Multiple Metalloproteinases Process Protransforming Growth Factor-α (ProTGF-α)†

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ABSTRACT: Shedding of TNF-α requires a single cleavage event, whereas the ectodomain of proTGF-α is cleaved at N-proximal (N-terminal) and membrane proximal (C-terminal) sites to release mature TGF-α. Tumor necrosis factor-α converting enzyme (TACE) was shown to have a central role in the shedding of both factors. Here we show that cleavage of the proTGF-α C-terminal site, required for release of mature growth factor, is less sensitive to a panel of hydroxamates than TNF-α processing. Recombinant TACE cleaves TNF-α and N-terminal TGF-α peptides 50-fold more efficiently than the C-terminal TGF-α peptide. Moreover, fractionation of rat liver epithelial cell membranes yields two populations: one contains TACE and cleaves peptides corresponding to TNF-α and both proTGF-α processing sites, while the other lacks detectable TACE and cleaves only the C-terminal proTGF-α processing site. Activities in both fractions are inhibited by hydroxamates and EDTA but not by cysteine, aspartate, or serine protease inhibitors. Both membrane fractions also contain ADAM 10. ADAM 10 correctly cleaves peptides and a soluble form of precursor TGF-α (proTGFecto) at the N-terminal site but not the C-terminal site. However, the kinetics of N-terminal peptide cleavage by ADAM 10 are 90-fold less efficient than TACE. Our findings indicate that while TACE is an efficient proTGF-α N-terminal convertase, a different activity, distinguishable from TACE, exists that can process proTGF-α at the C-terminal site. A model that accounts for these findings and the requirement for TACE in TGF-α shedding is proposed.

Extracellular domains of various cell surface proteins are released by proteolytic cleavage in a phenomenon known as ectodomain shedding (*1*−*4*). Targets of this process include growth factors and cytokines (EGF family ligands, TNF-α,[1] CSF-1), receptors (ErbB4, TNF receptors, interleukin receptors, PDGF-R, Notch, growth hormone receptor), enzymes (angiotensin converting enzyme), adhesion proteins (L-selectin, L1, CD30, CD44), and ectodomains of unknown function (*β*-amyloid precursor protein, cellular prion protein) (reviewed in refs *3* and *5*−*8*). Cleavage of these various proteins contributes to mitogenesis, cell migration, differentiation, and the pathology of disease states such as inflammation, tumorigenesis, spongiform encephalopathies, and Alzheimer's disease (*2*−*4*, *6*, *8*−*13*). Regulation of proteolysis is a key step in several cellular processes (*2*−*4*, *6*, *8*−*13*) and is likely to be complex given the diversity of ectodomain shedding stimulants, including phorbol esters (*14*−*20*), calcium ionophores (*21*, *22*), serum factors (*23*), and phosphatase inhibitors (*24*, *25*). Multiple enzymes, including zinc-dependent metalloproteinases, have been implicated in ectodomain shedding (*2*, *6*, *8*−*13*, *18*, *26*−*33*). Notably, RANKL(TRANCE), a TNF family member, is cleaved by at least two separate metalloproteinases (*34*), and HB-EGF, an EGF family ligand, may be cleaved by multiple metalloproteinases including ADAMs 9 (*35*), 10 (*36*), 12 (*37*), and 17 (*27*, *38*) and MMPs 3 (*39*) and 7 (*40*).

TGF-α, another member of the epidermal growth factor receptor (EGFR) ligand family, is a well-studied model of ectodomain shedding. TGF-α is synthesized as a biologically active, integral membrane precursor, which may be subsequently cleaved in the extracellular domain at two sites to release soluble, mature growth factor. The soluble form also activates EGFR (*41*−*44*), but the downstream biological consequences may be different from those mediated by the transmembrane protein (*41*, *42*, *44*−*50*). Membrane-anchored growth factor mediates juxtacrine signaling, whereas proteolytic conversion of TGF-α to its soluble form presumably switches the growth factor to an autocrine or paracrine activator of EGFR and allows signaling to occur at more distal sites (*41*, *42*, *44*, *49*). Although additional physiological roles of proTGF-α are still being studied, mature TGF-α, a

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[1] Abbreviations: TGF-α, transforming growth factor-α; TACE, tumor necrosis factor-α converting enzyme; TNF-α, tumor necrosis factor-α; R1 cells, ras-transformed rat liver epithelial cells; hPBMCs, human peripheral blood mononuclear cells; EGFR, epidermal growth factor receptor; ADAM, a disintegrin and metalloprotease; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase; rhADAM 10, recombinant human ADAM 10.

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potent cell mitogen in vitro, may have physiological roles in cell migration (*51*, *52*).

Intriguingly, although the two TGF-α cleavage sites contain the Ala-Val dipeptide at their cores (N-terminal, SPVAAA/VVSHFN; C-terminal, HADLLA/VVAASQ),[2] distinct convertases may carry out the N- and C-terminal cleavage events. The N-terminal site is processed more rapidly than the C-terminal site in transfected CHO cells (*15*, *53*). Conversely, many transformed cell lines and tumors secrete larger forms of TGF-α that retain the N-terminus, indicating inefficient or altered processing (*50*, *54*−*56*). Much emphasis has been placed on efforts to purify the enzymes responsible for TGF-α processing. ProTGF-α cleavage has been shown to be mediated by zinc-dependent metalloproteinases (*1*), and candidate activities have been identified (*17*, *56*). However, despite significant effort, candidates for the physiological enzyme(s) have remained elusive until the identification and purification of the TNF-α converting enzyme, TACE/ADAM-17 (*30*, *32*), a zinc-dependent metalloproteinase.

TACE is one member of the ADAM family of disintegrin metalloproteinases. It was purified on the basis of its ability to accurately cleave a peptide substrate spanning the cleavage site of TNF-α and was shown to process the full-length precursor protein (*30*−*32*). Subsequently, TACE was found to be a proTGF-α convertase on the basis of similarity in phenotypes between mice homozygous for a protease-inactivating mutation and those lacking TGF-α, including open eyes at birth, wavy hair, and aberrant skin architecture (*49*, *51*, *57*). Furthermore, fibroblasts (*38*, *57*) and primary keratinocytes (*38*) established from these mice were impaired 10−20-fold in their ability to shed TGF-α. Recombinant TACE also cleaves full-length and soluble forms of TGF-α at the correct N-terminal processing site in vitro. C-Terminal cleavage of the full-length form by TACE in vitro, however, does not occur, but processing of a soluble TGF-α construct occurs at both an incorrect and correct cleavage site (*38*). These results indicate that TACE is required for efficient TGF-α release, but it remains to be determined whether TACE directly cleaves proTGF-α in vivo. Residual TGF-α release by TACE-deficient cells suggests the existence of other enzymes with proTGF-α converting enzyme activities. Consistent with this, a recent report by Merlos-Suárez and colleagues recently demonstrated that APMA induced metalloproteinase-dependent proTGF-α cleavage in TACE- or shedding-deficient cells (*27*).

