Amino-terminal TACE prodomain attenuates TNFR2 cleavage independently of the cysteine switch

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TACE plays an important role in both lung development and the pathogenesis of pulmonary disease. TACE is expressed by a variety of cells in the lung, including alveolar macrophages, bronchial epithelial cells, and vascular smooth muscle cells. Lungs from embryonic TACE-deficient mice display impaired branching morphogenesis, inhibited epithelial cell proliferation and differentiation, and delayed vasculogenesis, thereby demonstrating a role for TACE in normal lung maturation (49). TACE also regulates mucin production by human airway epithelial cells. Activation of TACE by phorbol ester, Pseudomonas aeruginosa, or lipopolysaccharide catalyzes the cleavage of pro-TGF-α into soluble mature TGF-α, which then binds to and induces the phosphorylation of the epidermal growth factor receptor (EGFR), with resultant MUC5AC expression (39). Cigarette smoke, via a process that may involve oxygen free radicals, also activates TACE with resultant ligand-dependent EGFR phosphorylation and MUC5AC production (38). After activation, TACE undergoes stimulation-dependent internalization, which may downregulate catalytic activity at the plasma membrane (11). This may be relevant to the pathogenesis of community-acquired pneumonia as epithelial lining fluid cells from infected lungs have downregulated cell surface TACE expression compared with cells obtained from uninvolved lungs (16).

ADAM family zinc metalloproteases, including TACE, have a conserved structure that includes, from NH2 to COOH terminus, a signal sequence, prodomain, metalloprotease domain, disintegrin domain, cysteine-rich domain containing an EGF-like repeat, a transmembrane domain, and an intracytoplasmic tail (3, 23, 24, 35, 36). An important function of the prodomain is to retain the proenzyme in an inactive state. The formation of an intramolecular bond between a cysteine in the prodomain and a zinc atom in the catalytic site had been thought to mediate this inhibitory activity via a cysteine-switch mechanism. However, we recently reported that the amino terminus of the TACE prodomain might contribute to the ability of the prodomain to maintain TACE in an inactive state independently of a cysteine-switch mechanism. We synthesized a 37-amino acid peptide corresponding to TACE amino acids 18–54 (N-TACE18–54) and assessed whether it possessed TACE inhibitory activity. In an in vitro model assay system, N-TACE18–54 attenuated TACE-catalyzed cleavage of a TNFR2:Fc substrate. Furthermore, N-TACE18–54 inhibited constitutive TNFR2 shedding from a human monocytic cell line by 42%. A 19-amino acid, leucine-rich domain, corresponding to TACE amino acids 30–48, demonstrated partial inhibitory activity. In summary, we have identified a subdomain within the amino terminus of the TACE prodomain that attenuates TACE catalytic activity independently of a cysteine-switch mechanism, which provides new insight into the regulation of TACE enzymatic activity.

