules. In addition to an essential role in development and maintaining normal physiology, ectodomain shedding has been implicated as a pathological mechanism in disease, and both TACE and ADAM10 have been explored as targets for therapeutic drugs in cancer and inflammatory disease [11,12].

TACE and ADAM10 have distinct roles in biology, which probably reflects processing of a unique substrate repertoire by each protease. The detailed mechanisms by which these two metalloproteases achieve their substrate specificity have begun to be elucidated, but are still not entirely understood. In many cases, a particular substrate can be cleaved by either TACE or ADAM10, and the specific protease involved is dictated by the activating stimulus [13]. For example, treatment of cultured cells with phorbol esters potently induces ectodomain shedding of many cell surface proteins, generally in a TACE-dependent manner. Conversely, treatments that promote cellular calcium influx, including calcium ionophores, stimulate ADAM10-dependent shedding [14]. However, specific substrates do exist for each protease. Among EGFR ligands, for example, TGF-α, amphiregulin and ephiregulin are specifically processed by TACE, whereas BTC (betacellulin) and EGF are processed exclusively by ADAM10 [7]. A number of potential mechanisms could give rise to substrate discrimination between TACE and ADAM10. For example, targeting of protease and substrate to particular subcellular locales or membrane microdomains may be involved in promoting cleavage by TACE [15–17]. Specific complementary interactions between the protease and substrate also appear to be important for targets by ADAMs. ADAM10 recognition of ephrin substrates involves formation of a ternary complex between the ephrin substrate, its Eph receptor, and non-catalytic domains of the protease, suggesting exosite interactions as being important for ADAM substrate recognition [18].

Key words: disintegrin, ectodomain shedding, enzyme specificity, metalloproteinase, peptide library, tumour necrosis factor.
The general importance of active-site interactions in mediating protease substrate recognition would suggest that cleavage site recognition may also play a role in promoting ADAM cleavage and substrate specificity. However, evidence for a role for cleavage site sequence specificity among ADAM proteases has been equivocal. For example, point mutations at ADAM substrate cleavage sites often have marginal effects on shedding in cultured cells [19–24]. While juxtamembrane segment exchange experiments do suggest that the cleavage site sequence contributes to overall shedding efficiency, it is unclear whether a specific sequence will direct cleavage by a particular ADAM [25–28]. Mapping of TACE and ADAM10 cleavage sites in protein substrates has not established a clear consensus motif, which has led to the perception that ADAM proteases generally lack sequence specificity [18]. However, analysis of the cleavage rates of individual peptide substrates [29–31] and focused peptide library screening [32] have suggested that TACE has well-defined cleavage site specificity, primarily driven by a preference for aliphatic hydrophobic residues at the P1′ position. However, preferences of TACE for particular amino acid residues outside of the P1′ and P2 positions have not been studied in a systematic way. Furthermore, there have been no reported studies of the cleavage site specificity of ADAM10, it is not known whether it is distinct from that of TACE.

In the present manuscript, we demonstrate by peptide library screening and analysis of individual consensus substrates that TACE and ADAM10 have distinct amino acid preferences at multiple positions surrounding the substrate cleavage site. Furthermore, we show through mutagenesis that three amino acid residues within the S1′ pocket play a major role in dictating these differences in specificity. These observations suggest ways in which ADAM proteases may be targeted to distinct substrates in vivo, and provide insight into the design of ADAM-selective and broad spectrum inhibitors of these proteases.

**MATERIALS AND METHODS**

**Recombinant proteases**

Recombinant TACE ectodomain used for peptide library screening was obtained from Roy Black (Amgen, Seattle, WA, U.S.A.). TACE used for all other experiments, and ADAM10 for all studies, were produced in insect cells by baculoviral expression as truncated constructs consisting of the signal sequence, pro- and catalytic domains fused to a C-terminal His6 (hexahistidine) tag (residues 1–480 of mouse TACE or residues 1–460 of mouse ADAM10). Baculoviral expression vectors were prepared by PCR-based cloning into the plasmid pFastBac1, and point mutants were made by nested PCR amplification using overlapping mutagenic primers. Baculovirus was generated using the Bac-to-Bac system (Invitrogen) as recommended by the manufacturer. Proteases were expressed in Hi5 cells and purified from cell supernatants by immobilized metal affinity chromatography on Talon resin (BD Biosciences; 40 μl/15-cm-diameter plate of cells) by standard procedures. Proteases were eluted in 250 mM imidazole, 50 mM Tris/HCl, pH 7.4, and 250 mM NaCl, and dialysed into 10 mM Hepes, pH 7.4, and 100 mM NaCl with 10% glycerol prior to storage in aliquots at −80 ºC.