Here, we show that the release of TNF-α and TGF-α from cellular membranes can be differentiated through a series of hydroxamic acid inhibitors. We also demonstrate that partial fractionation experiments yield two separate activities, one that correctly processes a peptide substrate corresponding to the C-terminal cleavage sequence of proTGF-α and another that correctly processes N- and C-terminal TGF-α and TNF-α peptide substrates. Inhibition profiles for these fractions in peptide assays and kinetic measurements with recombinant forms of TACE and ADAM 10 on the peptide substrates confirm that TACE is the most efficient N-terminal convertase. Although ADAM 10 is present in both fractions, and purified ADAM 10 correctly cleaves the N-terminal site of proTGF-α in vitro, it does not cleave the correct C-terminal site, pointing to an unidentified proTGF-α C-terminal converting activity. A model that combines the demonstrated requirement for TACE with these observations is proposed.

[2] The TGF-α membrane proximal site is referred to as the C-terminal site and the TGF-α N-proximal site is referred to as the N-terminal site for the remainder of the paper.

EXPERIMENTAL PROCEDURES

Materials. The following peptides were synthesized by the University of North Carolina Peptide Synthesis Facility and were used in subsequent experiments: TNF-α, DNP-SPLAQAVRSSSR-$CONH_2$; N-terminal TGF-α, DNP-SPVAAAVVSHFN-$CONH_2$; and C-terminal TGF-α, DNP-HADLLAVVAASQ-$CONH_2$. The antibodies were anti-FLAG (Sigma, St. Louis, MO), anti-HA (Covance, Richmond, CA), anti-TACE/ADAM 17 (Chemicon International, Temecula, CA), and anti-ADAM 10 (Oncogene Research Products, Boston, MA). The furin inhibitor, decanoyl-Arg-Val-Lys-Arg chloromethyl ketone, was from Bachem Bioscience Inc. (King of Prussia, PA). Recombinant human ADAM 10 (rhADAM 10) was from R&D Systems (Minneapolis, MN). Bovine brain was purified essentially as described by Glynn and Howard (*58*).

Cell Lines and Cell Culture. Ras-transformed rat liver cells (R1 cells) and the proTGF-α-expressing stable clone derivative (*59*) were grown in minimal essential media (MEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) gentamicin (Gibco BRL). Human peripheral blood mononuclear cells (hPBMCs) were isolated by Ficoll gradient centrifugation with 1-Step Polymorphs (Accurate Chemical & Scientific Corp., Westbury, NY) from the heparinized blood of a volunteer donor. Purified hPBMCs were suspended at 8×10^6 cells/mL in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% antibiotics.

Cell-Based Inhibitor Assays. Aliquots of 100 μL (4×10^5 cells) of hPBMCs were added to 96-well microtiter plates. Compounds or DMSO vehicle was added at doses ranging from 0.04 to 10 μM (final DMSO concentration in culture medium was 0.05%) for 1 h before addition of lipopolysaccharide (LPS) (100 ng/mL) and PHA (50 μg/mL) (Sigma) (*60*−*62*). After incubation at 37 °C and 5% CO_2 for 48 h, cell-free conditioned media were collected by centrifugation at 800*g*. Media levels of soluble human TGF-α and TNF-α were determined using Quantikine immunoassay (R&D Systems, Minneapolis, MN) and ELISA (Oncogene Research Products, Cambridge, MA), respectively.

Twenty-four hours prior to addition of inhibitor, R1 cells were seeded in 60 mm dishes at 1.875×10^5 cells/mL. Plates were washed with phosphate-buffered saline (PBS) and fresh media added containing either DMSO vehicle or hydroxamic acid inhibitor at 1 nM−100 μM for IC_{50} measurements or 1 nM−10 μM for Western blots (final DMSO concentration in culture medium was 1%). After incubation at 37 °C and 5% CO_2 for 24 h, conditioned media and cells were harvested. Cells were disrupted in lysis buffer including protease inhibitors [50 mM Tris−base, 150 mM NaCl, 1% (v/v) Triton X-100, 0.01 mg/mL leupeptin, 0.1 mg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM EDTA]. Clarified lysates were analyzed for total protein content by the BCA protein assay (Pierce, Rockford,

IL). Conditioned media was treated with 1 mM PMSF, 1 mM EDTA, and 0.05% (v/v) trifluoroacetic acid (TFA), concentrated on a Sep Pak C-18 cartridge (Waters), and lyophilized in a speed vac. The media were resuspended in lysis buffer plus protease inhibitors. Media were analyzed by radioimmunoassay (RIA) as described previously (*63*). Lysate and media were also analyzed by Western blot.

Immunoblotting. For cell-based inhibitor studies, 20 μg of cell lysate and equivalent amounts of concentrated conditioned media were resolved by 15% SDS−PAGE. For analysis of membrane content, 20−30 μg of total homogenate or purified bovine ADAM 10 and 12−20 μg of membrane fractions from R1 cells were resolved by 10% SDS−PAGE. The gels were transferred to Immobilon-P PVDF membrane (Millipore). Membranes were blotted in 5% milk block [1× Tris-buffered saline and 0.1% Tween 20 (TBS−Tween) and 5% (w/v) nonfat dry milk] and the appropriate antibodies, followed by incubation with HRP-conjugated secondary antibodies, and developed by chemiluminescence and autoradiography. Blots were directly captured, films were densitometrically quantitated on a Kodak Digital Science Image Station 440CF, and data were analyzed using the Kodak 1D Image Analysis software. The fractional inhibition was calculated by taking the intensities of the bands in the DMSO control, I_o, and subtracting from the band intensities in the presence of inhibitor, I_i, and then dividing by the DMSO control intensity I_o according to the equation $(I_o - I_i)/I_o$. The average of three experiments and the standard deviation were calculated using Microsoft Excel.

