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

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Abstract

The metalloproteinases TACE (ADAM17) and ADAM10 are the primary enzymes responsible for catalyzing release of membrane anchored proteins from the cell surface in metazoan organisms. While the repertoire of protein substrates for these two proteases is partially overlapping, each one appears to target a subset of unique proteins *in vivo*. The mechanisms by which the two proteases achieve specificity for particular substrates are not completely understood. We have used peptide libraries to define the cleavage site selectivity of TACE and ADAM10. The two proteases have distinct primary sequence requirements at multiple positions surrounding the cleavage site in their substrates, which allowed us to generate peptide substrates that are highly specific for each of these proteases. The major difference between the two protease specificities maps to the P1' position (immediately downstream of the cleavage site) of the substrate. At this position, TACE is selective for smaller aliphatic residues, while ADAM10 can accommodate aromatic amino acids. Using mutagenesis we identify three residues in the S1' pockets of these enzymes that dramatically influence specificity for both peptide and protein substrates. Our results suggest that substrate selectivity of TACE and ADAM10 can be at least partly rationalized by specific features of their active sites.

Keywords

metalloproteinase; disintegrin; peptide library; enzyme specificity; tumor necrosis factor; ectodomain shedding

INTRODUCTION

ADAM (a disintegrin and metalloproteinase) proteases are a family of type I transmembrane proteins with a modular structure consisting of a prodomain and metzincin type metalloproteinase 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 (tumor 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 about 50% sequence similarity within the catalytic domain [2]. TACE was originally identified as the enzyme responsible for processing the inflammatory cytokine tumor necrosis factor α (TNF) 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 epidermal growth factor (EGF) receptor

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family, including transforming growth factor α (TGF- α) and heparin binding EGF (HB-EGF) [5-7]. While ADAM10 is also the dedicated processing enzyme for particular EGFR ligands [8], its critical function in development appears to be to regulate Notch signaling, potentially through cleavage of Notch and its cognate ligand delta-like ligand-1 (Dll-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 likely 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 (AR), and epiregulin (EPR) are specifically processed by TACE, while betacellulin (BTC) 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 targeting 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].

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, as 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.

Here, 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). 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 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 plate of cells) by standard procedures. Proteases were eluted in 250 mM imidazole, 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, and dialyzed into 10 mM HEPES, pH 7.4, 100 mM NaCl with 10% glycerol prior to storage in aliquots at -80 °C.

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-XXXXXXXXXXXX-amide (where X is an equimolar mixture of the 19 amino acids excluding Cys) for 2 hr at 37 °C in 10 mM HEPES, pH 7.4, 50 mM NaCl, followed by Edman sequencing on an Applied Biosystems Procise 494 Automated Protein Sequencer. These conditions result 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 MAXXXXLARGAARE(K-biotin)-amide and MGPXXXLKGGGKK(K-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 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 7-methoxycoumarin-4-acetyl (Mca) fluorophores at their N termini and 2,4-dinitrophenyl quenchers conjugated to diaminopropionic acid (Dap(Dnp)) 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 ($\epsilon_{328} = 12,900 \text{ M}^{-1} \text{ cm}^{-1}$). Peptide cleavage was monitored in black 96-well plates in a Tecan Safire plate reader over 30 min at 37 °C 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, 0.01% Brij 35) containing 1% DMSO carrier. Cleavage was quantified using TACEtide or TENTide exhaustively cleaved with TACE or ADAM10, respectively, as a standard. At 2 µM peptide, cleavage rates were linear over substrate concentration, indicating

that $[S] \ll K_M$. Specificity constants (k_{cat}/K_M) were thus calculated directly from initial rates according to the equation $V = [E][S]k_{cat}/K_M$. No peptide cleavage was detectable against any substrate by inactive mutant proteases (TACE E406A and ADAM10 E385Q).