**Peptide library screening**

Cleavage site specificity for TACE and ADAM10 was determined by digestion of mixture-based peptide libraries (synthesized at Tufts University Core Facility) followed by automated Edman sequencing of the reaction products as previously described [33]. To determine selectivity C-terminal to the cleavage site, ADAM10 (60 μg/ml) or TACE (170 μg/ml) was incubated with 1 mM acetyl-XXXXXXXXXXXXX-ami(de (where X is an equimolar mixture of the 19 amino acids excluding Cys) for 2 h at 37 ºC in 10 mM Hepes, pH 7.4, and 50 mM NaCl, followed by Edman sequencing on an Applied Biosystems Proceiss 494 Automated Protein Sequencer. These conditions resulted in cleavage of 5–10% of the peptides within the mixture as judged by sequencing yield. For positions N-terminal to the cleavage site, the peptide mixtures MAXXXXXXLRAAGK-biotin)-amide and MGPXXXXXKLGGK(GK-biotin)-amide were digested under similar conditions for the P5 and P4–P1 positions respectively [where K-biotin is ε-(biotinamidohexanoyl)lysine]. Following digestion, biotinylated peptides were treated with avidin–agarose and unbound N-terminal fragments were recovered and sequenced as described. Edman sequencing data were normalized by dividing the molar fraction of each residue within a given cycle by the molar fraction of that residue present within the starting mixture. After this transformation, the average value within a cycle is 1, and positively selected residues have values > 1.

**Cleavage assay of individual consensus peptide substrates**

Fluorescent peptide substrates were prepared by Genemed Synthesis or AnaSpec. All peptides were intramolecularly quenched fluorogenic substrates bearing Mca (7-methoxycoumarin-4-acetyl) fluorophores at their N-termini and 2,4-dinitrophenyl quenchers conjugated to dianisopropionic acid at their C-termini [34]. Peptide stock solutions were made by dissolving in DMSO and adjusting the concentration to 10 mM based on coumarin absorbance (ε328 = 12 900 M⁻¹·cm⁻¹). Peptide cleavage was monitored in black 96-well plates in a Tecan Safire plate reader over 30 min at 37 ºC at 30 s intervals using excitation and emission wavelengths of 325 nm and 393 nm respectively. Peptides were assayed at 2 μM concentration with 1–2 nM TACE or 1–5 nM ADAM10 in ADAM reaction buffer (10 mM Hepes, pH 7.4, 100 mM NaCl and 0.01% Brij 35) containing 1% DMSO carrier. Cleavage was quantified using TACEtide or TENtide (see Figure 2) exhaustively cleaved with TACE or ADAM10 respectively, as a standard. At 2 μM peptide, cleavage rates were linear over substrate concentration, indicating that [S] ≪ Kₘ. Specificity constants (Kᵥ/Kₘ) were thus calculated directly from initial rates according to the equation V = [E][S][Kᵥ/Kₘ]. No peptide cleavage was detectable against any substrate by inactive mutant proteases (TACE E406A and ADAM10 E385Q).

**Expression and purification of recombinant AP (alkaline phosphatase)-tagged proteins from mammalian cells**

The mammalian WT (wild-type) TNF expression vector [35,36] (a gift from Dr Carl Blobel, Abellis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY, U.S.A.) produces TNF with a FLAG epitope tag at its N-terminus and an AP (alkaline phosphatase) tag followed by Myc and His₆ tags at its C-terminus. Substrate mutants were prepared by PCR using mutagenic primers using standard protocols. To produce recombinant TNF, the plasmid was transiently transfected into HEK-293T cells [human embryonic kidney 293 cells expressing the large T-antigen of SV40 (simian virus 40)] using Lipofectamine™ 2000 as recommended by the manufacturer (Invitrogen). At 48 h later, cells were washed once with ice-cold PBS, and lysed in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A and 10 μg/ml aprotinin.
TACE and ADAM10 cleavage site motifs