Tumor necrosis factor-α-converting enzyme (TACE) or ADAM17 (a disintegrin and metalloprotease-17), a member of the disintegrin and metalloprotease family of zinc metalloproteases, is an important regulator of inflammation, immune regulation, and cellular proliferation as a consequence of its ability to process cell surface integral membrane proteins to soluble forms (2, 4, 24). TACE was originally identified as the enzyme that cleaves the membrane-bound precursor of tumor necrosis factor-α (TNF-α), as well as the type II 75-kDa TNF receptor (TNFR2, TNFRSF1B, CD120b), transforming growth factor (TGF)-α, and L-selectin (3, 23, 24, 29). Other cell surface proteins that have been identified as substrates for TACE include cytokines, chemokines, growth factors, adhesion molecules, and cytokine and growth factor receptors, as well as the cellular prion protein and the amyloid precursor protein (1, 6–8, 14, 17, 20, 29, 31–33, 37, 42, 43, 47, 48). TACE is synthesized as a latent proenzyme that is retained in an inactive state via an interaction between its prodomain and catalytic domain. Although the formation of an intramolecular bond between a cysteine in the prodomain and a zinc atom in the catalytic site had been thought to mediate this inhibitory activity, it was recently reported that the cysteine-switch motif is not required. Here, we hypothesized that the amino terminus of the TACE prodomain might contribute to the ability of the prodomain to maintain TACE in an inactive state independently of a cysteine-switch mechanism. We synthesized a 37-amino acid peptide corresponding to TACE amino acids 18–54 (N-TACE18–54) and assessed whether it possessed TACE inhibitory activity. In an in vitro model assay system, N-TACE18–54 attenuated TACE-catalyzed cleavage of a TNFR2:Fc substrate. Furthermore, N-TACE18–54 inhibited constitutive TNFR2 shedding from a human monocytic cell line by 42%. A 19-amino acid, leucine-rich domain, corresponding to TACE amino acids 30–48, demonstrated partial inhibitory activity. In summary, we have identified a subdomain within the amino terminus of the TACE prodomain that attenuates TACE catalytic activity independently of a cysteine-switch mechanism, which provides new insight into the regulation of TACE enzymatic activity.
other ADAM family members, cleavage of the TACE prodomain typically occurs COOH-terminal to a consensus proprotein-convertase sequence [RX(K/R)R] (12). Removal of the TACE prodomain is catalyzed by furin and other proprotein-convertases, such as PC7, in the late Golgi compartment (5, 12, 28, 35).

In the present study, we hypothesized that the amino-terminal region of the TACE prodomain might contribute to the ability of the TACE prodomain to maintain TACE in an inactive state independently of a cysteine-switch mechanism (15). We synthesized a 37-amino acid peptide that corresponds to TACE amino acids 18–54 (N-TACE18–54) but does not contain the consensus cysteine-switch motif (PKVCGY186) (21). N-TACE18–54 attenuated TACE-catalyzed TNFR2 cleavage in a model assay system and constitutive TNFR2 shedding from the human U-937 monocytic cell line. A 19-amino acid, leucine-rich domain, which corresponds to N-TACE amino acids 30–48, possessed partial TACE inhibitory activity. Therefore, we propose that a subdomain within the amino terminus of the TACE prodomain attenuates TACE catalytic activity independently of the cysteine-switch mechanism. This study provides new insight into the ability of the TACE prodomain to regulate TACE enzymatic activity.

METHODS

Characterization of N-TACE18–54 inhibitory activity. A model assay system was developed to assess the ability of N-TACE18–54 to attenuate TACE enzymatic activity. Recombinant human TACE (rhTACE), as well as the recombinant human 55-kDa type I TNF receptor and TNFR2 fusion proteins (rhTNFR1:Fc and rhTNFR2:Fc, respectively) were purchased from R&D Systems (Minneapolis, MN). Both TNFR1:Fc and TNFR2:Fc are recombinant human chimeric proteins that encode the extracellular receptor domain, fused to a carboxy-terminal 6×-histidine-tagged Fc region of human IgG1 via a linker peptide (IEGRMD). rhTACE corresponds to the mature form after removal of the prodomain and has an apparent molecular size of 70 kDa. N-TACE18–54 was synthesized by Sigma-Genosys (The Woodlands, TX). N-TACE18–54 truncation mutants were also synthesized: an amino-terminal mutant corresponding to N-TACE amino acids 18–29, a middomain mutant corresponding to N-TACE amino acids 30–42, a carboxy-terminal mutant corresponding to amino acids 43–54, and an extended middomain mutant corresponding to N-TACE amino acids 30–48 (N-TACE30–48). A scrambled peptide containing the N-TACE18–54 amino acids in a random order was also synthesized by Sigma-Genosys. Chou-Fasman analysis was performed using MacVector (Accelrys, Burlington, MA). Vasoactive intestinal peptide (VIP) and α-defensin were purchased from Bachem (Torrance, CA). TNF-α proteinase inhibitor (TAPl-2) was purchased from Peptides International (Louisville, KY). 1,10-Phenanthroline monohydrate and zinc chloride (ZnCl2) were purchased from Sigma-Aldrich (St. Louis, MO).