Expression and purification of recombinant AP-tagged proteins from mammalian cells

The mammalian WT TNF expression vector [35,36] (a gift from Dr. Carl Blobel) produces TNF with a FLAG epitope tag at its N terminus and an alkaline phosphatase (AP) 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 HEK293T cells using Lipofectamine 2000 as recommended by the manufacturer (Invitrogen). 48 hr later, cells were washed once with cold PBS, and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml pepstatin A, 10 µg/ml aprotinin (250 µl per 10 cm plate). Cell lysates were cleared by centrifugation (10 min at 16,000 × g) and then incubated with M2 anti-FLAG affinity gel (Sigma, 50 µl beads/plate) pre-equilibrated in FLAG wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X100) for 2 hr at 4 °C. Beads were washed twice with lysis buffer and twice with FLAG wash buffer. FLAG-tagged proteins were eluted with 0.5 mg/ml FLAG peptide (Sigma) in wash buffer and subjected to dialysis against 10 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Triton X100 with 10% glycerol. Purified protein was quantified from Coomassie-stained SDS-polyacrylamide gels by densitometry using Quantity One software (Bio Rad) and BSA standards.

TNF cleavage assay

For analysis by immunoblotting, purified AP-tagged TNF (15 µg/ml) was incubated at 37 °C with protease (5 – 10 nM) in ADAM reaction buffer. At various times, aliquots were removed and quenched with 6X SDS-PAGE loading buffer. Samples were fractionated by SDS-PAGE followed by immunoblotting with anti-His₅ antibody (Qiagen). For quantitative measurements, purified AP-tagged TNF (2.5 µg) was first bound to 50 µL M2 anti-FLAG resin. Resin was washed twice with FLAG wash buffer, resuspended in 50 µl of ADAM assay buffer and preincubated at 37 °C for 15 min. Proteolysis was initiated by addition of 5 µl of WT or mutant TACE or ADAM10 (5 – 10 nM). Reactions were run in duplicate and stopped after 1 hr at 37 °C, and the released product bearing the AP moiety was separated from the resin by centrifugation. Uncleaved resin-bound substrate was recovered by elution with 0.5 mg/ml FLAG peptide. AP activity present in supernatants was quantified using the colorimetric substrate 4-nitrophenyl phosphate [36], using pure uncleaved protein substrate as a standard. In all cases, less than 33% of the substrate was cleaved under these conditions. All experiments were repeated at least three times with similar results.

RESULTS AND DISCUSSION

TACE and ADAM 10 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 matrix metalloproteinases (MMPs) [33,37]. Accordingly, we subsequently used a secondary library MAXXXXXX-LRGAREK (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 Pro at the P5 position for both TACE and ADAM10 and an approximately equal selection for Pro 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, MGPXXXX-LKGEEK(biotin). Using this library, we identified further determinants of specificity at the P4 through 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 Pro at the P3 position for TACE, suggesting that a Pro 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 having Pro fixed at the P3 position, which revealed no selection for peptides with Pro at P5 (not shown).

Although the TACE and ADAM10 cleavage site motifs as determined by peptide library screening are similar to one another overall, there are notable differences between them 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 Val being the most favored. Our data show that ADAM10 by contrast prefers larger residues, primarily Leu, but can also accommodate aromatic residues and even Gln. 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 Leu and Val, to be overrepresented at the P1' position. Consistent with the peptide library data, the presence of Val at P1' was particularly frequent at TACE cleavage sites (8 out of 23 sites) in comparison to ADAM10 sites (3 out of 18 sites). Though we did note the presence of Gln 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 Gln. The most striking feature of the established cleavage sites that to our knowledge has not been previously noted was the preponderance of small residues (Gly, Ser and Ala) at the P3' position, consistent with the peptide screening data. Interestingly, small residues at the P3' position occurred with equal frequency (~60%) as hydrophobic residues at P1'. We also noted residues overrepresented at cleavage sites to a less dramatic extent that concurred with the peptide library screen, including Pro at P5 (6 out of 34 sites), Ala at P3 (7 sites) Glu at P2 (8 sites), Ala at P1 (5 sites) and Val (6 sites) and basic residues (6 sites) at P2'. We also noted that Arg was frequently present at the P1 position (7 sites), though this was not selected from the peptide libraries. Taken together, this analysis suggests that TACE and ADAM10 cleavage sites share tendencies in their primary sequence, despite the lack of an absolute requirement for specific residues at any position surrounding the cleavage site. Among currently known substrates, the presence of Val at P1' is the only clearly apparent feature that could influence substrate cleavage by TACE as opposed to ADAM10. Selectivity at P1' also distinguishes TACE from MMPs, all of which are selective for larger aliphatic and/or aromatic residues at that position. In addition, MMPs select Pro exclusively at the P3 position and not at P5 [33]. Having a Val residue at P1' or a Pro residue at the P5 position (as opposed to P3) could function to prevent inappropriate cleavage of TACE substrates by MMPs.