RESULTS AND DISCUSSION

TACE and ADAM10 have significant differences in their cleavage motifs

To determine whether cleavage site sequence specificity might contribute to differences in protein substrate recognition by TACE and ADAM10, we applied an iterative peptide library screening approach to identify cleavage motifs for the two proteases [33]. Initially, amino acids preferred by TACE and ADAM10 at positions downstream of the cleavage site were identified using a random dodecamer library (Figure 1). Our results indicate that both TACE and ADAM10 strongly preferred hydrophobic residues at the P1’ position, and had weaker selectivity for basic and aliphatic residues at P2’ and small residues at P3’ (Figure 1 and Table 1). This motif is generally similar to that preferred by MMPs (matrix metalloproteinases) [33,37]. Accordingly, we subsequently used a secondary library MAXXXXXLRGAARE(K-biotin), which we previously used to profile MMP cleavage specificity [33], to determine preferences at the positions upstream of the cleavage site for both proteases. Screening of this library revealed a strong selection for proline at the P5 position for both TACE and ADAM10 and an approximately equal selection for proline at the P3 position for TACE exclusively (Figure 1). In order to determine the cleavage specificity at the remaining upstream positions, we carried out an additional round of screening with an ADAM-specific tertiary library, MGPXXXXLKGGGKK(K-biotin). Using this library, we identified further determinants of specificity at the P4–P1 positions, which include selectivity for small residues such as alanine at the P1, P2 and P3 positions by both enzymes. Use of this library did not reveal a preference for proline at the P3 position for TACE, suggesting that a proline residue upstream of the scissile bond promotes cleavage by TACE if present at either P5 or P3 but not both. This observation was substantiated by analysis of TACE specificity using a peptide library with a proline residue fixed at the P3 position, which revealed no selection for peptides with proline at P5 (results not shown).

Figure 1  TACE and ADAM10 display distinct cleavage site motifs

(A) Peptide cleavage selectivity for TACE and ADAM10. The distribution of amino acid residues at positions C-terminal to the ADAM cleavage site was determined by sequencing a partial digest of an N-terminally blocked random dodecamer library. Secondary and tertiary libraries used to determine specificity at the indicated positions upstream of the cleavage site had free N-termini and C-terminal biotin groups. The amino acid distribution of the substrate pool was determined by Edman sequencing of the digest following removal of the biotinylated C-termini using immobilized avidin. Positively selected residues for each primed and unprimed position are indicated by heat map according to the scale on the bottom of the panel. Data were normalized such that for a value of 1 (black) corresponds to the average quantity per amino acid in a given sequencing cycle and would indicate no selectivity, whereas residues having a value greater than 1 (red) are positively selected by the protease. Due to interfering peaks on the sequencer, data is missing for the following residues at specific upstream positions: arginine at P5, glycine from P5 to P3, glutamine from P4 to P1, and lysine at P2 and P1. Heat maps were generated using MapleTree using quantified data provided in Supplementary Table S1 (at http://www.BiochemJ.org/bj/424/bj4240099add.htm). (B) Comparison of P1’ selectivity of TACE and ADAM10. Selectivity values used to generate the heat map in (A) are depicted as a bar graph.
Table 1  Residues selected by TACE and ADAM10 from peptide libraries

Selectivity values (in parentheses) were calculated as described in the legend to Figure 1. Only residues with selectivity values greater than 1.4 are shown.