Membrane Fractionation. Thirty-four 150 mm dishes of confluent R1 cells were washed in PBS, scraped, and pelleted. R1 cells were resuspended in 0.25 M sucrose, 2 mM $MgCl_2$, 10 mM HEPES, and 1 mL/20 g wet weight cells protease inhibitor cocktail (Sigma). Cells were broken in a ball-bearing homogenizer to preserve membrane and protein integrity. The homogenate was then centrifuged successively: 2000*g*, 5 min, 4 °C; supernatant spun at 32000*g*, 10 min, 4 °C; supernatant spun at 45000*g*, 60 min, 4 °C. The resulting pellet was resuspended in 2.5 mL of 0.25 M sucrose, 10 mM HEPES, and protease inhibitor cocktail, layered on top of a 33−38.7% discontinuous sucrose gradient, and centrifuged at 98000*g* for 12−18 h at 4 °C, forming two membrane pellets: one at the bottom of the tube (G1) and the other at the 33−38.7% sucrose interface (G2). The G2 pellet was removed with a 3 cm^3 syringe and a 25 g needle, mixed with 10 mM HEPES, and centrifuged at 98000*g* for 30 min to remove excess sucrose. Both membrane pellets were resuspended in 0.25 M sucrose, 10 mM HEPES, and protease inhibitor cocktail. The membrane fractions were frozen in liquid nitrogen and stored at −80 °C. This procedure has been used previously to characterize TACE activity (*31*, *64*, *65*).

Peptide Assays. Peptides (20−35 μM) were incubated with 5 μL (approximately 1 μg) of each membrane fraction for 20 min (TNF-α), 2 h (N-terminal), or 2.5 h (C-terminal) in the presence and absence of inhibitors. The reactions were quenched with 1% heptafluorobutyric acid (HFBA) to a final volume of 100 μL, and cleavage products were analyzed by high-performance liquid chromatography (HPLC) on a Hewlett-Packard LC 1090 or by liquid chromatography/mass spectrometry (LC/MS) using a Hewlett-Packard HP1090 liquid chromatograph coupled to a Sciex API-III triple quadrupole mass spectrometer. UV absorbance was monitored at 350 nm. Uncleaved substrate and cleavage products were identified by respective molecular masses and turnover calculated on the basis of UV absorbance or mass spectrometric signal intensity.

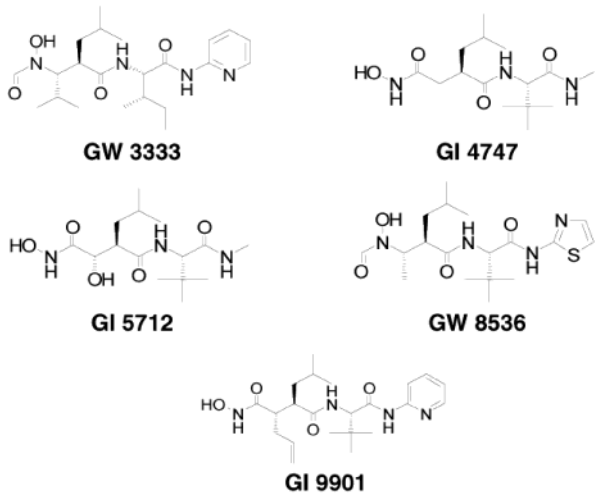

FIGURE 1: Structures of hydroxamate inhibitors used in these studies.

Specificity constants were determined for the peptides using homogeneously pure forms of TACE expressed in insect cells (*65*, *66*). Peptides (20−35 μM) were incubated with each form of the enzyme and separated by HPLC, and k_{cat}/K_m values were calculated according to the equation $\{[P/(S + P)]/100\}/([E] \times \text{time})$, where P is the area under the product peak, S is the area under the substrate peak, and [E] is the concentration of enzyme used.

Using the fluorescent substrate Dabcyl-LAQAHomopheR-SC(Fluorescein)-NH_2, rhADAM 10 was first titrated with TIMP-1 in order to obtain specificity constants for the DNP-peptide substrates. Peptides were incubated with rhADAM 10, and product formation was measured by HPLC. Specificity constants, k_{cat}/K_m values, were calculated for the peptides according to the same formula used for TACE.

In Vitro Cleavage of ProTGF-α Proteins by ADAM 10. Aliquots of concentrated media from COS-1 cells transfected with the proTGFecto construct (*38*) were incubated with 100 ng of rhADAM 10 or 10 mM Tris, pH 8.0 at 37 °C, over a 4 h time period. The reactions were stopped by addition of SDS−PAGE sample buffer, separated by SDS−PAGE, and Western blotted as described above using antibodies to the FLAG and HA epitope tags. Sequence of the cleavage products was performed by the UNC Proteomics and Mass Spectrometry Facility according to a recently described procedure (*38*).

RESULTS

Differential Inhibition of TGF-α vs TNF-α Release by Hydroxamates. We compared the efficiency of several hydroxamic acid inhibitors (Figure 1) to determine if TACE is solely responsible for cleavage of TGF-α in cells by comparing TNF-α versus TGF-α release from human PBMCs stimulated with PHA and LPS (*28*, *60*−*62*, *64*, *67*). Since cell-based assays have large inherent variabilities, the ratio of TGF-α/TNF-α release in a single experiment was compared, and the assay was repeated several times (n =

Table 1: IC_{50} Measurements for Hydroxamic Acid Inhibitors[a]

compound	TACE K_i (μM)	IC_{50} (μM) hPBMCs TGF-α[b]	hPBMCs TNF-α[b]	TGF-α/TNF-α
GW 3333	0.041	12.1	1.4	9.2 ± 3.2
GI 4747	0.110	23.3	12.2	2.2 ± 0.44
GI 5712	0.017	8.9	2.3	4.7 ± 1.1
GW 8536	0.019	3.8	0.75	6.3 ± 2.2
GI 9901	0.015	1.2	0.14	8.8 ± 1.5

[a] Inhibitors were added in concentrations to obtain a dose−response curve for TNF-α or TGF-α as measured by ELISA (hPBMC). [b] IC_{50}s were calculated and averaged from three experiments, in which levels of secreted TGF-α and TNF-α from hPBMCs were determined in parallel for the same media samples. TGF-α/TNF-α ratios were calculated from IC_{50} data of each individual experiment and averaged. The standard error represents the intrinsic experimental variability.