Assays (50 μl) were performed in 50 mM Tris-HCl and 25 mM NaCl, pH 8.0, and incubated at 30°C for 30 min. Proteins were separated by SDS-PAGE using 4–12% Bis-Tris NUPAGE gels (Invitrogen, Carlsbad, CA) and visualized with the SilverQuest Silver Staining kit (Invitrogen). For Western blot analysis, proteins were separated via SDS-PAGE, electrophoresed onto nitrocellulose membranes, and incubated overnight (4°C) with a murine IgG1 monoclonal antibody (200 ng/ml) directed against the 6×-histidine tag (TetraHis; Qiagen, Valencia, CA), which reacts with the COOH terminus of the rhTNFR2:Fc fusion protein. A rabbit polyclonal antibody generated against N-TACE amino acids 18–54 (Sigma-Genosys) was utilized for Western blotting at a 1:1,000 dilution. Detection was by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies.

Quantification of TNFR2 shedding. U-937 cells purchased from ATCC (Manassas, VA) were maintained in RPMI 1640 medium with 10% fetal bovine serum. U-937 cells were plated in six-well plates at a density of 2 × 105 cells/well in 1 mL of media. Release of TNFR2 into U-937 cell culture medium during a 24-h period was quantified by a sandwich ELISA (R&D Systems). Cellular apoptosis and necrosis were measured using the TACS Annexin V-FITC Apoptosis Detection kit (R&D Systems) and an XL-MCL flow cytometer (Beckman-Coulter, Miami, FL). Statistical analysis was performed by a paired Student’s t-test with a Bonferroni correction for multiple comparisons and by single-factor ANOVA. Differences were considered significant at a P value ≤ 0.05.

RESULTS

N-TACE18–54 attenuates TACE-mediated TNFR2 cleavage. Experiments were conducted to assess whether the amino terminus of the TACE prodomain can regulate TACE catalytic activity independently of the cysteine-switch mechanism. A peptide corresponding to amino acids 18–54 of the TACE coding sequence (N-TACE18–54), which lacks the hydrophobic signal peptide sequence and the cysteine-switch consensus motif, was synthesized (Fig. 1). The N-TACE18–54 amino acid sequence was deduced from amino acid residues 18–54 of the TACE protein. The synthesized N-TACE18–54 peptide corresponds to TACE amino acids 18–54. The signal peptide (underlined), encoded by TACE amino acids 1–17, is not included in the N-TACE18–54 sequence. The 19-amino acid, leucine-rich inhibitory domain is denoted by the double underline.

Fig. 1. Characterization of a 37-amino acid peptide that corresponds to TNF-α-converting enzyme amino acids 18–54 (N-TACE18–54). A: TACE protein structure. The TACE prodomain comprises TACE amino acids 18–214 and encodes a consensus cysteine-switch motif. B: N-TACE18–54 amino acid sequence. The synthesized N-TACE18–54 peptide corresponds to TACE amino acids 18–54.
The inhibitory activity of N-TACE\textsuperscript{18–54} was assessed in an in vitro assay system utilizing rhTACE and TNF chimeric receptor fusion proteins as model substrates. Because rhTNFR1:Fc and rhTNFR2:Fc each contain the entire extracellular domain of the receptor, we reasoned that they might be susceptible to cleavage by rhTACE. As shown in Fig. 2A, rhTACE cleaved the rhTNFR2:Fc model substrate, generating two predominant cleavage products, which were detected by silver staining. Furthermore, the TACE-catalyzed cleavage of the TNFR2:Fc model substrate was attenuated by 80 \( \mu \)M N-TACE\textsuperscript{18–54}. In contrast, rhTACE did not cleave the rhTNFR1:Fc model substrate. Because both rhTNFR1:Fc and rhTNFR2:Fc encode the same linker and Fc region of IgG1, we conclude that rhTACE cleaves TNFR2, but not TNFR1 or the IgG1 chimera. Therefore, in subsequent experiments, rhTNFR2:Fc was used as a substrate to assess the ability of N-TACE\textsuperscript{18–54} to attenuate rhTACE activity. The ability of rhTACE to cleave the rhTNFR2:Fc model substrate was also shown to be zinc dependent, which is consistent with the classification of TACE as a member of the ADAM family of zinc metalloproteases. As shown in Fig. 2B, incubation with the predominantly zinc-specific chelator 1,10-phenanthroline significantly attenuated rhTACE-mediated TNFR2:Fc cleavage, which was partially restored by the addition of 25–100 \( \mu \)M ZnCl\(_2\). As has been described for other zinc metalloproteases, a further increase in ZnCl\(_2\) concentration resulted in a decline in enzyme activity (10).