<table>
<thead>
<tr>
<th>Cleavage position</th>
<th>Enzyme</th>
<th>P5</th>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P1′</th>
<th>P2′</th>
<th>P3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACE</td>
<td>P (1.7)</td>
<td>R</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>V</td>
<td>K</td>
<td>G</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>H (1.6)</td>
<td>V</td>
<td>S</td>
<td>L</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>K</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>T (1.4)</td>
<td>T</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>K</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M (1.4)</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM10</td>
<td>P (2.1)</td>
<td>T</td>
<td>T</td>
<td>S</td>
<td>A</td>
<td>L</td>
<td>K</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>A (1.5)</td>
<td>A</td>
<td>E</td>
<td>Y</td>
<td>R</td>
<td>A</td>
<td>E</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H (1.5)</td>
<td>H</td>
<td>Q</td>
<td>T</td>
<td>T</td>
<td>K</td>
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</tbody>
</table>

Although the TACE and ADAM10 cleavage site motifs as determined by peptide library screening are similar to one another overall, there are notable differences at multiple positions surrounding the cleavage site. These differences are most pronounced at the P1′ position, at which both enzymes are most highly selective. Consistent with previous observations on cleavage of peptide substrates [29–32], TACE prefers aliphatic hydrophobic residues at P1′, with a valine being the most favoured. Our data show that ADAM10, in contrast, prefers larger residues, primarily leucine, but can also accommodate aromatic residues and even glutamine. These results indicate that TACE and ADAM10 display distinct cleavage site motifs that may play a significant role in protein substrate specificity in vivo.

Because TACE and ADAM10 are frequently described as lacking a definable consensus cleavage motif, we compared our peptide library results to sequences that have been mapped as ADAM cleavage sites in vivo or in cultured cells (Table 2). As previously noted, we found hydrophobic residues, primarily leucine and valine, to be over-represented at the P1′ position. Consistent with the peptide library data, the presence of valine at P1′ was particularly frequent at TACE cleavage sites (eight out of 23 sites) in comparison with ADAM10 sites (three out of 18 sites). Though we did note the presence of glutamine at the P1′ position of two ADAM10 sites, we did not observe an aromatic residue at P1′ for any substrates. It is possible that additional ADAM10 cleavage sites exist that remain to be identified that have larger P1′ residues, as has been observed for deep-pocket MMPs [37]. Alternatively, selectivity for aromatic residues by ADAM10 may be a by-product of having a pocket that deselects smaller aliphatic residues while selecting larger aliphatic residues and glutamine. The most striking feature of the established cleavage site motifs is the preference for hydrophobic residues at P1′.
Figure 2  Cleavage kinetics for peptide substrates based on the predicted optimal cleavage motifs for TACE and ADAM10

(A) Consensus decapeptide substrates were designed and synthesized based on the data from Figure 1. Peptides were dually labelled with a fluorophore and quencher, allowing cleavage to be monitored by fluorescence increase as described in the Materials and methods section. The arrow indicates the site of cleavage. (B) The kinetics of cleavage ($k_{cat}/K_m$ values) of each fluorescent peptide substrate by TACE and ADAM10 were determined directly from the initial rate at a single substrate concentration ($2 \mu M$; [S]) under conditions where $K_m \gg [S]$. (C) Cleavage rates are shown as a percentage of the $k_{cat}/K_m$ value of the peptide that was cleaved the fastest by a given protease (the ‘L’ substrate is identical to ADAMtide from B). Error bars reflect the standard deviation from at least three separate experiments.

Evaluation of individual consensus peptide substrates for TACE and ADAM10

To analyse ADAM cleavage specificity more quantitatively and to generate tools for further study, a series of decameric peptides based on the TACE and ADAM10 consensus motifs were designed and synthesized (Figure 2A). One peptide substrate, termed ADAMtide, comprised residues selected by both TACE and ADAM10. We have also generated a pair of substrates called TACEtide and TENtide, designed to be selective for TACE and ADAM10 respectively, by incorporating residues that are favoured by one protease over the other. As a reference, we also evaluated a peptide derived from the TACE cleavage site in human TNF (TNFtide), which is similar to peptide substrates typically used to assay TACE [31,38]. All peptides were generated as intramolecularly quenched fluorescent substrates, with N-terminal Mca fluorophores and C-terminal dinitrophenyl quenching groups [34]. Cleavage rates were monitored continuously by following the increase in coumarin fluorescence over time (Figure 2B). Although ADAMtide was cleaved efficiently by both TACE and ADAM10, each protease was highly selective for its respective substrate. For example, TENtide was cleaved efficiently by ADAM10 but was not detectably cleaved by TACE under the conditions used. Although TACEtide was cleaved by TACE with comparable efficiency to TNFtide, TACEtide is clearly more selective for TACE, as it is cleaved approx. 2.5-fold less efficiently than TNFtide by ADAM10. These results confirm the peptide library results by establishing that we can generate highly selective substrates for TACE and ADAM10. Owing to their high degree of selectivity, TACEtide and TENtide may be useful for analysis of TACE and ADAM10 activity in more complex systems, such as intact cells or crude cell lysates. In addition, as efficient fluorescent substrates, these peptides have potential applications as the basis for high-throughput screens to identify ADAM protease inhibitors.

