The transmembrane domain of TACE regulates protein ectodomain shedding

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Numerous membrane proteins are cleaved by tumor necrosis factor- α converting enzyme (TACE), which causes the release of their ectodomains. An ADAM (a disintegrin and metalloprotease domain) family member, TACE contains several noncatalytic domains whose roles in ectodomain shedding have yet to be fully resolved. Here, we have explored the function of the transmembrane domain (TM) of TACE by coupling molecular engineering and functional analysis. A TM-free TACE construct that is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI)-binding polypeptide failed to restore shedding of transforming growth factor- α (TGF- α), tumor necrosis factor- α (TNF- α) and L-selectin in cells lacking endogenous TACE activity. Substitution of the TACE TM with that of the prolactin receptor or platelet-derived growth factor receptor (PDGFR) also resulted in severe loss of TGF- α shedding, but had no effects on the cleavage of TNF- α and L-selectin. Replacement of the TM in TGF- α with that of L-selectin enabled TGF- α shedding by the TACE mutants carrying the TM of prolactin receptor and PDGFR. Taken together, our observations suggest that anchorage of TACE to the lipid bilayer through a TM is required for efficient cleavage of a broad spectrum of substrates, and that the amino-acid sequence of TACE TM may play a role in regulatory specificity among TACE substrates.

Keywords: transmembrane domain, TACE/ADAM17, ectodomain shedding, transforming growth factor- α , tumor necrosis factor- α , L-selectin

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Introduction

Since its identification about 10 years ago as the protease that produces the circulating cytokine tumor necrosis factor (TNF)- α from its transmembrane precursor [1, 2], TNF- α converting enzyme (TACE), which is also known as ADAM (a disintegrin and metalloprotease domain)17, has emerged as a major physiological shed-dase that cleaves the ectodomains of some 50 transmembrane proteins. Substrates of TACE can be either type I or type II membrane proteins. They belong to families of proteins with diverse structures and functions ranging from precursors of mitogenic growth factors to those of proinflammatory/proapoptotic cytokines, and from cell adhesins to viral receptors and ectoenzymes [3, 4]. The

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Abbreviations: transforming growth factor- α (TGF- α); tumor necrosis factor- α (TNF- α); TNF- α converting enzyme (TACE); a disintegrin and metalloprotease domain (ADAM); cysteine-rich/disintegrin domain (CRD); transmembrane domain (TM); glycosylphosphatidylinositol (GPI); platelet-derived growth factor receptor (PDGFR); Concanavalin A (Con A); ethylenediamine tetraacetic acid (EDTA); phosphate-buffered saline (PBS); PBS supplemented with 5 mM EDTA, 10 mM 1,10-phenanthroline, 2% bovine serum albumin and 0.1% NaN₃ (PEB); phycoerythrin (PE); enhanced green fluorescence protein (GFP); the hemagglutinin epitope (HA); fluorescence resonance energy transfer (FRET); cytoplasmic domain-truncated human CD4 (hCD4); methyl- β cyclodextrin (M β CD)

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biological activities of these molecules are greatly influenced by TACE-mediated ectodomain shedding. Thus, as a result of cleavage by TACE, transforming growth factor (TGF- α), a paracrine mitogen, is converted into a freely diffusible growth factor that is able to activate the epidermal growth factor receptor in non-adjacent cells. Elevated levels of soluble autocrine TGF- α are critical for cancer development [5]. Likewise, free TNF- α released by TACE plays an important role in the clearance of intracellular pathogens, and in the pathogenesis of cachexia and a broad range of inflammatory diseases including arthritis, colitis and heart failure, while transmembrane TNF- α has an insignificant role in these processes [6]. In addition, TACE-mediated ectodomain cleavage of L-selectin, a leukocyte surface marker that binds vascular endothelial cells, affects recruitment of circulating leukocytes to sites of inflammation [7].

Because of the medical importance of the reactions catalyzed by TACE, the mechanism underlying the regulation of TACE activity has been a focus of many investigations. On the one hand, studies have found that phorbol 12-myristate 13-acetate is a general shedding inducer for all TACE substrates. In addition, growth factors and ionophores are efficient shedding stimuli for all substrates examined [8, 9]. On the other hand, shedding by TACE in response to a variety of stimuli is strongly inhibited when Erk and/or p38 MAP kinase-mediated signaling pathways are blocked [10, 11]. These suggest that diverse membrane proteins share regulatory mechanisms that control their cleavage by TACE. An important question remains as to whether and how shedding of distinct substrates can be specifically regulated.

The primary sequence of the biosynthesized TACE polypeptide consists of an amino-terminal prodomain, a catalytic metalloprotease domain, a cysteine-rich/disintegrin domain (CRD), a transmembrane domain (TM) and a cytoplasmic domain [1, 2], as illustrated in Figure 1. The prodomain binds to the catalytic center of the metalloprotease domain and thus keeps the protein in the inactive zymogen state until the prodomain is removed by a proprotein convertase in a late-Golgi compartment [12]. Studies using insect cells as an expression system suggest that the prodomain also functions as an intramolecular chaperone along the secretory pathway [13]. The metalloprotease domain of TACE cannot be replaced functionally by the catalytic domain of ADAM10 despite a high degree of sequence homology between the two ADAM family members [14].