3). If TACE was the only enzyme involved in processing both growth factors, the ratio of the IC_{50}s for TGF-α/TNF-α release should remain constant. The results shown in Table 1 indicate that this was not the case. For example, the ratio of IC_{50}s for TGF-α versus TNF-α release was 8−9-fold for GI 9901 while only 2−3-fold for the GI 4747 inhibitor. (The ratios of the IC_{50}s for TGF-α/TNF-α were used as a comparison rather than the individual IC_{50} measurements, which can vary widely between experiments in cell-based assays.) These ratios were experimentally different as assessed by the standard errors associated with each value, suggesting that multiple convertases are involved in the processing of TGF-α.

Inhibition of ProTGF-α Cleavage in R1 Cells Reveals Distinctions in N- and C-Terminal Cleavage. If the N- and C-terminal cleavage events of proTGF-α are carried out by different enzymes, and thus differentially inhibited, then media from a TGF-α inhibitor release assay could contain both the mature TGF-α and a C-terminally but not N-terminally cleaved species. We addressed this possibility by using the ras-immortalized rat liver epithelial cell line, R1, which is well characterized for its ability to process tagged precursor TGF-α [schematic Figure 2A (*59*)]. IC_{50} values for the various inhibitors against TGF-α release in this cell line are all approximately micromolar (GW 3333, 5.3 μM; GI 4747, 2.8 μM; GI 5712, 1.9 μM; GW 8536, 1.6 μM; GI 9901, 0.95 μM). Cells were incubated with doses of two of these inhibitors (GI 9901 and GI 4747) in concentrations of 1 nM, 100 nM, and 10 μM. These two inhibitors respectively have the largest and smallest ratio in IC_{50}s from the hPBMCs for TGF-α versus TNF-α release. Representative blots of the results are shown in Figure 2B. R1 cells that were treated with DMSO vehicle alone showed prominent FLAG-reactive TGF-α forms in the cell lysate that correspond to previously characterized species (*59*). Thus, 25 and 36 kDa forms in the lysate are nascent and fully glycosylated proTGF-α, respectively, while a 16 kDa species corresponds to the residual cytoplasmic tail resulting from C-terminal cleavage. Soluble HA-reactive TGF-α forms in the conditioned media included a 6 kDa form corresponding to mature TGF-α, cleaved at both the N- and C-termini, as well as higher molecular mass species of 15 and 29 kDa that likely correspond to TGF-α forms cleaved at the C-terminus only, confirming our hypothesis that the secreted TGF-α measured in the ELISA is a composite of multiple species.

Analysis of lysate and media from hydroxamate-treated R1 cells reveals differential effects on the various TGF-α species present in conditioned media. Importantly, the 6 kDa media form was considerably more sensitive to hydroxamate inhibition than the 29 kDa soluble forms (100 nM inhibitor, lanes 3 and 6), consistent with preferential inhibition of N-terminal cleavage as opposed to the C-terminal processing. In fact, when cells were treated with 10 μM GI 9901 (lane 4), the media contained neither the mature TGF-α (6 kD) nor the C-terminally only (29 kDa) cleaved products, indicating inhibition of C-terminal processing, consistent with the IC_{50} data in Table 1.

We used the Western results from three trials to calculate the fractional inhibition by GI 9901 and GI 4747 for the release of the three proteins (6, 15, and 29 kDa) into the media (see Experimental Procedures). These findings, displayed graphically for inhibitors GI 9901 and GI 4747 in Figure 2C, show that disappearance of the 6 kDa protein (mature TGF-α) proceeds at lower inhibitor concentrations than for the 29 kDa protein. For inhibitor concentrations of 100 nM, the 6 kDa band almost disappears, whereas the 29 kDa species still persists strongly. With the poorer inhibitor, GI 4747, statistically meaningful differences were also observed at 10 μM. The results with the 15 kDa form are comparable to the 29 kDa form. However, due to the variability between experiments, the results for this form were not statistically different (data not shown). The data from both the 6 and 29 kDa analysis demonstrate that disappearance of the bands is dependent on different inhibitor concentrations.

Peptide Cleavage by Recombinant TACE. We have distinguished the N- and C-terminal proTGF-α cleavage events on the basis of their differential sensitivities to hydroxamate inhibitors in cell-based assays. Therefore, the specificity constants (k_{cat}/K_m) of peptide substrates based on the N-terminal and C-terminal cleavage sites of TGF-α were compared with a TNF-α peptide substrate to further examine the possibility that separate enzymes cleave the two sites of proTGF-α. Specificity constants (k_{cat}/K_m) provide an indication as to whether a particular enzyme/substrate interaction is physiologically relevant. For example, TACE and ADAM 10 both cleave a TNF-α peptide substrate, but TACE does so with a much higher specificity constant (10^5 vs 10^3 M^{-1} s^{-1}) (*30*, *32*, *68*). Underscoring the importance of this difference, while both enzymes cleave precursor TNF-α in vitro, only TACE is an actual physiological TNF-α converting enzyme (*30*−*32*). Both recombinant catalytic (CAT) and catalytic-disintegrin (CAT-DIS) domains of TACE efficiently cleave the TNF-α and N-terminal TGF-α peptide substrates with almost identical k_{cat}/K_m (Table 2). In contrast, the k_{cat}/K_m for TACE cleavage of the C-terminal TGF-α peptide substrate was approximately 2 orders of magnitude lower.

Enzymatic Activities of G1 and G2. We examined whether R1 cells contain multiple convertases using a membrane fractionation procedure previously used to screen TACE inhibitors (see Experimental Procedures) (*31*, *64*, *65*). Consistent with previous results, G2 fractions from R1 cells contained all immunodetectable TACE (Figure 3A) and were equivalent to the previously published membrane fraction (*31*, *64*, *65*). G2 fractions accurately cleaved TNF-α, as well as N- and C-terminal TGF-α cleavage site peptides, albeit with markedly different efficiencies. The TNF-α convertase