To quantitatively evaluate the extent of S1' pocket selectivity of either ADAM within a fixed sequence context, we examined three ADAMtide variants in which the P1' residue was substituted with valine, tyrosine or alanine, but the remaining sequence was unchanged (Figure 2C). For peptides in this series, the rank order
Identificaon of active site deormators of ADAM speciﬁciten

Clear diﬀerences in peptide cleavage speciﬁcity for TACE and ADAM10, namely at the P1′ position, suggest diﬀerences in the S1′ site within the catalytic domain of these proteases. The S1′ pocket of TACE has been deﬁned through X-ray crystallography of the catalytic domain in complex with peptidomimetic inhibitors (Figure 3A) [39,40]. Interestingly, despite the diﬀerences in selectivity, ADAM10 and TACE have a high degree of sequence conservation within their S1′ pockets (Table 3). Alignment of the TACE and ADAM10 catalytic domains indicates that, of the seven residues that line the S1′ pocket, three are identical between the two proteases, whereas the remaining four residues have relatively conservative substitutions. Among residues that diﬀer between the two proteases, Leu401 and Val402 are in closest proximity to the P1′ leucine mimetic in the crystal structure of TACE bound to a peptide hydroxamate inhibitor. We thus hypothesized that these two residues in particular were largely responsible for the diﬀerence in P1′ speciﬁcity between TACE and ADAM10. To test this hypothesis, we generated point mutants in each protease that exchange these two S1′ pocket residues with those found at the corresponding position in the other protease. These mutants were ﬁrst evaluated for cleavage selectivity against the series of ADAMtide substrates with variable P1′ residues.

We initially generated the ADAM10 I380L/T381V double mutant. Based on the cleavage proﬁle of the ADAMtide series, this mutant had P1′ peptide substrate selectivity indistinguishable from TACE, suggesting that one or both of the mutated residues are indeed the critical determinants of S1′ site speciﬁcity for ADAM10 (Figure 3B). We also generated the ADAM10 T381V single mutant and assayed it using our panel of peptides (Figure 3B). Mutation of Thr381 alone also increased the relative cleavage rate of the P1′ valine substrate in comparison to the WT enzyme, and was accompanied by a decrease in P1′ tyrosine substrate cleavage. However, the magnitude of these changes was smaller than seen with the double mutant, suggesting

![Figure 3 Determinants of TACE and ADAM10 P1′ speciﬁcity](image-url)

**Figure 3** Determinants of TACE and ADAM10 P1′ speciﬁcity

(A) Structure of the TACE active site. Co-ordinates were taken from the X-ray crystal structure of TACE in complex with a hydroxamic acid peptide-based inhibitor ([39]; PDB code 1BKC). For clarity, only the P1′ leucine mimetic and hydroxamate group of the bound inhibitor are shown (in light green). The hydroxamate metal chelating group co-ordinates the active site zinc ion (dark grey). The seven amino acid residues that comprise the S1′ pocket are labelled and shown in magenta in stick representation. The Figure was prepared using PyMOL (DeLano Scientiﬁc; http://www.pymol.org).

(B) Cleavage parameters for ﬂuorescent peptide substrates by ADAM10 I380L/T381V (left panel) and ADAM10 T381V (right panel) mutants. (C) Cleavage parameters for TACE L401I/V402T (left panel), TACE V402T (middle panel) and TACE V402T/V440T (right panel). Values were determined from measuring initial cleavage rates at 2 μM substrate as for the data shown in Figure 2.