The cytoplasmic domain of TACE was thought to be involved in the regulation of ectodomain cleavage in response to intracellular signaling events such as receptor protein tyrosine kinase and MAP kinase activation [10,

Figure 1 Schematic drawing of the domain structures of zymogen (upper, amino acids 18-827) and mature TACE (lower, amino acids 215-827). Pro, prodomain; Cat, catalytic domain; CRD, cysteine-rich disintegrin domain; TM, transmembrane domain; and CT, cytoplasmic domain. The open circle in the catalytic domain signifies the active site.

11]. Interestingly, the cytoplasmic domain is phosphorylated in response to shedding activators [9, 15]. Nevertheless, TACE constructs lacking the entire cytoplasmic domain are fully functional in shedding [9, 14, 16]. Therefore, the precise role for the cytoplasmic domain in ectodomain shedding remains to be defined.

The CRD in TACE likely plays a regulatory role in ectodomain shedding. Accordingly, a single point mutation in this domain [16] was found to be responsible for the inactivation of ectodomain shedding of numerous membrane proteins in a mutant cell line [17]. Furthermore, in the absence of the CRD, cleavage of synthetic substrates by the catalytic domain of TACE is more effectively inhibited by its prodomain [13, 18] and the tissue inhibitor of metalloprotease-3 (TIMP-3) [19]. Finally, a study has suggested that the CRD may be involved in substrate recognition [14].

The TM is the only domain in TACE whose function has not been specifically examined. However, TMs frequently encode information required for the functions of many other proteins (e.g. [20-23]). Interestingly, secreted soluble TACE constructs made by deletion of the TM (and the cytoplasmic domain) have been found to be defective in shedding in cell-based functional assays ([24], and Fan, unpublished data). Nevertheless, previous research has not addressed whether the TM simply serves to retain the protease on the plasma membrane where the substrates are located or it has an additional role(s) in the regulation of ectodomain shedding. Here we demonstrate that a plasma membrane anchorage signal, a glycosylphosphatidylinositol (GPI)-binding sequence, could not functionally replace the TACE TM in the cleavage of transmembrane TGF- α , TNF- α and L-selectin. Substitution of the TM with that of the prolactin receptor or platelet-derived growth factor receptor (PDGFR) also strongly inhibited TGF- α shedding, but had little effect

on TNF- α and L-selectin ectodomain cleavage. Furthermore, the native TACE TM sequence was no longer required for ectodomain cleavage of TGF- α after the substrate's TM was replaced with the TM of L-selectin. Thus, it appears that TM-mediated plasma membrane anchorage is of general importance for the shedding of a variety of substrates and that the amino-acid sequence of TACE TM encodes information for regulatory specificity among them.

Materials and Methods

Reagents

Dulbecco-modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS), methyl- β -cyclodextrin (M β CD) and the monoclonal anti-Flag M2 antibody were purchased from Sigma-Aldrich (St Louis, MO). Concanavalin A (Con A)-conjugated Sepharose and the ECL kit were purchased from Amersham (Piscataway, NJ). The monoclonal anti-Myc epitope 9E10 antibody was purchased from Covance (Princeton, NJ). The monoclonal anti-L-selectin DREG56 antibody was kindly provided by Dr TK Kishimoto (Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT) [25]. Phycoerythrin (PE)-conjugated DREG56 was obtained from Biolegend (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human CD4 (hCD4) antibody was a generous gift from Dr Y Ron (Robert Wood Johnson Medical School) [26]. Alexa Fluor 488-conjugated anti-M2 and Alexa 555-conjugated anti-hemagglutinin epitope (HA) (clone 12CA5) were prepared using the Alexa Fluor 488 and 555 Monoclonal Antibody Labeling Kits (Invitrogen, Carlsbad, CA).

Expression vectors

A total of 34 TACE expression vectors carrying different membrane anchors were used in this study. All TACE vectors except extracellular Flag-tagged wild-type TACE (eFlag-WT TACE) were constructed on the basis of the Δ C construct, which contained the native ectodomain (Met1-Asn671), the TM (Ile672-Val694), and the first three amino acids (Asp695-Lys697), but not the rest of the cytoplasmic domain of murine TACE (Figure 2A). The T-GPI construct contained the ectodomain of TACE and the last 43 amino acids of human secreted placental alkaline phosphatase (Tyr493-Pro535) (Figure 2A). The carboxyl-terminal fragment of the phosphatase is a GPI anchor [27]. The T-PLR construct contained the ectodomain of TACE, the TM of rabbit prolactin receptor (Thr230-Lys253) and the three juxtamembrane amino acids of the TACE cytoplasmic domain (Figure 2A). The T-PDGFR construct contained the ectodomain of TACE, the TM of human PDGFR β (Lys531-Gln557) and the three juxtamembrane amino acids of the TACE cytoplasmic domain (Figure 2A). The PD-TA construct contained the ectodomain of TACE, the first half of the PDGFR β TM (Lys531 through Val543), the second half of TACE TM (Trp684-Val694) and the three juxtamembrane amino acids of the TACE cytoplasmic domain (Figure 2A). The TA-PD construct contained the ectodomain of TACE, the first half of TACE TM (Ile672-Phe683), the second half of the PDGFR β TM (Leu544-Gln557) and the three juxtamembrane amino acids of the TACE cytoplasmic domain (Figure 2A). For scanning mutagenesis, 22 of

the 23 TM amino acids were individually mutated to tryptophan, while Trp684 was replaced with Ala. All TACE constructs were carboxyl-terminally tagged with a c-Myc epitope to facilitate detection. In addition, an eFlag-tagged version of T-GPI, T-PLR and T-PDGFR was constructed. Similar to eFlag-WT TACE, in eFlag-tagged TM mutants, the epitope was placed near the N-terminus of the catalytic domain (between Ala 216 and Asp217) [16].

