The Tumor Necrosis Factor-α Converting Enzyme (TACE): A Unique Metalloproteinase with Highly Defined Substrate Selectivity†

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ABSTRACT: TNFα converting enzyme (TACE) processes precursor TNFα between Ala76 and Val77, yielding a correctly processed bioactive 17 kDa protein. Genetic evidence indicates that TACE may also be involved in the shedding of other ectodomains. Here we show that native and recombinant forms of TACE efficiently processed a synthetic substrate corresponding to the TNFα cleavage site only. For all other substrates, conversion occurred only at high enzyme concentrations and prolonged reaction times. Often, cleavage under those conditions was accompanied by nonspecific reactions. We also compared TNFα cleavage by TACE to cleavage by those members of the matrix metalloproteinase (MMP) family previously implied in TNFα release. The specificity constants for TNFα cleavage by the MMPs were approximately 100−1000-fold slower relative to TACE. MMP 7 also processed precursor TNFα at the correct cleavage site but did so with a 30-fold lower specificity constant relative to TACE. In contrast, MMP 1 processed precursor TNFα between Ala74 and Gln75, in addition to between Ala76 and Val77, while MMP 9 cleaved this natural substrate solely between Ala74 and Gln75. Additionally, the MMP substrate Dnp-PChaGC(Me)HK(NMA)-NH_2 was not cleaved at all by TACE, while collagenase (MMP 1), gelatinase (MMP 9), stromelysin 1 (MMP 3), and matrilysin (MMP 7) all processed this substrate efficiently. All of these results indicate that TACE is unique in terms of its specificity requirements for substrate cleavage.

One of the most promising targets for the treatment of inflammatory diseases is the TNFα[1] converting enzyme (TACE or ADAM 17). TACE is a zinc metalloproteinase that cleaves 26 kDa precursor TNFα to its mature, 17 kDa form (*1*, *2*). Precursor TNFα exists as a membrane-bound form in certain cell types, while mature TNFα is secreted into the extracellular compartment (*3*). If the precursor form of TNFα contains a mutated cleavage site, it does not get released from cells, indicating that a processing event is required for TNFα to be secreted (*4*, *5*). Inhibitors of TACE may therefore be therapeutically useful in the treatment of any disease state where inhibition of TNFα release has been validated as a target, such as arthritis and Crohn's disease (*6*−*8*).

A class of inhibitors known as hydroxamates have been shown to inhibit TNFα secretion from a variety of cell types (*9*−*11*). These inhibitors were previously demonstrated to inhibit a class of enzymes known as the matrix metalloproteases (MMPs). A microsomal activity that processes precursor TNFα in vitro was purified from membrane preparations, and the enzyme was cloned and expressed (*1*, *2*). TACE, while inhibited by matrix metalloprotease inhibitors, belongs to the disintegrin family of metalloproteases or ADAMs (*12*).

TACE appears to be the physiological TNFα converting enzyme since TNFα release is reduced by 90% in cells derived from $TACE^{-/-}$ mice (*1*). Surprisingly, it was later found that the shedding of other ectodomains was also blocked in these knockout cells. Those ectodomains affected so far include the amyloid precursor protein (APP; *13*), L-selectin (*14*), TNFα receptors I and II (TNFR-I and -II;

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[1] Abbreviations: TNFα, tumor necrosis factor-α; TACE, TNFα converting enzyme; mTACE, microsomal TACE; rTACE, mature ectodomain of recombinant TACE; MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; GW 9471, (2*R*,3*S*)-2-(2-methyl-1-propyl)-3-*N*-hydroxycarbamoyl-4-phenylsulfanylbutanoic acid [(1*S*)-1-methylcarbamoyl-2-phenyl-1-ethyl]amide; MMP 1, human fibroblast collagenase; MMP 3, stromelysin 1; MMP 7, matrilysin; MMP 9, 92 kDa gelatinase; Dnp, 2,4-dinitrophenyl; Nma, *N*-methylanthranilic acid; NBD, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-hexanoic acid; DMC, 7-dimethylaminocoumarin-4-acetate; DMSO, dimethyl sulfoxide; Cha, *β*-cyclohexylalanyl; C(Me), *S*-methylcysteine; Nva, norvaline; HFBA, heptafluorobutyric acid; NR, no reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); APP, amyloid precursor protein; TNFR, TNFα receptor; TGFα, transforming growth factor-α; HER4, erbB4/HER4 epidermal growth factor receptor isoform JM-a; IL-6R, interleukin-6 receptor; M-CSFR, macrophage colony-stimulating factor receptor; GHR, growth hormone receptor; PrP^c, cellular prion protein; KL-1, c-kit ligand; RANKL, receptor of activated nuclear factor κ-B (NFκB) ligand; MT1-MMP, membrane-type 1 matrix metalloproteinase.

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14, *15*), transforming growth factor-α (TGFα; *14*), the erbB4/HER4 epidermal growth factor receptor isoform JM-a (HER4; *16*), the interleukin-6 receptor (IL-6R; *17*), fractalkine (*18*, *19*), the macrophage colony-stimulating factor receptor (M-CSFR; *20*), CD30 (*21*), the growth hormone receptor (GHR; *22*), the Notch 1 receptor (Notch; *23*) and the cellular prion protein (PrP^c; *24*).