**Table 3** Residues comprising the ADAM S1′ pocket

<table>
<thead>
<tr>
<th>Protease</th>
<th>Residue (TACE numbering)</th>
<th>348</th>
<th>401</th>
<th>402</th>
<th>405</th>
<th>434</th>
<th>439</th>
<th>440</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACE</td>
<td>Leu</td>
<td>Leu</td>
<td>Leu</td>
<td>Val</td>
<td>His</td>
<td>Val</td>
<td>Ala</td>
<td>Val</td>
</tr>
<tr>
<td>ADAM10</td>
<td>Leu</td>
<td>Ile</td>
<td>Thr</td>
<td>His</td>
<td>Val</td>
<td>Ala</td>
<td>Val</td>
<td>Val</td>
</tr>
</tbody>
</table>

of cleavage rates is identical to that anticipated from the peptide library screens (P1′ V > L > Y > A for TACE and P1′ L > Y > V > A for ADAM10). These data also conﬁrm that substitution of an aromatic residue at P1′ leads to a more substantial decrease in activity for TACE (10-fold) than for ADAM10 (a 20 % decrease) in comparison with substrates bearing optimal P1′ residues. Furthermore, although a P1′ valine residue is clearly suboptimal for ADAM10, the rate of cleavage is decreased by only a factor of 3 in comparison with the peptide with P1′ Leu. This may explain why some in vivo protein substrates of ADAM10 are able to accommodate valine residues at the P3 position and of methionine at the P3′ position.
that Thr$^{381}$ and Ile$^{380}$ together determine the P1′ specificity of ADAM10.

To evaluate further the contribution of these two residues to the ADAM P1′ selectivity, we generated the TACE L401I/V402T double mutant and characterized its cleavage of the ADAMtide peptide series (Figure 3C). As anticipated, mutation of these two residues conferred upon TACE a P1′ cleavage profile more similar to ADAM10, in particular with respect to peptides having leucine or valine residues at P1′. However, in contrast to the ADAM10 double mutant, there was not an absolute switch of specificity in that the P1′ tyrosine peptide was cleaved at a lower rate than would be anticipated. The cleavage profile of the TACE V402T single mutant was essentially identical to the L401I/V402T double mutant (Figure 3C). Collectively, these data suggest that, although Leu$^{401}$ and Val$^{402}$ are necessary and sufficient for determining the P1′ cleavage specificity of TACE, the corresponding residues on ADAM10 are necessary but not sufficient. Hypothesizing that residues found deeper within the S1′ pocket were likely to promote cleavage of peptides with aromatic residues at the P1′ position, we generated the TACE V402T/V440T double mutant. Indeed, we found that combined mutation of these two residues dramatically enhances cleavage of peptides with a tyrosine residue at P1′, suggesting that Val$^{440}$ is a critical determinant of S1′ specificity for TACE (Figure 3C).

Interestingly, this mutational analysis suggests that the structural basis for S1′ pocket selectivity differs between ADAMs and MMPs. Variability among MMPs in P1′ selectivity maps largely to a single residue within the S1′ pocket (equivalent to Leu$^{401}$ of TACE; Table 3). So-called ‘deep-pocket’ MMPs, such as MMP3, have a leucine residue at this position, whereas ‘shallow-pocket’ MMPs have either an arginine residue (MMP1) or a tyrosine residue (MMP7) at this position that partly occludes the pocket and hinders access of larger aromatic residues at P1′ [41,42]. This mechanism of pocket occlusion cannot account for differences in specificity between ADAMs, since TACE, which prefers smaller residues at P1′, has a leucine residue at this position as seen with deep pocket MMPs. In addition, the residue equivalent to Val$^{402}$ of TACE, which co-operates with Leu$^{401}$ in dictating S1′ accessibility, is nearly invariant as valine among all MMPs regardless of S1′ pocket size.

**Active site contribution to protein substrate cleavage by TACE**

TACE appears to be the major physiological processing enzyme for TNF, and ADAM10 expression cannot reconstitute defective TNF shedding in TACE-deficient cells. Experiments with TACE/ADAM10 chimaeras indicate that the ability to cleave TNF maps to the TACE catalytic domain [43]. However, it is not known to what extent this is due to active-site recognition of the TNF cleavage site as opposed to distal exosite interactions, which could potentially diminish the importance of active-site interactions by enhancing the affinity of the protease for its substrates. To assess the contribution of the active site in protein substrate recognition, we evaluated the impact of mutagenesis of both TACE and TNF on cleavage of the full-length protein substrate in vitro.