Evaluation of individual consensus peptide substrates for TACE and ADAM10

To analyze ADAM cleavage specificity more quantitatively and to generate tools for further study, a series of decaeric peptides based on the TACE and ADAM10 consensus motifs were designed and synthesized (Figure 2A). One peptide substrate, termed ADAMtide, was comprised of 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 favored 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 amino-terminal 7-methoxycoumarin-4-acetyl fluorophores and carboxy-terminal dinitrophenyl quenching groups [34]. Cleavage rates were monitored continuously by following the increase in coumarin fluorescence over time (Figure 2B). While 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. While TACEtide was cleaved by TACE with comparable efficiency to TNFtide, TACEtide is clearly more selective for TACE, as it is cleaved approximately 2.5-fold less efficiently than TNFtide by ADAM10. These data 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 either Val, Tyr, or Ala, but the remaining sequence was unchanged (Figure 2C). For peptides in this series, the rank order 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. Further, while a P1' Val residue is clearly suboptimal for ADAM10, the rate of cleavage is decreased by only a factor of three in comparison to the peptide with P1' Leu. This may explain why some *in vivo* protein substrates of ADAM10 are able to accommodate Val residues at the P1' position. Notably, changing the P1' residue alone is insufficient to produce substrates that are fully selective for either protease, indicating that residues found at other positions within the peptide make important contributions to the selectivity of TACEtide and TENTide. For example, ADAM10 appears to be more tolerant than TACE of aromatic residues at the P3 position and of Met at the P3' position.

Identification of active site determinants of ADAM specificity

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 seven residues that line the S1' pocket, three are identical between the two proteases, while the remaining four residues have relatively conservative substitutions. Among residues that differ between the two proteases, Leu-401 and Val-402 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 Thr-381 alone also increased the relative cleavage rate of the P1' Val substrate in comparison to the WT enzyme, and was accompanied by a decrease in P1' Tyr substrate cleavage. However, the magnitude of these changes were smaller than seen with the double mutant, suggesting that Thr-381 and Ile-380 together determine the P1' specificity of ADAM10.