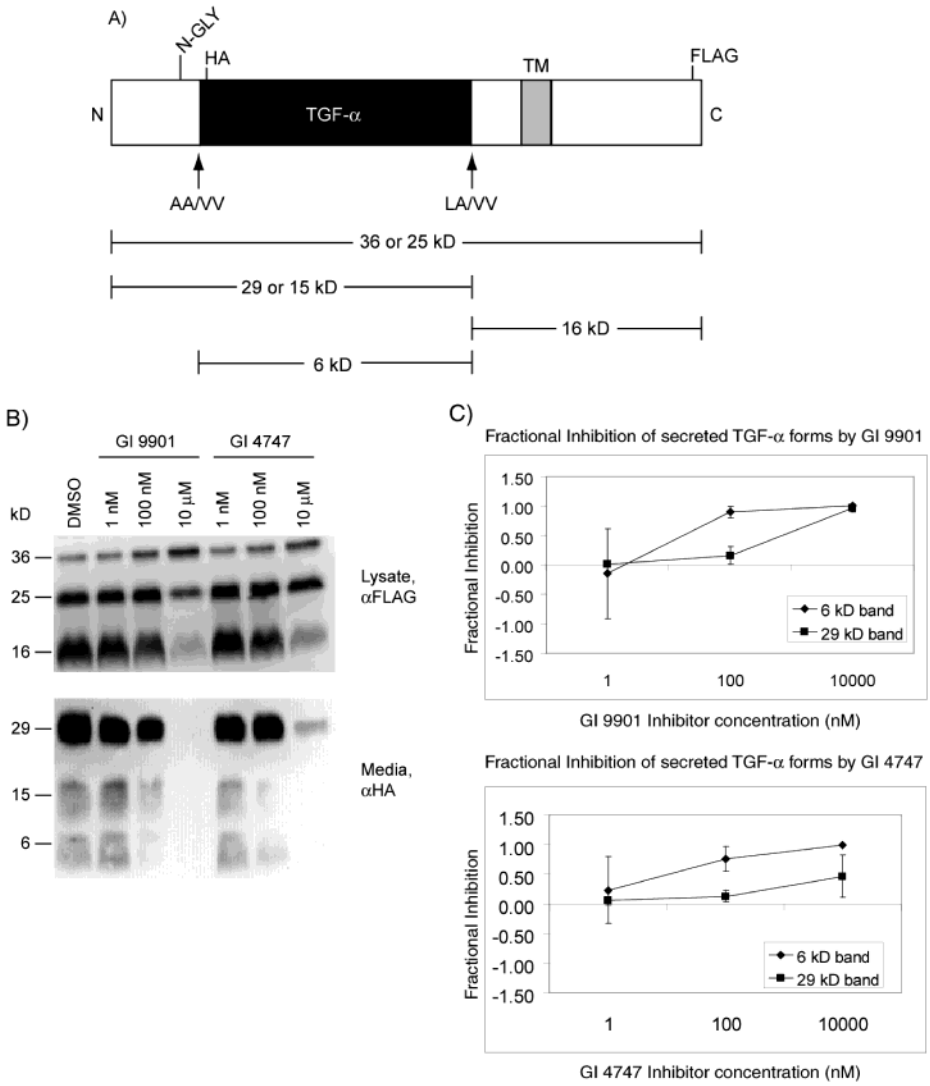

FIGURE 2: Forms of TGF-α produced in response to hydroxamate inhibitors. (A) Schematic of dual epitope-tagged proTGF-α. Arrows indicated cleavage sites, with flanking sequences shown, and the locations of HA and FLAG epitopes, as well as an N-glycosylation site, are indicated. The relationships between various TGF-α species observed in Western analyses are shown. The molecular mass difference between the 36 and 25 kDa pro forms was previously found to be due to differential glycosylation (*73*). (B) R1 cells, stably overexpressing the construct shown in (A), were incubated with the GI 9901 and GI 4747 at the indicated hydroxamate concentrations for 24 h prior to harvest. Lysate (50 μg) and equivalent media concentrations (standardized for the amount of lysate protein) were separated by SDS−PAGE, and blots of lysate and media species were probed with anti-HA (media) or anti-FLAG (lysate). Representative blots ($n = 5$) are shown. (C) The fractional inhibition of media bands observed in (B) was determined by densitometric analysis of short exposure films or direct detection of chemiluminescence on the Kodak Image Station 440 CF using Kodak 1D software and plotted in relation to the same band detected in DMSO-treated samples. Results from GI 9901- and GI 4747-treated cultures are shown ($n = 3$).

Table 2: Kinetics of Peptide Cleavage by Recombinant TACE[a]

	k_{cat}/K_m (M^{-1} s^{-1})	
peptide	CAT	CAT-DIS
TNF-α	1.7×10^5	1.2×10^5
TGF-α N-terminus	1.3×10^5	1.0×10^5
TGF-α C-terminus	2.7×10^3	2.0×10^3

[a] Each peptide was incubated with recombinant TACE catalytic domain (CAT) or recombinant TACE catalytic-disintegrin domains (CAT-DIS). k_{cat}/K_m values were calculated using the equation $\{[P/(S + P)]/100\}/([E] \times \text{time})$, where P is the area under the product peak, S is the area under the substrate peak, and [E] is the concentration of enzyme used. Errors are less than 30%.

and N-terminal TGF-α activities in G2 fractions were comparable while the C-terminal TGF-α convertase activity was slower [TNF-α, 23.5 ± 13.1 mg fraction^{-1} min^{-1} (n = 6); N-terminal TGF-α, 1.82 ± 1.43 mg fraction^{-1} min^{-1} (n = 6); and C-terminal TGF-α, 0.383 ± 0.245 mg fraction^{-1} min^{-1} (n = 4)]. In contrast, G1 fractions contained no immunodetectable TACE, and using comparable amounts of total protein, TNF and N-terminal TGF-α processings were not detectable, whereas C-terminal TGF-α processing clearly occurred [0.189 ± 0.185 mg fraction^{-1} min^{-1} (n = 6)].

The different activities contained in G1 and G2 fractions were further characterized by examining the inhibition profiles of the two membrane fractions (Table 3). TNF-α peptide cleavage was used as a control for TACE activity in these experiments. The G1 and G2 fractions are not substantially inhibited by cysteine, aspartate, or serine protease inhibitors (Sigma protease inhibitor cocktail; "protease" in Table 3). However, the furin inhibitor decanoyl-Arg-Val-Lys-Arg chloromethyl ketone inhibited 20−40% of the total TNF-α activity of G2. This was not due to TACE inhibition, since decanoyl-Arg-Val-Lys-Arg chloromethyl