TNF mutants were prepared in which the WT P1′ residue (valine) was replaced with leucine or tyrosine. TNF and its mutants were expressed as AP fusion proteins and purified by FLAG epitope affinity chromatography from HEK-293T cells. Purified AP–TNF migrated on SDS/PAGE as a doublet at approx. 100 kDa. Peptide N-glycanase treatment caused the doublet to collapse into a single polypeptide, indicating that that the two species arose from differential N-glycosylation and not partial proteolytic cleavage (results not shown). Relative cleavage rates of the various mutants were determined by immunoblotting.
it did not reach statistical significance. Similarly, WT ADAM10 could also cleave TNF in vitro, with the anticipated specificity profile (P1' L ≈ Y > V). As observed with peptide substrates, the ADAM10 I380L/T381V double mutant had a cleavage profile for TNF resembling that of TACE (P1' V > L > Y). Overall, both WT and S1' pocket mutant ADAMs have cleavage selectivity on full-length TNF that parallels what we have observed for short peptide substrates. These results indicate that active-site selectivity contributes significantly to the efficiency of TACE towards an authentic protein substrate.

The role of the active site in substrate selectivity by ADAMs

Typically, consensus cleavage motifs for proteases are deduced from the sequences surrounding the natural cleavage sites of in vivo protein substrates, but this approach has been unsuitable for understanding ADAM substrate specificity. Inspection of substrate cleavage sites for TACE and ADAM10 has failed to reveal a well-defined consensus sequence, despite tendencies for specific residues to be present at several positions (see Table 2).

We used peptide library screening to determine the cleavage site selectivity for TACE and ADAM10. Our approach involved the analysis of peptide mixtures rather than individual substrates, so that each subsite is examined independently of the others. Although this has the disadvantage of obscuring co-operative interactions between subsites (the influence of a particular residue at one position on selectivity at another position), it does allow the impact of substituting each residue at each position within the peptide to be analysed systematically and exhaustively. This allowed us to identify both pronounced and subtle differences between the two proteases that we exploited in the design of highly selective substrates. By using multiple peptide libraries with partially fixed sequence, we were able to uncover negative co-operativity between the S5 and S3 sites of TACE, where proline is selected by either site only if it is absent from the other.

The importance of cleavage site recognition in proteolysis of natural protein substrates by ADAMs has been controversial. Based on our results in the present study, we conclude that the relative contribution of the cleavage site to overall cleavage efficiency is likely to be substrate-dependent. For example, although TNF is processed by TACE and not by ADAM10, a chimaeric protease incorporating the catalytic domain of TACE and the remaining sequence of ADAM10 cleaves TNF efficiently in cultured cells [43]. The close match between the TNF sequence and the TACE consensus together with the efficient cleavage of TNFdiite by TACE indicates that the TACE active site plays a dominant role in recognition of TNF. We have shown here that mutation of either the TNF processing site or the TACE S1’ pocket has an impact on in vitro cleavage of TNF in a manner quantitatively similar to cleavage of peptide substrates. Early observations that mutation of individual residues near the TNF cleavage site do not have an impact on processing in cultured cells probably reflects the high levels of TNF produced by transient overexpression, as well as the presence of residues at virtually every position that promote cleavage by TACE, including but not restricted to the P1’ position [19, 24]. Among EGFR ligands, replacement of the juxtamembrane sequence of EGF, an exclusive substrate of ADAM10, with that of TGF-α is sufficient to convert it into a TACE substrate [14]. In contrast, cleavage of some substrates is likely to be more dominated by exosite interactions involving non-catalytic domains within the proteases. For example, in cultured cells, the catalytic domain of ADAM10 alone is insufficient for its ability to cleave EGF [14], which has a cleavage site sequence that is highly divergent from the consensus (Table 2). Presumably other domains within ADAM10 are important for direct interactions with EGF to enhance substrate affinity in the absence of favourable contacts surrounding the cleavage site. Cleavage of BTC by ADAM10 also appears to involve interactions outside of the active site, since replacement of its juxtamembrane region with that of TGF-α is insufficient to direct cleavage by TACE. In addition, chimaeric proteases replacing any region of ADAM10 with that of TACE are inactive as BTC sheddases [14]. Based on its processing site sequence, we would predict BTC to be cleaved efficiently by either TACE or ADAM10, and in this case exsorite interactions may be important in limiting cleavage by TACE [35] rather than promoting cleavage by ADAM10. The cleavage site motifs presented here should prove useful in dissecting the relative contributions of active site versus exosite or scaffolding interactions to the processing of endogenous ADAM substrates in multiple systems.