The expression vectors for human transmembrane TGF- α [28] and Flag-tagged transmembrane TNF- α [16] have been described previously. The mammalian expression vector for transmembrane L-selectin was kindly provided by Dr TK Kishimoto [25]. Exchange of the TMs between transmembrane TGF- α (Ile100-His122) and L-selectin (Pro333-Leu354) resulted in the chimeric TGF-LS-TGF and LS-TGF-LS substrates (Figure 2B). The expression vector for HA-tagged TGF- α was kindly provided by Dr DC Lee (University of North Carolina) [29]. The expression vector for cytoplasmic domain-truncated human CD4 (hCD4) was generously supplied by Dr R Medzhitov (Yale University) [30]. The expression vector for the proprotein convertase furin, pcDNA3-furin, was kindly provided by Dr AJ Klein-Szanto (Fox Chase Cancer Center) [31].

For constructing TACE and substrate variants, cDNA fragments containing desired mutations were generated by polymerase chain reactions using the high fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA), digested with *Bam*HI and *Sal*I, and cloned into the RK5 plasmid, in which the expression of a gene of interest is driven by a cytomegalovirus promoter. Sequence authenticity of the inserts in the final expression vectors was verified by automated DNA sequencing performed by the Robert Wood Johnson Medical School DNA Core Facility.

Cell lines and culture conditions

The shedding-defective M1 cell line [32] was used to stably express human transmembrane TGF- α , L-selectin, TNF- α , TGF-LS-TGF and LS-TGF-LS (Figure 2B). M1 cells were transfected with expression vectors for the TACE substrates, and selected with puromycin [16, 33]. Expression clones were identified by immunostaining and Western blotting. The resulting M1-TGF- α , M1-TNF- α , M1-L-selectin, M1-TGF-LS-TGF and M1-LS-TGF-LS cell lines as well as parental M1 cells were maintained in DMEM, which was supplemented with 8% FBS and the antibiotics penicillin and streptomycin.

Assays of TGF- α , TNF- α and L-selectin ectodomain shedding

Two-color flow cytometry was used to assess TGF- α , TNF- α and L-selectin ectodomain shedding [33]. M1-TGF- α , M1-TNF- α , M1-L-selectin, M1-TGF-LS-TGF and M1-LS-TGF-LS cells were transiently cotransfected with a TACE expression vector (or the control RK5 vector) and a mammalian expression plasmid for enhanced green fluorescence protein (GFP) at a ratio of 3:1. At 20h after transfection, cell culture plates were placed on ice and the medium was replaced with cold phosphate-buffered saline (PBS), supplemented with 10 mM 1,10-phenanthroline (a metalloprotease inhibitor), 5 mM ethylenediamine tetraacetic acid (EDTA), 2% bovine serum albumin and 0.1% NaN₃ (PEB). Cells were scraped off the plastic with a Cell Lifter (Corning Inc., Corning, NY), collected and centrifuged at 1 000 rpm at 4 °C for 5 min. Following the removal of the supernatant, cells were resuspended in 50 μ l PEB

Figure 2 Schematic presentation of selected TACE constructs (A) and chimeric substrates (B) used in this study with different lipid membrane anchors. Amino-acid sequences of membrane anchors (either the GPI-binding signal or TMs) in TACE (A) and substrates (B) are shown. The membrane-proximal juxtamembrane sequences downstream of the TACE cleavage sites in wild-type transmembrane TGF-α and L-selectin (B) are also shown.

containing 200 ng anti-TGF- α or 250 ng anti-Flag or 500 ng anti-L-selectin antibody, incubated on ice for 30 min with gentle agitation, washed twice with PEB and reacted with 50 μ l PE-conjugated goat anti-mouse IgG (whole molecule, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in PEB (1:200) for 30 min. After another two washes with PEB, cells were fixed in 500 μ l of 1% paraformaldehyde (prepared in PEB) and were immediately analyzed by flow cytometry using a Coulter Epics XL.MCL flow cytometer (Beckman Coulter, Miami, FL). GFP and PE signals were simultaneously detected through the FL1 and FL2 channels, respectively. For each analysis, unstained stable cells (M1-TGF- α or M1-TNF- α or M1-L-selectin) transfected with GFP and the control RK5 vector without an insert, and immunostained stable cells transfected with RK5 only (i.e. no GFP), were used to set up the instrument in order to obtain optimal GFP-PE signal compensation. Mock-stained parental M1 cells were used to verify the final compensatory conditions under which ~98% of the cells were recognized as GFP- and PE-doubly negative cells.