As shown by SDS-PAGE and silver staining (Fig. 3A), N-TACE\textsuperscript{18–54} attenuated the proteolytic cleavage of 0.95 \( \mu \)M rhTNFR2:Fc by 0.5 \( \mu \)M rhTACE in a concentration-dependent fashion between 20 and 160 \( \mu \)M. The identity of rhTNFR2:Fc and its cleavage products was confirmed by immunoblotting utilizing an anti-\( \delta \)-X-histidine antibody, which reacts with the COOH-terminal \( \delta \)-X-histidine tag of the rhTNFR2:Fc chimeric protein (Fig. 3B). Together, these experiments demonstrate that N-TACE\textsuperscript{18–54} can attenuate TACE proteolytic activity toward TNFR2.

We next performed experiments utilizing irrelevant peptides, \( \alpha \)-defensin and VIP, to assess the specificity of N-TACE\textsuperscript{18–54} attenuation of TACE-catalyzed TNFR2 cleavage. As shown in Fig. 4, neither \( \alpha \)-defensin nor VIP affected the ability of TACE to proteolytically cleave rhTNFR2:Fc. In contrast, TACE-catalyzed rhTNFR2 cleavage was partially inhibited by 80 \( \mu \)M N-TACE\textsuperscript{18–54} and completely inhibited by 25 \( \mu \)M TAPI-2, a hydroxamic acid-based zinc metalloprotease inhibitor. These experiments are consistent with the conclusion that the inhibitory activity of N-TACE\textsuperscript{18–54} is not a nonspecific peptide effect. We also assessed whether N-TACE\textsuperscript{18–54} is a substrate for TACE catalytic activity. There was no decrease in the quantity of N-TACE\textsuperscript{18–54} by immunoblotting after incubation with rhTACE for 4 h (data not shown), suggesting that N-TACE\textsuperscript{18–54} is not a substrate for TACE.

Characterization of N-TACE\textsuperscript{18–54} inhibitory activity. Experiments were next performed to characterize the N-TACE domains that mediate its inhibitory activity. Truncation mutants were synthesized corresponding to the amino-terminal, middle, and carboxy-terminal domains of N-TACE, not including the signal peptide. The amino-terminal mutant corresponded to TACE amino acids 18–29, the middomain mutant corresponded to TACE amino acids 30–42, and the carboxy-terminal mutant corresponded to amino acids 43–54. As shown in Fig. 5, none of these truncation mutants (80 \( \mu \)M) attenuated the ability of TACE to proteolytically cleave rhTNFR2:Fc. This demonstrates that these N-TACE\textsuperscript{18–54} truncation mutants do not possess TACE inhibitory activity. Furthermore, since these truncation mutants were synthesized in a fashion identical to N-TACE\textsuperscript{18–54}, this experiment also demonstrates that the ability of N-TACE\textsuperscript{18–54} to function as a TACE inhibitor is not an artifact related to its synthesis and/or purification.

To characterize further the structural requirements for inhibitory activity, another truncation mutant corresponding to N-TACE amino acids 30–48 (N-TACE\textsuperscript{30–48}) was synthesized. N-TACE\textsuperscript{30–48} is predicted to have a helical structure by Chou-Fasman analysis and is leucine rich, which may be important for its ability to attenuate TACE activity. As shown in Fig. 6, both N-TACE\textsuperscript{18–54} and N-TACE\textsuperscript{30–48} attenuated the TACE-catalyzed proteolytic cleavage of rhTNFR2:Fc. The inhibitory activity of N-TACE\textsuperscript{30–48}, however, was less than that of N-TACE\textsuperscript{18–54}. These experiments demonstrate that the domain...
corresponding to amino acids 30–48 of N-TACE partially mediates the TACE inhibitory activity of N-TACE18–54.