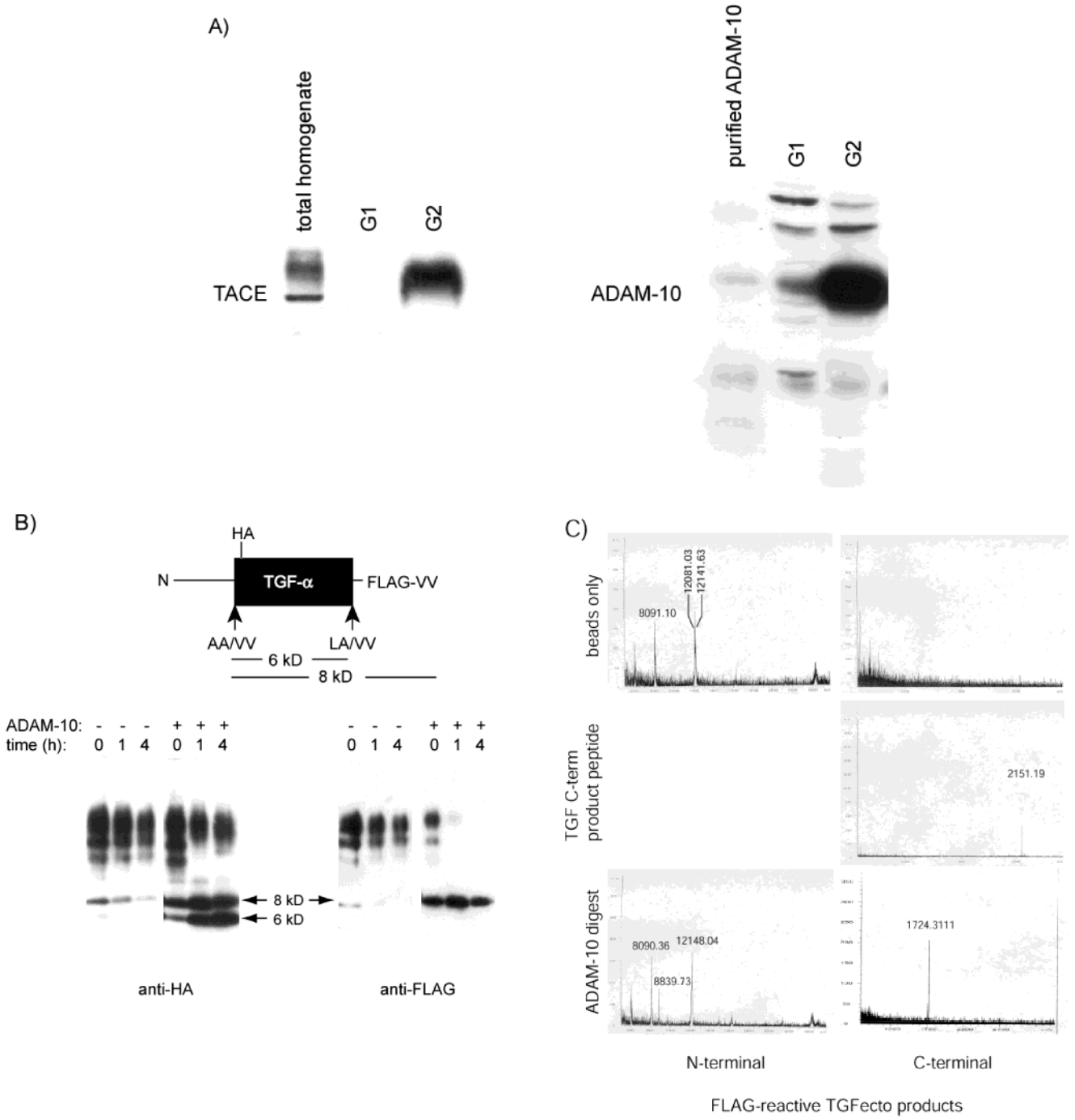

FIGURE 3: In vitro cleavage of proTGFecto by ADAM 10. (A) Western blots of TACE and ADAM 10 in G1 and G2 membrane fractions. For TACE immunoblotting, 20−30 μg of total homogenate and 12−20 μg of G1 or G2 were separated by SDS−PAGE and blotted using antiserum to the cytoplasmic domain of TACE. For ADAM 10 immunoblotting, 20−30 μg of purified bovine ADAM 10 and 12−20 μg of G1 or G2 were separated by SDS−PAGE and blotted using antiserum to the cytoplasmic domain of ADAM 10. (B) ProTGFecto (*38*) schematic shown above blots with cleavage sites and expected products indicated. ProTGFecto was incubated with 1 μg of recombinant human ADAM 10 or buffer over a 4 h time course at 37 °C. Reactions were stopped by the addition of SDS−PAGE sample buffer, separated by SDS−PAGE, and blotted for HA and FLAG. Addition of ADAM 10 resulted in the generation of an 8 kDa product that is both HA and FLAG reactive and a 6 kDa product that is only HA reactive. (C) Mass spectrometry of cleavage products was performed as described previously (*38*). Top panels: anti-FLAG M2 affinity gel control (left, *m*/*z* 6000−24000; right, *m*/*z* 700−2500). Middle panel: synthetic peptide corresponding to the expected C-terminal cleavage product VVAASQKKQDYKDDDDKVV (*m*/*z* 2151). Bottom panels: products of the rhADAM 10 reaction (left, N-terminal products, *m*/*z* 6000−24000; right, C-terminal products, *m*/*z* 700−2500).

ketone did not inhibit recombinant TACE (data not shown). This indicates that TACE does not need to be activated by furin-dependent removal of the prodomain in our assays. Interestingly, inhibition of the cleavage activities by EDTA was differential. Inhibition of the TNF-α and C-terminal TGF-α activities in G2 were nearly complete at 50 mM EDTA. However, the N-terminal TGF-α activity in G2 and the activity in G1 were only partially inhibited by this same concentration of EDTA.

Addition of varying amounts of the five structurally diverse hydroxamates discussed above indicated that the least potent TACE inhibitors (e.g., GW 3333 and GI 4747) were the most

Table 3: Inhibition of Enzyme Activity against Peptide Substrates[a]

inhibitor	G1[b] C-term	G2 TNF-α	G2 N-term	G2 C-term
vehicle	0	0	0	0
furin	0	39	2	20
protease	20	12	15	19
EDTA				
50 mM	44	88	50	98
10 mM	0	82	43	69
GW 3333				
200 μM	>95	–	–	–
10 μM	35	–	–	33
1 μM	13	–	–	–
100 nM	–	65	61	–
10 nM	–	32	38	–
2 nM	–	19	22	–
GI 4747				
200 μM	>95	–	–	–
10 μM	67	–	–	>95
1 μM	57	–	–	–
100 nM	–	72	67	–
10 nM	–	48	42	–
2 nM	–	17	8	–
GI 5712				
200 μM	>95	–	–	–
10 μM	66	–	–	>95
1 μM	21	–	–	–
100 nM	–	72	67	–
10 nM	–	61	34	–
2 nM	–	41	14	–
GW 8536				
200 μM	40	–	–	–
10 μM	27	–	–	20
1 μM	9	–	–	–
100 nM	–	86	59	–
10 nM	–	58	54	–
2 nM	–	37	34	–
GI 9901				
200 μM	43	–	–	–
10 μM	48	–	–	57
1 μM	28	–	–	–
100 nM	–	88	68	–
10 nM	–	59	60	–
2 nM	–	43	50	–