In experiments determining whether M β CD influences the shedding activity of T-GPI, M1-L-selectin cells were cotransfected with T-GPI and an hCD4 expression vector as a transfection marker (in place of GFP). At 24 h after transfection, cells were switched into a medium containing 1% M β CD or fresh DMEM. After 1 h incubation at 37 °C, cells were stained with the FITC-conjugated anti-hCD4 and PE-conjugated DREG56 antibodies. Flow cytometry was performed as described above.

TACE Western blotting

For detecting the expression of Myc-tagged TACE constructs, M1 cells grown on 6-well plates were transiently transfected with indicated TACE expression plasmids. Twenty hours after transfection, cells were lysed with ice-cold Tris-buffered saline (pH 7.5) containing 1% NP-40, 5 mM EDTA, 10 mM 1,10-phenanthroline and the “Complete” protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). After 15 min centrifugation at 14 000 rpm in an Eppendorf benchtop minifuge with a 30-place rotor, supernatant was collected. The protein concentration of the supernatant was determined using a BCA protein assay kit (Pearce, Rockford, IL). Glycosylated proteins were precipitated from a 600 μ g total cellular protein sample adjusted to 1 ml with 50 μ l of Con A Sepharose and resolved by SDS-PAGE [16, 33]. Two sets of precipitates were performed. After electrophoresis on different gels, one set of samples were blotted onto PVDF membrane and sequentially reacted to the anti-Myc 9E10 antibody and horseradish peroxidase-conjugated goat anti-mouse IgG [16, 33]. Recombinant TACE proteins were visualized by chemiluminescence using an ECL kit. The other gel was stained with Coomassie blue to reveal total amounts of Con A-precipitated glycosylated proteins.

Immunofluorescence staining of cell surface TACE

We followed a previously described protocol to detect the mature TACE on the cell surface [16]. Briefly, M1 cells were transfected with eFlag-tagged constructs with or without pcDNA3-furin. At 24 h after transfection, the culture medium was replaced with 200 μ l cold, serum-free DMEM supplemented with 2% bovine serum albumin plus the anti-Flag antibody (final concentration: 5 μ g/ml). After a 30 min incubation on ice, coverslips were washed three times with cold PBS, fixed for 10 min with 100% methanol and reacted with FITC-conjugated goat anti-mouse IgG

diluted in PBS (1:400) for 30 min. After three additional washes, cell nuclei were stained with 4',6-diamidine-2-phenyl indole (DAPI) and viewed with an Olympus IX51 epifluorescent microscope. For quantitative analyses, images of five randomly selected fields were acquired under FITC and DAPI channels. Cells with surface TACE and total number of DAPI-stained cells in the fields were scored.

Fluorescence resonance energy transfer (FRET) analysis

M1 cells grown on coverslips were cotransfected with an eFlag-tagged TACE expression vector, HA-TGF- α and pcDNA3-furin [31]. Cell surface staining was performed 24 h after transfection using Alexa Fluor 488-conjugated anti-Flag (M2) and Alexa Fluor 555-conjugated anti-HA as described above. In addition, single fluorochrome staining was performed for correspondent controls. After staining, cells were washed twice with cold PBS and fixed for 15 min with 4% paraformaldehyde prepared in PBS, and mounted on microscope slides. Cells stained with Alexa Fluor 488 and Alexa Fluor 555 were imaged using Zeiss LSM510 META microscope. The emission spectrum from 500 to 628 nm was registered when a 488 nm laser was used to excite Alexa Fluor 488. For each TACE construct, FRET was calculated with data obtained from a total of 25 regions of interest in the plasma membrane of 5 cells (5 regions per cell), taking into consideration donor and acceptor spectral bleedthrough using the following equation: relative FRET = $I_{DA} - a(I_{AA} - cI_{DD}) - d(I_{DD} - bI_{AA})$, where I_{DA} is the fluorescence intensity of acceptor (TGF- α) emission caused by donor (TACE) excitation; I_{AA} is the fluorescence intensity of acceptor emission due to acceptor excitation; and I_{DD} is the fluorescence intensity of the donor emission resulted from donor excitation. Coefficients a and b are the acceptor bleedthrough in the I_{DA} and I_{DD} filter sets, whereas c and d are the donor bleedthrough in the I_{DA} and I_{AA} filter sets [34].