To further evaluate 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 Leu or Val at P1'. However, in contrast to the ADAM10 double mutant, there was not an absolute switch of specificity in that the P1' Tyr peptide was cleaved at a slower 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 while 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 Tyr at P1', suggesting that Val440 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 Leu residue at this position, while "shallow pocket" MMPs have either an Arg residue (MMP1) or a Tyr 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 Leu residue at this position as seen with deep pocket MMPs. In addition the residue equivalent to Val-402 of TACE, which cooperates with Leu-401 in dictating S1' accessibility, is nearly invariant as Val 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 chimeras 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 (Val) was replaced with Leu or Tyr. TNF and its mutants were expressed as AP fusion proteins and purified by FLAG epitope affinity chromatography from HEK293T cells. Purified AP-TNF migrated on SDS-PAGE as a doublet at around 100 kDa. Peptide N-glycanase treatment caused the doublet to collapse into a single polypeptide, indicating that the two species arose from differential N-glycosylation and not partial proteolytic cleavage (not shown). Relative cleavage rates of the various mutants were determined by immunoblotting (Figure 4A). Because it was difficult to accurately determine initial cleavage rates by immunoblotting, we took advantage of the fused alkaline phosphatase to quantify the extent of cleavage. For these assays, the TNF-AP fusion was first bound to anti-FLAG antibody coated beads. After treatment with protease, the level of AP activity both released into the supernatant and remaining on the beads was quantified by colorimetric assay (Figure 4B). Cleavage of TNF and its mutants by WT TACE followed the same pattern as anticipated based on cleavage preferences on peptide substrates (P1' Val > Leu >> Tyr). Quantitatively, the relative cleavage rates of the various mutants as determined using the alkaline phosphatase assay were similar to peptides with identical substitutions at the P1' position. Furthermore, the TACE L401I/V402T double mutant preferred Leu over Val at P1' in the full length TNF protein; while there was a tendency towards increased cleavage of protein with Tyr at P1' compared to WT TACE, it did not reach statistical significance. Similarly, WT ADAM10 could also cleave TNF *in vitro*, with the anticipated specificity profile (P1' Leu ~ Tyr > Val). As observed with peptide substrates, the ADAM10 I380L/T381V double mutant had a cleavage profile for TNF resembling TACE (P1' Val > Leu > Tyr). 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. While this has the disadvantage of obscuring cooperative 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 analyzed 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 cooperativity between the S5 and S3 sites of TACE, where Pro 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, we conclude that the relative contribution of the cleavage site to overall cleavage efficiency is likely to be substrate dependent. For example, while TNF is processed by TACE and not ADAM10, a chimeric 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 TNFtide 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 impacts *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 impact processing in cultured cells likely reflect 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]. By 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 favorable 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, chimeric proteases replacing any region of ADAM10 with that of TACE are inactive as betacellulin sheddases [14]. Based on its processing site sequence, we would predict betacellulin to be cleaved efficiently by either TACE or ADAM10, and in this case exosite 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADAM, a disintegrin and metalloproteinase
 AP, alkaline phosphatase
 AR, amphiregulin
 BTC, betacellulin
 Dap, diaminopropionic acid
 Dll-1, delta-like ligand 1
 Dnp, 2,4-dinitrophenyl
 EGF, epidermal growth factor
 EPR, epiregulin
 HB-EGF, heparin binding epidermal growth factor
 Mca, 7-methoxycoumarin-4-acetyl
 MMP, matrix metalloproteinase

TACE, tumor necrosis factor α converting enzyme
 TGF- α , transforming growth factor α
 TNF, tumor necrosis factor α
 WT, wild-type

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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 amino 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 to a value of 1 (black) corresponds the average quantity

per amino acid in a given sequencing cycle and would indicate no selectivity, while 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: Arg at P5, Gly from P5 to P3, Gln from P4 to P1, and Lys at P2 and P1. Heat maps were generated using MapleTree using quantified data available online as Supplementary Table S1. **(B)** Comparison of P1' selectivity of TACE and ADAM10. Selectivity values used to generate the heat map in panel **A** are depicted as a bar graph.

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 labeled with a fluorophores and quencher, allowing cleavage to be monitored by fluorescence increase as described under Experimental Procedures. 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 μ M) 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 panel B). Error bars reflect the standard deviation from at least three separate experiments.

Figure 3. Determinants of TACE and ADAM10 P1' specificity

(A) Structure of the TACE active site. Coordinates were taken from the X-ray crystal structure of 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 coordinates the active site zinc ion (dark gray). The seven amino-acid residues that comprise the S1' pocket are labeled and shown in magenta in stick representation. The figure was prepared using Pymol. (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 μ M substrate as for the data shown in Figure 2.

Figure 4. Active site contribution to protein substrate cleavage by TACE

(A) Time course for cleavage of TNF and its mutants by WT TACE (upper panel) and by TACE L401I/V402T (lower panel). Protein substrate was incubated with enzyme at 37 °C for the indicated times. Reactions were quenched and subjected to SDS-PAGE followed by immunoblotting with anti-His₆. (B) Cleavage rates of AP-fused substrates by WT TACE (black) or TACE L401I/V402T (grey) were measured after 60 min incubation at 37 °C. The amount of immobilized substrate released from FLAG beads was quantified using a colorimetric assay for AP. The results show a single representative of three independent experiments.

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.