[a] Data are expressed as percent inhibition of peptide cleavage by each fraction from at least three preparations as analyzed by HPLC. [b] G1 does not cleave TNF-α or N-term peptide substrates. Errors are generally less than 30%. Dashes indicate no data.

potent against the C-terminal TGF-α cleavage activity and vice versa (e.g., GI 9901) (Table 3). Furthermore, the hydroxamate inhibition profiles for TNF-α and N-terminal TGF-α activities in G2 were similar, and the profiles for C-terminal TGF-α cleavage by both G1 and G2 were similar to, although distinct from, the TNF-α and N-terminal TGF-α profiles. The in vitro inhibitory activities measured here may not be comparable to the TGF-α IC_{50} numbers since the cell-based numbers represent multiple cleavage products. Nevertheless, this indicates that similar enzymes, distinct from TACE, present in both fractions may cleave the C-terminal proTGF-α peptide. The sensitivity of this activity to tissue inhibitor of metalloproteases (TIMP) was also measured. TIMP3 but not TIMPs 1 or 2 inhibited cleavage of the C-terminal proTGF-α peptide at concentrations that inhibit MMPs (data not shown) (*68*). Furthermore, we tested several MMPs to rule out their involvement as the C-terminal activity. None of the MMPs, including MT-MMP1, cleaved the C-terminal proTGF-α peptide solely at the Ala/Val site, as some of the MMPs process the peptide at two places (data not shown).

These results are consistent with the involvement of an ADAM other than TACE in this cleavage event. Western analysis was performed to determine if ADAM 9, 10, 12, and 15 were possible candidates for this activity. Only ADAM 10 was found to be present in both fractions (Figure 3A).

In Vitro Cleavage of ProTGF-α by ADAM 10. TACE has recently been shown to efficiently process an epitope-tagged soluble substrate based on the full-length precursor TGF-α , proTGFecto (*38*). Since both G1 and G2 have TGF-α converting activities, we tested the membrane fractions and recombinant human ADAM 10 (rhADAM 10) for their ability to cleave FLAG-affinity-purified proTGF-α. G1, G2, and rhADAM 10 cleaved this protein at the N-terminal site only, as was recently found for TACE (data not shown) (*38*). To observe C-terminal cleavage, we tested rhADAM 10 for its ability to cleave both sites of proTGFecto (Figure 3A) (*38*) in vitro. In the presence of rhADAM 10, 6 and 8 kDa species were observed with a concomitant decrease in higher molecular mass species over a 4 h time course. The 8 kDa FLAG- and HA-reactive species are consistent with cleavage at the N-terminal site, while the 6 kDa HA-reactive species are consistent with cleavage at both sites (Figure 3B). In fact, these are the same species observed when this substrate is incubated in the presence of TACE in parallel reactions (data not shown). We were unable to cleave proTGFecto with G1 and G2 because both contain ADAM 10 as well as other activities, and any results could not be attributed to a single activity.

We subjected samples that had been incubated with rhADAM 10 for 4 h to analysis by MALDI/TOF-MS (*38*) to identify the cleavage sites used by ADAM 10 (Figure 3B). The N-terminally cleaved product, 8 kDa, was *m*/*z* 8839.73 (bottom left panel) and was identical to the product produced by TACE (data not shown), which corresponds to cleavage at the physiological Ala-Ala/Val-Val bond. The correct FLAG-tagged product generated by cleavage at the C-terminal Leu-Ala/Val-Val site is *m*/*z* 2151 and is represented by the spectra of a synthetic peptide (middle panel). A single major FLAG-tagged C-terminal cleavage product of *m*/*z* 1724.31 was obtained from the ADAM 10 digest (bottom right panel), which corresponds to cleavage of the Ser/Gln bond five amino acids C-terminal to the physiological Leu-Ala/Val-Val bond. In some experiments, an additional minor C-terminal product of *m*/*z* 1811 product was detected, which corresponds to cleavage of the Ala/Ser bond four amino acids C-terminal to the physiological Leu-Ala/Val-Val bond (data not shown). Note that incubation of proTGFecto with TACE also generates the *m*/*z* 1811 product in addition to the *m*/*z* 2151 product (*38*); however, there is no evidence that this is a physiological product. ADAM 10 does not cleave the physiological Leu-Ala/Val-Val bond to generate the *m*/*z* 2151 product, indicating that our C-terminal specific activity is a novel enzyme, distinct from both TACE and ADAM 10.

We also cleaved the TNF-α and N-terminal and C-terminal TGF-α peptides with rhADAM 10 and confirmed our results with proTGFecto. rhADAM 10 accurately cleaved the TNF-α and N-terminal TGF-α peptides but failed to accurately cleave the C-terminal TGF-α peptide (data not shown). Since the N-terminal peptide was accurately cleaved by rhADAM 10, k_{cat}/K_m values were measured for this peptide using the TNF-α peptide as a comparison. The kinetics of N-terminal

cleavage were essentially identical to those measured for TNF-α (1.33×10^3 M^{-1} s^{-1} and 1.25×10^3 M^{-1} s^{-1}, respectively). These values were 2 orders of magnitude lower than those measured for TACE (Table 2), confirming that TACE was the most efficient proTGF-α N-terminal convertase using peptide substrates.

DISCUSSION

Ectodomain shedding of cell surface proteins is required for normal development and may have important roles in the pathology of diseases (*3*, *6*, *11*). Determining the mechanism and specificity of shedding is crucial to understanding these cellular processes. Regulated proteolysis affects signaling in a variety of ways, including cell motility by cleavage of adhesion proteins and altering the strength, duration, or spatial context of signaling by cleavage of growth factors and their receptors (reviewed in refs *4* and *12*). Thus, developing a detailed view of shedding mechanisms is an important goal.