Results

Previous studies have shown that truncation constructs of TACE, which lack the cytoplasmic domain, are fully functional in ectodomain shedding [9, 14, 16]. However, further deletion of the TACE TM resulted in inactivation of the cleavage activity in TACE [24]. Nevertheless, soluble TACE forms lacking the TM and the cytoplasmic domain can correctly cleave detergent-extracted transmembrane proteins or synthetic peptide substrates *in vitro* (albeit very inefficiently) [3, 18, 35]. These observations suggest that plasma membrane anchorage may be important for TACE to cleave substrates in the cellular context. We therefore generated a TACE construct that is attached to the cell surface by deleting the TM and cytoplasmic domain and linking the GPI-binding signal of placental alkaline phosphatase [27] to the end of the CRD (Figure 2A). We determined the ectodomain shedding activity in the resulting T-GPI construct by analyzing levels of transmembrane TGF- α on the surface of M1-TGF- α cells after transient transfection with the TACE mutant. The M1-TGF- α cell line was derived from the

M1 cell line by stable transfection with transmembrane TGF- α [16]. Therefore, like the parental M1 cell line, M1-TGF- α cells lack endogenous TACE activity due to the expression of a single TACE form with the mutation of the conserved Met435 to Ile in the catalytic center [16]. Cell surface TGF- α was detected by flow cytometry. Even though the average transfection efficiency was about 60%, we were able to specifically analyze cells expressing recombinant TACE because they were cotransfected with GFP (see the Material and Methods section for details). Consistent with our previously published data [9, 16], the cytoplasmic domain-deleted TACE Δ C construct efficiently cleaved transmembrane TGF- α . As a result, there were significant decreases in cell surface TGF- α levels in M1-TGF- α cells transfected with the Δ C construct as compared with cells transfected with the control RK5 vector (and GFP) (Figure 3). However, the T-GPI construct was totally inactive in TGF- α shedding (Figure 3). This suggests that lipid membrane insertion of TACE may be required for ectodomain shedding of TGF- α . To test this, we constructed a chimeric TACE in which its TM was substituted by the TM of the prolactin receptor. The resulting T-PLR chimera was ineffective in restoring TGF- α shedding (Figure 3). Similarly, replacement of the TACE TM with the TM of PDGFR caused a strong reduction in TGF- α cleavage (Figure 3).

To determine whether T-GPI, T-PLR and T-PDGFR were defective in expression and maturation, we used Con A Sepharose to precipitate glycosylated proteins in cells transfected with the TACE constructs. Western blot analysis detected two glycosylated Myc-tagged TACE forms in the extracts of transfected cells. According to published data [12], the large form corresponds to the

zymogen, while the smaller form corresponds to the mature protein without the prodomain (Figure 4A). Evidently, the TGF- α -shedding-defective T-GPI, T-PLR and T-PDGFR constructs are synthesized at levels that are similar to that of the shedding-proficient Δ C construct. In addition, they were also processed to mature forms like Δ C. Noticeably, in all the constructs tested, the mature form represents only a small fraction compared with the zymogen form.

Since mature TACE but not the zymogen is found on the cell surface [12, 36], we performed live cell immunostaining to corroborate the above Con A precipitation-western blotting results. To circumvent the problem that no antibody is currently available for staining the ectodomain of rodent TACE, we placed a Flag epitope tag (eFlag) into the amino-terminus of the catalytic domain in the TM mutants and performed immunostaining in live cells with the monoclonal anti-Flag M2 antibody [16]. We previously demonstrated that the eFlag does not affect the enzyme activity of TACE. Thus, eFlag-WT TACE was fully active in cleaving TNF- α and TGF- α , whereas eFlag-M435I and eFlag-C600Y TACE constructs were inactive like their untagged versions [16]. Likewise, functional analyses confirmed that the eFlag-GPI, eFlag-T-PLR and eFlag-T-PDGFR constructs are all defective in TGF- α shedding (data not shown). Clearly, eFlag-GPI, eFlag-T-PLR, eFlag-T-PDGFR as well as eFlag-WT TACE could be detected on the cell surface (Figure 4B, left). Nevertheless, only about 4% of the cell population in the eFlag-WT TACE-transfected culture showed positive cell surface staining (Figure 4B, right), which was significantly below its ~60% transfection efficiency, as revealed by anti-Flag staining performed after

Figure 3 TACE TM is required for efficient shedding of TGF- α . The shedding-defective M1-TGF- α cells, which stably express transmembrane TGF- α , were transiently cotransfected with the transfection marker GFP and indicated TACE vectors or the control RK5 plasmid at a ratio of 1:3. Transmembrane TGF- α on the surface of live unfixed cells was immunostained with anti-TGF- α and a PE-conjugated secondary antibody, and was detected by flow cytometry. GFP-positive cells were gated as cells expressing the cotransfected TACE construct. Solid lines, TACE constructs; dotted lines, RK5.

Figure 4 Lack of adverse effects of TM deletion and substitution on biosynthesis and maturation of TACE. **(A)** M1 cells were transfected with indicated expression vectors. Glycosylated proteins were precipitated with Con A Sepharose, resolved by SDS-PAGE, and subjected to Coomassie blue staining to reveal total glycosylated proteins (top), and Western blotting to detect mature and zymogen TACE forms using the anti-Myc 9E10 antibody (bottom). **(B)** M1 cells were transfected with eFlag-tagged TACE constructs and pcDNA3-furin, and subjected to surface staining with anti-Flag. The percentages of positively stained cells from a total of five randomly selected fields (average $\pm$ standard deviation) for each TACE construct are presented in the bar graph. * $p < 0.05$ when compared with eFlag-WT TACE; ** $p < 0.01$. Note that similar results were obtained without furin cotransfection (data not shown).

cells were fixed and permeated with methanol (data not shown). The percentages of cells with positive surface staining in eFlag-T-GPI-, eFlag-T-PLR- and eFlag-T-PDGFR-transfected cultures ranged from 6.6% to 10% (Figure 4B, right), which are significantly higher than those of the eFlag-WT TACE-transfected culture but

still below their ~50-60% transfection efficiencies (data not shown). Together, the data presented in Figure 4 are in agreement with previously published results suggesting that TACE maturation is an inefficient process [12, 16, 37]. Despite this low maturation efficiency, it appears very unlikely that TM deletion or substitution had a negative impact on TACE maturation to affect TGF- α ectodomain shedding.