REFERENCES


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SUPPLEMENTARY ONLINE DATA

Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10

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Table S1  Quantitative selectivity values for TACE and ADAM10 peptide library data

Values reflect the normalized molar fraction of each residue present within a given sequencing cycle after correction for amino acid bias present in the library. Data handling was carried out as described in the Materials and methods section of the main text.

<table>
<thead>
<tr>
<th>Library Position</th>
<th>TACE</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.26 0.76 0.92 1.21 1.11 3.10 1.82 2.85 1.11 0.98 0.94 0.08 0.52 0.65 0.45 0.31 0.35 0.38</td>
</tr>
<tr>
<td>P2</td>
<td>0.36 1.09 1.03 1.05 1.54 1.45 1.37 0.79 1.23 0.88 1.17 0.80 1.55 1.23 1.09 0.51 0.37 0.80</td>
</tr>
<tr>
<td>P3</td>
<td>0.67 1.60 1.34 1.60 1.66 0.74 0.67 0.56 0.85 0.67 0.78 0.70 1.52 1.21 1.31 0.76 0.68 0.86</td>
</tr>
<tr>
<td>P4</td>
<td>1.33 1.18 0.92 1.04 2.03 0.84 0.85 0.91 0.85 0.85 0.98 0.73 1.03 1.10 1.08 0.88 0.81 1.18</td>
</tr>
<tr>
<td>MAXXXXX-LRGAREK(biotin)</td>
<td>1.65 1.19 1.12 0.98 0.72 0.66 0.60 0.84 0.86 0.98 0.88 0.34 1.12 0.97 1.05 0.67</td>
</tr>
<tr>
<td>P5</td>
<td>1.86 1.44 0.99 0.53 0.93 0.75 0.83 1.20 0.95 1.17 0.93 0.30 1.33 0.80 1.05 1.13 0.70</td>
</tr>
<tr>
<td>MGPXXXX-LKGGEEK(biotin)</td>
<td>0.71 0.94 0.96 1.40 1.14 0.84 0.66 1.18 0.41 0.80 1.55 1.21 1.31 0.76 0.68 0.86</td>
</tr>
<tr>
<td>P4</td>
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</tr>
<tr>
<td>P3</td>
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<tr>
<td>P2</td>
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</tbody>
</table>

<table>
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<tr>
<th>Library Position</th>
<th>ADAM10</th>
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</thead>
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</tr>
<tr>
<td>P2</td>
<td>0.32 0.93 0.88 0.91 1.42 1.49 1.16 1.03 1.18 0.94 1.23 0.98 1.78 1.59 1.16 0.62 0.29 0.55</td>
</tr>
<tr>
<td>P3</td>
<td>0.60 2.13 1.59 1.23 1.00 0.85 0.64 0.69 1.28 0.89 0.98 0.81 0.90 1.30 1.27 0.76 0.61 0.73</td>
</tr>
<tr>
<td>P4</td>
<td>1.16 1.32 1.09 0.89 0.95 0.86 0.65 0.84 0.87 0.87 0.93 0.80 0.91 1.21 1.08 0.92 0.91 1.04</td>
</tr>
<tr>
<td>MAXXXXX-LRGAREK(biotin)</td>
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<tr>
<td>MGPXXXX-LKGGEEK(biotin)</td>
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</tr>
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<td>P4</td>
<td>0.82 1.49 1.24 1.62 1.03 0.74 0.66 1.10 0.76 1.33 1.43 1.18 1.18 0.76 0.44 0.64</td>
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