ErbB receptor ligands, including EGF and neuregulin family members, are prominent targets of ectodomain shedding, and ADAMs, particularly TACE, are implicated in these events. For example, current evidence points to an important role for shedding of TGF-α, an EGF family member, by TACE. The phenotypic similarities between the TGF-α- and TACE-targeted mice are clear. Both exhibit open eyes at birth and wavy hair (*51*, *52*). Also, cells derived from the $TACE^{-/-}$ mice are significantly impaired in their ability to shed TGF-α (*38*, *57*), and reintroduction of TACE into TACE-deficient cells restores correct processing (*38*). Moreover, recombinant TACE directly cleaves the N-terminus of authentic proTGF-α, as well as the C-terminal site of a truncated, secreted form of proTGF-α that includes the juxtamembrane region followed by an epitope sequence (*38*). Thus, evidence of a role for TACE is compelling, but these findings do not exclude the possibility that other enzymes cleave proTGF-α in some physiological contexts or that the role for TACE is indirect, at least with respect to the C-terminal releasing cleavage.

The results presented here address the potential role for other enzymes in proTGF-α processing by providing evidence for a separate C-terminal proTGF-α enzyme activity. IC_{50} measurements for a series of hydroxamate inhibitors indicate that TGF-α shedding, a process dependent on cleavage at the C-terminal site, has a different inhibition profile than TNF-α secretion, which is presumably mediated by TACE. Moreover, TGF-α/TNF-α IC_{50} ratios for the five compounds are not consistently increased or decreased, as would be expected if the same enzyme were responsible for cleavage of both proteins. Although the IC_{50} values for TGF-α release are a composite of cleavage products, the K_i values for TACE are not consistent with the inhibition profile we observed for TGF-α. Supporting this conclusion, low doses of hydroxamates selectively impair release of the fully processed 6 kDa growth factor compared to larger secreted forms. These findings are consistent with the micromolar IC_{50} measurements found for prevention of native TGF-α release from R1 cells by the hydroxamic acids and support preferential inhibition of the N-terminal/C-terminal cleavage reactions. Furthermore, these findings distinguish the mechanisms for N-terminal and C-terminal cleavage and are consistent with a distinction in the kinetics of N- and C-terminal cleavage in CHO cells that prompted speculation that different enzymes act on these two proTGF-α sites (*15*, *53*). However, we cannot exclude the possibility that these data represent coordinate action of multiple enzymes.

Biochemical analysis of the membrane fractions that are depleted of (G1) or contain (G2) TACE activity provides clear evidence of at least one additional proTGF-α converting enzyme. In particular, the G1 fraction is depleted of TACE immunoreactivity, and yet it contains a metalloproteinase activity (Table 3, EDTA and hydroxamic acid inhibition) that accurately and specifically cleaves the TGF-α C-terminal peptide. Although EDTA inhibition of the membrane fractions is variable, this could be attributed to the complexity of the fractions or possibly the relative amounts of each activity in the fraction. TACE, for example, is not inhibited completely by 50 mM EDTA at higher enzyme concentrations (data not shown). Nevertheless, the composite inhibition profiles indicate that the cleavage activities we examined are metalloproteinases. Moreover, the TIMP inhibition data, and lack of correct cleavage by MMPs, indicate that the G1 enzyme is likely an ADAM.

The G2 fraction, on the other hand, contains TACE and accurately cleaves TNF-α and both TGF-α peptides, albeit with very different efficiencies. The presence of TACE in G2 indicates that it is likely the predominant activity responsible for cleavage of the TNF-α and TGF-α N-terminal peptides, since the inhibition profiles for these events are highly similar. The C-terminal TGF-α G2 activity is unlikely due to TACE as it is markedly less sensitive to the hydroxamates (Table 3; GW 3333, GW 8536, GI 9901) than either the TNF-α or N-terminal TGF-α peptides. However, C-terminal TGF-α cleavage by G1 and G2 was similarly sensitive to the hydroxamates. The decreased sensitivity of C-terminal TGF-α cleavage by G1 (200 μM inhibitor is required for complete inhibition) also correlates with the persistence of the 29 kDa C-terminal cleavage product obtained when R1 cells were treated with hydroxamate inhibitors.

The kinetics of C-terminal cleavage by TACE were much less efficient than either N-terminal or TNF-α convertase activities, providing additional evidence against ADAM 17 as a major C-terminal converting enzyme. The above finding along with the results in Sunnarborg et al., where TACE processes proTGFecto at two sites in vitro (*38*), raises significant doubt as to whether direct cleavage of the C-terminal site of the membrane-anchored protein by TACE, an event essential for growth factor release, occurs in vivo (*30*, *32*, *68*). A caveat is that our assays utilized peptide substrates; hence, we cannot exclude the possibility that TACE cleaves the C-terminal site in intact proTGF-α with greater efficiency. Conceivably, the dynamics of cleavage of integral membrane proTGF-α by integral membrane TACE are different.

Further characterization of the membrane fractions indicates that ADAM 10 was present in both fractions and positioned to be the C-terminal-specific enzyme. However, ADAM 10 correctly cleaves only the N-terminal site in peptide assays or in vitro cleavage reactions with the proTGFecto substrate, excluding it as the C-terminal activity we identified. It is also not likely that ADAM 10 is an efficient N-terminal TGF-α convertase because the kinetics

of the TNF-α and N-terminal peptide cleavage reactions are 2 orders of magnitude lower than those measured for TACE. Thus, TACE is most likely the most efficient N-terminal TGF-α convertase, but we have uncovered compelling evidence for the existence of an unknown C-terminal-specific proTGF-α converting activity different from TACE.

One possible model to account for our data as well as the apparent requirement for TACE in TGF-α shedding, is that TACE cleaves the N-terminal site, removing steric hindrance due to N-glycosylation at an adjacent upstream site (*43*, *53*). This would in turn enable efficient C-terminal cleavage by this as yet unidentified enzyme. Precedence for this model comes from the processing of Notch and β-amyloid precursor proteins (*2*, *20*, *69*−*71*). TACE and ADAM 10 have been implicated in the processing of the same ectodomain site in each of these proteins (*2*, *3*, *71*), but both Notch and β-amyloid precursor protein also undergo additional cleavage in their transmembrane domains by a γ-secretase, possibly presenilin-2 (*3*, *72*). The cleavage of membrane proteins at distinct sites by multiple proteases possibly reflects a requirement for intricate regulation of ectodomain shedding. Our data indicate that proTGF-α, a well-studied model of ectodomain shedding, may be similarly subject to regulation via multiple proteases. Future efforts will be aimed at identifying the additional components that contribute to TGF-α release from the cell surface as an important step to unraveling the regulation of this important process.

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