We next assessed whether the TGF- α shedding-defective TACE mutants retained the ability to physically interact with transmembrane TGF- α . Initially, we attempted to address this by coimmunoprecipitation. We used antibodies against endogenous TACE and TGF- α , and also those against epitope tags (Myc and Flag for TACE, and HA for TGF- α) for immunoprecipitation. However, we were unable to detect transmembrane TGF- α in the immunoprecipitates of TACE with its native TM; similarly, we failed to detect wild-type TACE in the immunoprecipitates of TGF- α (data not shown). These results were not surprising because TACE has been shown to coprecipitate with few substrates. We then employed confocal laser scanning FRET analysis. Since it occurs only when the separation distance of two molecules is ≤ 10 nm, FRET is a powerful technique to detect protein-protein interactions [34]. M1 cells were transiently transfected with an eFlag-tagged TACE construct plus HA-tagged TGF- α . Cell surface staining of mature TACE and transmembrane HA-TGF- α was accomplished using Alexa Fluor 488-conjugated anti-Flag and Alexa Fluor 555-conjugated anti-HA, respectively. To prevent “hit and run” by eFlag-WT TACE, cells were cultured in the presence of GM6001, a general metalloprotease inhibitor. The images in Figure 5A demonstrate apparent colocalization of the TACE proteins and HA-TGF- α . Interestingly, significantly stronger FRET from the shedding-defective eFlag-GPI, eFlag-T-PLR and eFlag-T-PDGFR to HA-TGF- α was detected than from the shedding-proficient eFlag-WT TACE to HA-TGF- α (Figure 5B). The expression levels of the different TACE constructs in the foci randomly chosen for FRET quantification were similar (data not shown). Likewise, there was no significant difference in the expression levels of HA-TGF- α among cells transfected with different TACE constructs (data not shown). Taken together, data presented in Figures 3-5 suggest that TM deletion and substitution in TACE severely interferes with ectodomain cleavage of transmembrane TGF- α without decreasing TACE biosynthesis, maturation and physical interaction with the substrate.

It has been documented in a considerable number of cases that single amino-acid substitution in TMs can have detrimental effects on the functions of proteins

Figure 5 TGF- α shedding-defective TACE mutants retain the ability to interact with transmembrane TGF- α . M1 cells were co-transfected with indicated TACE constructs, HA-TGF- α and pcDNA3-furin. Cell surface TACE and HA-TGF- α were stained with Alexa Fluor 488-conjugated anti-Flag and Alexa Fluor 555-conjugated anti-HA, respectively. **(A)** Confocal images showing apparent colocalization of TACE and TGF- α . **(B)** Relative FRET from the TACE constructs to TGF- α . Data represent averages $\pm$ standard deviations of 25 regions of interest in the plasma membrane for each TACE construct. See “Materials and Methods” for details. ** $p < 0.01$ when compared with eFlag-WT TACE.

Figure 6 Replacing either half of the TACE TM with the corresponding region of the PDGFR TM has little impact on TGF- α shedding. Transfection, immunostaining and flow cytometry analysis were performed as described in Figure 3 legend.

(e.g. [20–22]). To identify possible “critical residues” in the TACE TM, we performed scanning mutagenesis in which 22 of the 23 amino acids in the TACE TM were individually substituted with tryptophan and Trp684 was replaced with alanine. Previously, tryptophan scan-

ning mutagenesis has been used to study other TMs (e.g. [38–40]). Functional analyses showed that all 23 resulting scanning mutants maintained the full capacity to release TGF- α (data not shown). Thus, the importance of TACE TM in TGF- α shedding may not be attributed to single

amino acids within this domain. We next constructed two subdomain chimeras in which the TACE TM was divided into two halves which were replaced with the corresponding segments of the PDGFR β (Figure 2A). As shown in Figure 6, the two sub-TM chimeric mutants showed little loss of TGF- α -cleavage activity. Therefore, it appears that either half of the TACE TM was able to compensate for the loss of the other half.

Around 50 transmembrane proteins have been identified as TACE substrates. Since the TACE TM appears to be critical for TGF- α shedding, we wanted to test whether this non-catalytic TACE domain is also required for ectodomain cleavage of other TACE substrates. We chose to analyze the shedding of TNF- α and L-selectin because these two proteins are from protein families that are structurally and functionally distinguishable from that of TGF- α . We took the same approach for analyzing TNF- α and L-selectin shedding as for TGF- α shedding. Accordingly, we derived M1 cell lines that stably express transmembrane TNF- α or L-selectin, and analyzed their

shedding by flow cytometry. As expected, transfection of ΔC into the TACE-deficient cells restored the shedding of both L-selectin and TNF- α (Figure 7). The TM-free, membrane-anchored T-GPI construct was defective in the cleavage of transmembrane TNF- α and L-selectin, similar to transmembrane TGF- α . However, both the T-PLR and T-PDGFR constructs efficiently restored shedding of TNF- α and L-selectin, which strikingly contrasts to TGF- α . Thus, substitution of the TACE TM with heterologous TMs had different effects on TGF- α , TNF- α and L-selectin shedding.