**N-TACE**<sup>18–54</sup> attenuates constitutive TNFR2 shedding from U-937 cells. We next assessed whether N-TACE<sup>18–54</sup> was capable of attenuating TNFR2 shedding in a cell-based system. The U-937 monocytic cell line was incubated with either N-TACE<sup>18–54</sup> (0.04–40 μM) or the amino-terminal mutant, corresponding to N-TACE amino acids 18–29 (40 μM), for 24 h. As shown in Fig. 3, the quantity of sTNFR2 present in medium from cells treated with N-TACE<sup>18–54</sup> was significantly reduced in a concentration-dependent fashion. Furthermore, 40 μM N-TACE<sup>18–54</sup> significantly attenuated TNFR2 shedding by 42% compared with cells treated with media alone (107.3 ± 3.3 vs. 184.6 ± 2.2 pg/ml, n = 6, P ≤ 10<sup>−8</sup>). In contrast, the amino-terminal mutant had no effect on TNFR2 shedding compared with cells treated with medium alone (186.5 ± 1.8 vs. 184.6 ± 2.2 pg/ml, n = 6, P = not significant). The ability of N-TACE<sup>18–54</sup> to decrease TNFR2 shedding was not a consequence of either apoptosis or necrosis, as assessed by annexin V binding and propidium iodide uptake (data not shown). Additional experiments were performed utilizing a scrambled 37-amino acid peptide that contained the N-TACE<sup>18–54</sup> amino acids in a random order to confirm that the inhibition of TNFR2 shedding by N-TACE<sup>18–54</sup> is dependent upon its amino acid sequence. The scrambled peptide did not attenuate TNFR2 shedding but instead was associated with a 4% increase in constitutive
TNFR2 shedding compared with cells treated with medium alone (115.7 ± 0.6 vs. 111.4 ± 1.7 pg/ml, n = 6, P = 0.038). These data demonstrate that N-TACE18–54 significantly attenuates constitutive TNFR2 shedding from U-937 cells.

Experiments were also performed to assess whether N-TACE30–48 inhibits TNFR2 shedding from U-937 cells. Treatment with 40 μM N-TACE30–48 inhibited TNFR2 shedding by 16% compared with cells treated with media alone (160.8 ± 3.4 vs. 190.4 ± 2.9 pg/ml, n = 6, P ≤ 10⁻⁴). This suggests that although N-TACE30–48 partially attenuates TNFR2 shedding, N-TACE amino acids 18–54 are required for a maximal effect in this cell-based system.

**DISCUSSION**

TACE can regulate inflammatory responses via the proteolytic cleavage and shedding of TNFR2 to function as a soluble TNF binding protein (29). The important role that soluble sTNFR2 plays in regulating TNF bioactivity is exemplified by virally encoded soluble TNF binding proteins that function as highly effective modulators of innate immune responses (9). For example, both the Shope fibroma and myxoma viruses express T2 proteins, which are structurally similar to TNFR2 and are secreted by infected cells to subvert TNF-dependent host defenses (40, 46). Similarly, a soluble human TNFR2-Ig fusion protein is utilized clinically to attenuate TNF bioactivity and disease severity in patients with inflammatory arthritides and psoriasis (26). Furthermore, sTNFR2 may modulate pulmonary inflammatory responses in the acute respiratory distress syndrome, asthma, sarcoidosis, bacterial pneumonia, and tuberculosis (18, 25, 27, 30, 44, 45).

Regulation of TACE enzymatic activity is important to prevent excessive or unanticipated cleavage of target proteins. TACE is synthesized as a latent proenzyme and is retained in an inactive state via an interaction between its prodomain and catalytic domain. Although this interaction was thought to be mediated via a cysteine-switch mechanism, it has recently been shown that the prodomain cysteine-switch motif is not required for this inhibitory activity (15). This is based upon the finding that a TACE prodomain variant containing a cysteine-to-alanine substitution at position 184 showed the same inhibitory activity toward a recombinant TACE catalytic-domain construct as the wild-type TACE prodomain (15).