Enzyme	Cleavage position							
	P5	P4	P3	P2	P1	P1'	P2'	P3'
TACE	P (1.7)	R (1.9)	A (1.6)	A (1.7)	A (1.5)	V (3.1)	K (1.6)	G (1.6)
		H (1.6)	V (1.5)	S (1.5)		L (2.9)	V (1.5)	S (1.6)
		T (1.4)	R (1.5)	R (1.5)		I (1.8)	I (1.4)	K (1.5)
ADAM10			M (1.4)	E (1.4)				
		T (2.2)	T (1.6)	S (1.6)	A (1.6)	L (3.6)	K (1.8)	G (2.1)
	P (2.1)	R (1.6)	A (1.5)	E (1.6)		Y (2.5)	R (1.6)	A (1.6)
	A (1.5)	H (1.5)	H (1.4)	A (1.6)		F (2.0)	V (1.5)	
						Q (1.9)	T (1.4)	

TACE and ADAM10 cleavage sites in protein substrates

Reported cleavage sites are shown for proteins established as either TACE or ADAM10 substrates through either genetic or knockdown approaches. In cases where proteins are cleaved at two distinct sites, both sites are listed. Abbreviations: EPR, epiregulin; TNFR, TNF receptor; IL-IRII, Type II interleukin-1 receptor; TR, transferrin receptor; GHR, growth hormone receptor; GP, glycoprotein; Ptpz, receptor-type protein tyrosine phosphatase Z; IL-6R, interleukin-6 receptor; HB-EGF, heparin binding EGF; APP, amyloid precursor protein; FasL, Fas ligand.

Cleavage position												
Protease	Substrate	Cleavage position										Reference
		P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'		
TACE	TNF	P	L	A	Q	A	-	V	R	S	S	[29]
	TGF- α	P	V	A	A	A	-	V	V	S	H	[7]
	TGF- α	A	D	L	L	A	-	V	V	A	A	[7]
	APR	V	R	V	E	Q	-	V	V	K	P	[28]
	APR	R	C	G	E	K	-	S	M	K	T	[28]
	EPR	P	R	V	A	Q	-	V	S	I	T	[7]
	EPR	E	H	F	F	L	-	T	V	H	Q	[7]
	L-selectin	Q	E	T	N	R	-	S	F	S	K	[44]
	p55 TNFR	P	Q	I	E	N	-	V	K	G	T	[29]
	p75 TNFR	P	P	A	E	G	-	S	T	G	D	[29]
	IL-1RII	K	E	A	S	S	-	T	F	S	W	[29]
	TR	T	E	C	E	R	-	L	A	G	T	[45]
	Collagen XVII	I	E	K	D	R	-	L	Q	G	M	[46]
	GHR	P	Q	M	S	P	-	F	T	C	E	[47]
Ebola GP	K	T	L	P	D	-	Q	G	D	N	[48]	
Ptpz	G	L	A	E	G	-	L	E	S	E	[49]	
Both	IL-6R	S	L	P	V	Q	-	D	S	S	S	[21]
	HB-EGF	G	L	T	L	P	-	V	E	N	P	[28]
	Notch	Y	K	I	E	A	-	V	K	S	E	[50,51]
	APP	V	H	H	Q	K	-	L	V	F	F	[52]
	Prion protein	T	N	M	K	H	-	M	A	G	A	[53]
	Prion protein	K	T	N	M	K	-	H	M	A	G	[53]
	Pmel17	V	V	S	T	Q	-	L	I	M	P	[54]
ADAM10	BTC	S	P	E	T	N	-	L	L	C	G	[7]
	BTC	V	D	L	F	Y	-	L	R	G	D	[7]
	EGF	H	Y	S	V	R	-	N	S	D	S	[7]
	EGF	W	W	E	L	R	-	H	A	G	H	[7]
	Dll-1	L	S	E	R	H	-	M	E	S	Q	[10]
	E-cadherin	R	K	A	Q	P	-	V	E	A	G	[55]
	N-cadherin	T	D	V	D	R	-	I	V	G	A	[56]
	CD23	A	E	Q	Q	R	-	L	K	S	Q	[57]
	CD23	G	D	Q	M	A	-	Q	K	S	Q	[57]
	FasL	H	T	A	S	S	-	L	E	K	Q	[58]
	FasL	S	S	L	E	K	-	Q	I	G	H	[58]

Table 3

Residues comprising the ADAM S1' pocket.

	Residue (TACE numbering)						
	348	401	402	405	434	439	440
Protease							
TACE							
ADAM10	Leu Leu	Leu Ile	Val Thr	His His	Val Ile	Ala Ala	Val Thr