We next examined whether the TM of a TACE substrate may determine the requirement of the TACE TM for shedding, by constructing TGF- α /L-selectin chimeras that carry each other's TM (Figure 2B). The TMs of TGF- α and L-selectin were chosen for domain swap because they are both type I membrane proteins. (In contrast, TNF- α is a type II membrane protein, and would not be appropriate for TM-switch with TGF- α). Similarly, we derived M1 sublines that stably express

Figure 7 Ectodomain shedding of L-selectin and TNF- α is impaired by substitution of the TACE TM with the GPI anchor, but is maintained by substitution with the TMs of prolactin receptor and PDGFR. TACE-deficient M1-L-selectin and M1-TNF- α cells, which stably express transmembrane L-selectin and TNF- α , respectively, were transiently cotransfected with an expression vector for enhanced GFP plus an expression vector for TACE or the control RK5 plasmid. The TACE substrates on the cell surface were immunostained with appropriate primary antibodies and a PE-conjugated secondary antibody, and were detected by flow cytometry. GFP-positive cells were gated as cells expressing the cotransfected TACE construct. Solid lines, TACE constructs; dotted lines, RK5.

Figure 8 Substitution of the TM of TGF- α with that of L-selectin enables TGF- α shedding by T-PLR and T-PDGFR, whereas replacement of the TM of L-selectin with that of TGF- α does not significantly affect L-selectin shedding by the TACE constructs. Shedding-defective M1 cells stably expressing either the TGF-LS-TGF or LS-TGF-LS chimeric substrate were transiently co-transfected with GFP and indicated TACE vectors or the control RK5 plasmid. TGF- α and L-selectin shedding was analyzed as in Figures 3 and 7.

Figure 9 Failure to recover ectodomain cleavage activity in T-GPI by M β CD. M1-L-selectin cells were cotransfected with T-GPI and hCD4. 24 h later, cells were treated with M β CD for 1 h, and then subjected to surface staining with FITC-conjugated anti-hCD4 and PE-conjugated anti-L-selectin. Surface L-selectin levels on hCD4-positive cells were determined by flow cytometry.

the chimeric TACE substrates. As expected, TGF- α and L-selectin ectodomain shedding by Δ C was not affected by swapping the TMs between the substrates (Figure 8). Evidently, substitution of the TM of TGF- α with that of L-selectin enabled TGF- α ectodomain shedding by T-PLR and T-PDGFR (Figure 8), which were defective in cleaving wild-type TGF- α (Figure 3). However, replace-

ment of the TM of L-selectin with that of TGF- α had little effect on L-selectin shedding by the TACE mutants. Thus, whether the TACE TM is required for shedding appears to be largely determined by the TM of TGF- α and the ectodomain of L-selectin.

Many membrane proteins are targeted to discrete microdomains in the plasma membrane. GPI-anchored

proteins are located in membrane rafts rich in cholesterol [41], whereas TACE appears to be a non-raft protein [42, 43]. Thus, microdomain mislocalization might underlie the shedding deficiency in T-GPI. To test this hypothesis, we determined whether the cholesterol-depleting reagent M β CD, which disrupts rafts and reorganizes membrane domains [44], would recover L-selectin shedding by T-GPI. The L-selectin shedding assay was performed as those presented above, except that a cytoplasmic domain-truncated hCD4 construct was used in place of GFP because we found that M β CD treatment had a detrimental effect on GFP signal (data not shown). As shown in Figure 9, M β CD treatment did not change the expression levels of L-selectin on the surfaces of T-GPI-transfected M1-L-selectin cells.

Discussion

In this report we have presented evidence for an important role of the TACE TM in ectodomain cleavage. It should be pointed out that it has been previously shown that a TACE construct containing the CRD and TM of ADAM10 failed to cleave the ectodomain of the type II receptor for interleukin 1 [14]. Although the inactivity in the TACE-ADAM10 chimera was attributed to the lack of the native CRD of TACE, it is also possible, in light of our work, that the substitution of the TM was fully or partially responsible for the loss of the cytokine receptor shedding.

The function of this noncatalytic domain in shedding appears to be twofold. First, it serves as a transmembrane anchor that may be broadly required for efficient cleavage of many substrates. Thus, its substitution with a peripheral membrane anchor, the GPI-linker of placental alkaline phosphatase, had detrimental effects on the shedding of all three TACE substrates tested. Further analyses with additional substrates are needed to determine whether a transmembrane anchor is indeed of general importance in shedding. Second, the particular amino-acid sequence of the TACE TM appears to encode information that is critical for the shedding of some, but not all, substrates. Accordingly, substitution of the TACE TM with the TM of prolactin receptor and PDGFR strongly inhibited TGF- α release but had minor (if any) effects on TNF- α and L-selectin shedding. Thus, the TACE TM may provide a mechanism for regulating the shedding of a subset of substrates specifically. Again, future studies with an expanded number of substrates are required to determine the degree of specificity of the dependence on the TACE TM for shedding.