Here we report that the amino terminus of the TACE prodomain also possesses TACE inhibitory activity that is independent of the cysteine-switch mechanism. We synthesized a 37-amino acid peptide that corresponded to the amino terminus of the TACE prodomain but did not include the consensus cysteine-switch motif. This peptide, termed N-TACE18–54, comprises amino acids 18–54 of the TACE protein and was demonstrated to attenuate TACE-catalyzed cleavage of TNFR2 in vitro. This inhibitory activity appeared
to be specific, as neither truncation mutants corresponding to the amino-terminal, middle, and carboxy-terminal domains of N-TACE nor irrelevant small proteins (VIP and α-defensin) possessed TACE inhibitory activity. N-TACE\(^{18-54}\) also attenuated by 42% constitutive TNFR2 shedding from the U-937 mononcytic cell line, which suggests that N-TACE may partially attenuate the activity of native, cell-associated TACE. This is consistent with a role for TACE in constitutive TNFR2 shedding, as was described in HEK293 cells expressing a dominant negative TACE (41). Neither the amino-terminal truncation mutant nor a scrambled peptide inhibited constitutive U-937 cell TNFR2 shedding, which suggests that N-TACE\(^{18-54}\) mediates this inhibitory activity in a sequence-specific fashion. Our findings, however, do not establish whether the ability of N-TACE\(^{18-54}\) to attenuate constitutive TNFR2 shedding in intact cells is specific for TACE alone, as N-TACE\(^{18-54}\) could conceivably inhibit other enzymes that also function as TNFR2 shedases. We propose that the amino-terminal region of the TACE prodomain can attenuate TACE catalytic activity independently of the cysteine-switch mechanism.

Interestingly, the TACE disintegrin/cysteine-rich domain has been reported to diminish the inhibitory potency of the prodomain for the catalytic domain (15). Although the full-length TACE prodomain was a potent inhibitor of a recombinant TACE catalytic domain construct (IC\(_{50} = 70\) nM), its inhibitory activity was significantly less against a construct that contained both the TACE catalytic and disintegrin/cysteine-rich domains (IC\(_{50} > 2\) μM) (15). Furthermore, the disintegrin/cysteine-rich domain appeared to decrease the ability of the prodomain to stably bind the catalytic domain (15). Thus it is possible that in our study, the disintegrin/cysteine-rich domain impaired the ability of N-TACE\(^{18-54}\) to inhibit TACE catalytic activity, since rhTACE corresponds to the mature TACE ectodomain. Furthermore, this may in part explain why micromolar concentrations of N-TACE\(^{18-54}\) were required to inhibit rhTACE-mediated TNFR2:Fc cleavage, as well as constitutive TNFR2 shedding from U-937 cells.

TNFR1 has been reported to represent a substrate for TACE based upon the demonstration of increased TNFR1 shedding following reconstitution of TACE-deficient cell lines (31). In our model system, rhTNFR2, but not rhTNFR1, served as a substrate for rhTACE enzymatic activity. Similarly, TACE has been reported to have no detectable activity against a TNFR1 model peptide substrate corresponding to the known TNFR1 cleavage site (22). The inability of rhTACE to cleave rhTNFR1:Fc raises the question as to whether TNFR1 serves as a substrate for TACE in vivo or, alternatively, whether there is a requirement for either or both proteins to be membrane anchored or whether additional regulatory proteins are required.

In conclusion, we have identified that a subdomain within the amino terminus of the TACE prodomain attenuates TACE catalytic activity toward TNFR2. We propose that the ability of N-TACE\(^{18-54}\) to inhibit TACE activity in vitro, as well as constitutive TNFR2 shedding in a cell-based system, provides a new insight into the mechanism by which the activity of a disintegrin metalloprotease might be attenuated by its prodomain independently of a cysteine-switch mechanism.

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