The activities of a number of transmembrane proteins are determined by single amino acids in their TMs. A

conserved glycine in the TM of the vesicular stomatitis virus G protein is essential for infection-mediated membrane fusion [22]. Point mutations in fibroblast growth factor receptor 3 lead to constitutive activation of the receptor, causing a common type of dwarfism [20, 21]. However, our scanning mutagenesis experiments failed to identify critical residues in the TACE TM. Furthermore, replacing either half of the TACE TM with corresponding regions of the PDGFR TM showed only slight decreases in TGF- α shedding, suggesting that segments within the TACE TM can compensate for its partial loss despite its overall requirement for TGF- α shedding.

The mechanism by which the TACE TM influences ectodomain shedding is yet to be established. TMs frequently determine the processing of membrane proteins in the secretory pathway. TACE undergoes glycosylation and prodomain removal before it reaches the plasma membrane, where it cleaves substrates [12]. Several lines of evidence suggest that the deletion and substitution of TM does not affect the processing and maturation of TACE. First, transfection with their expression vectors led to the generation of their mature forms as demonstrated by Con A precipitation coupled with Western blotting analysis. Second, immunostaining of live cells detected the mature proteins on the cell surface as shown by bright-field and confocal fluorescence microscopy. Finally, T-PLR and T-PDGFR constructs are active in TNF- α and L-selectin shedding although they are defective in TGF- α ectodomain cleavage. Nevertheless, these data do not rule out a potential role for the TACE TM in subcellular distribution as this activity in the deletion and substitution constructs may have been provided by the GPI linker and the heterologous TMs, respectively.

The plasma membrane in higher organisms is organized into microdomains including membrane rafts [45]. Compared with wild-type TACE, the TACE mutants created in this study might influence microdomain distribution. First, because GPI anchors are raft-targeting signals [46], the T-GPI constructs are expected to be in membrane rafts. Second, changes in amino acid sequence and consequential changes in the secondary structure in the TM chimeras may result in altered microdomain distribution. Finally, since it has been shown that the length of TMs influences protein-lipid interaction [47], replacing the 23-amino-acid TACE TM with the 27-residue PDGFR TM may also affect localization in the microdomains. However, it is difficult to attribute the TGF- α shedding deficiency in the T-GPI, T-PLR and T-PDGFR constructs to possible changes in microdomain distribution because (1) FRET analyses did not detect decreased association of the TGF- α shedding-defective T-GPI, T-PLR and T-PDGFR constructs with transmembrane TGF- α as

compared to that of wild-type TACE with the substrate, (2) the T-PLR and T-PDGFR constructs remain active in TNF- α and L-selectin shedding despite their defects in TGF- α ectodomain cleavage and (3) M β CD, which disrupts rafts and reorganizes membrane microdomains, fails to recover ectodomain shedding activity in T-GPI.

It is also possible that the TACE TM is involved in engaging substrates since interactions between some transmembrane proteins are mediated by TMs [22, 23, 48, 49]. Although our FRET data indicate that the TGF- α -shedding-defective mutants can interact with transmembrane TGF- α , these experiments do not predict the nature of physical interaction. Therefore, it is possible that the substituting TMs in the TACE mutants fail to engage the TM of TGF- α in such a way as to place the cleavage site in the juxtamembrane region of TGF- α to the catalytic center of TACE. Along this line of speculation, the TMs of L-selectin and TNF- α might have lower stringent requirement, as compared with the TM of TGF- α , for interacting with another TM, which therefore allows for cleavage of not only transmembrane L-selectin and TNF- α but also the chimeric TGF-LS-TGF substrate by the T-PLR and T-PDGFR constructs. The ectodomain of L-selectin appears to play a dominant role in determining the association of L-selectin with TACE. Thus, replacing the TM of L-selectin with that of TGF- α had little adverse effect on L-selectin ectodomain shedding.

Ectodomain shedding by TACE is regulated by intracellular activities [10, 11]. Other membrane proteins are likely to be involved in this signaling process because deletion of the cytoplasmic domain does not affect shedding [9, 14, 50]. Therefore, the possibility that the TACE TM interacts with regulatory proteins key to TACE function should also be considered.

About 50 membrane proteins with very diverse biological activities have been reported as TACE substrates. An intriguing question is whether and how shedding of different ectodomains by TACE can be specifically regulated. Since dysregulated shedding of inflammatory cytokines and growth factors by TACE plays important roles in the pathogenesis of inflammation and other diseases, TACE is also regarded as an important therapeutic target. We have discovered that TGF- α , TNF- α and L-selectin have contrasting requirements of the TACE TM sequence for ectodomain shedding. A previous study showed that, among four TACE substrates tested, only the type II receptor for interleukin 1 failed to be cleaved by a mutated TACE construct [14]. One would hope that, as a result of extensive investigations, strategies that selectively interfere with shedding of one or a narrow group of TACE substrates without affecting the shedding of ectodomains required for normal physiological processes can be de-

veloped for therapeutic purposes.

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