Here we show that while recombinant forms of TACE efficiently cleave a synthetic peptide containing the cleavage site of TNFα, little or no cleavage was observed for HER4, APP, L-selectin, c-kit ligand (KL-1; *25*, *26*), TNFR-I and -II, RANKL, Notch, and IL-6R, with k_{cat}/K_m values for specific products typically 100−1000-fold lower relative to the one observed for TNFα cleavage. Taking into consideration that all reactions were performed under conditions where substrate availability was not rate limiting, this suggests that within the membrane, where substrate availability may be rate limiting, TACE is less likely to process most of these precursors. Alternatively, accessory factors within the membrane, yet to be identified, may be required for presenting alternate substrates to TACE.

MMPs such as collagenase, stromelysin, gelatinase, and matrilysin can process precursor TNFα to yield bioactive TNFα (*9*, *27*, *28*). We also describe here the specificity constants of TACE and these MMPs for precursor TNFα cleavage. We demonstrate that only MMP 7 and TACE cleave both precursor TNFα and a synthetic peptide substrate correctly, between Ala76 and Val77, although MMP 7 is less efficient (k_{cat}/K_m is 30-fold lower relative to TACE). In addition, k_{cat}/K_m values are reported for the cleavage of the synthetic precursor TNFα substrate, Dnp-SPLAQAVRSSSR-NH_2, and the MMP substrates Dnp-PChaGC(Me)HK(NMA)-NH_2 and NBD-RPKPLANvaWK(DMC)-NH_2 by native and recombinant TACE, MMP 1, MMP 3, MMP 7, and MMP 9.

MATERIALS AND METHODS

Media and Reagents. All cell culture reagents were from Life Technologies (Gibco/BRL). GW 9471 was synthesized as described (*29*). Synthetic peptides were custom synthesized by Zeneca, CRB. Protease inhibitors were purchased from Sigma or CalBiochem. The 19 kDa MMP 1 was prepared as previously described (*30*). MMP 3 was expressed in *Escherichia coli* and purified in a similar manner as described for truncated MMP 1. A truncated 50 kDa version of MMP 9 was expressed in insect cells from a baculovirus vector and purified as described below. Recombinant forms of TACE Met1 to Arg473 and Met1 to Arg651 were obtained as described in Milla and co-workers (*31*). All other chemicals and reagents were purchased from commercial sources.

Purification of TACE. MonoMac 6 (*32*) cells were grown in RPMI media supplemented with L-glutamine, 10% fetal bovine serum, and pennicillin/streptomycin. All lysis and purification steps were performed at 4 °C. Cells were resuspended in buffer containing 10 mM HEPES, pH 7.5, 0.25 M sucrose, 5 mM $MgCl_2$, and the following protease inhibitors: leupeptin (10 μM), AEBSF (1 mM), E-64 (10 μM), pepstatin (1 μM), DCI (10 μM), and phosphoramidon (10 μM). Cells were lysed and membrane fractions were purified by sucrose density gradient centrifugation as described before (*33*).

*Expression and Purification of FLAG-Tagged ProTNF*α. A cDNA encoding proTNFα was obtained from plasmid pIP860. The insert was amplified by PCR and included an N-terminal FLAG tag. The 5′ primer (5′ CGG CGG CGC CAT ATG GAC TAC AAG GAC GAC GAT GAC AAAG AGC ACT GAA AGC ATG ATC) consisted of a *Nde*I restriction site followed by the FLAG octapeptide sequence and the eight N-terminal residues of proTNFα. The 3′ primer (5′ CGG CGG CGC GGA TCC TTA CAG GGC AAT GAT CCC AAA GTA 3′) consisted of the sequence encoding the seven C-terminal residues of the proTNFα and an ochre termination codon followed by a *Bam*HI site. The amplified DNA sequence was cloned into pET11b at the *Nde*I−*Bam*HI sites creating the plasmid pET11b/proTNFα(5′-FLAG). The complete DNA sequence of the insert was confirmed by automated DNA sequencing. Cultures were grown at 25 °C. After induction with 0.5 mM IPTG for 3 h, cells were harvested by centrifugation, and the expressed proTNFα was isolated from inclusion bodies by extraction with 6 M guanidine hydrochloride in 50 mM Tris, pH 7.5. The denatured protein was refolded by overnight dialysis against 20 mM Tris, pH 7.5, and 150 mM NaCl. Purified protein was obtained by passing the dialyzed material over an M2 antibody affinity column (Sigma Aldrich) specific for the FLAG sequence, according to the manufacturer's instructions.

*Cleavage of ProTNF*α *by MMPs and TACE.* To initially assess cleavage of precursor TNFα by MMPs and TACE, enzymes were added to separate Eppendorf tubes at final concentrations of 34 nM (MMP 1), 100 nM (MMP 3), 57 nM (MMP 7), 120 nM (MMP 9), or 0.2 nM (microsomal TACE) in buffer containing 10 mM HEPES, pH 7.5, and 0.25 M sucrose (buffer B). Enzymes were then mixed with 1 μg of FLAG-tagged precursor TNFα in a final reaction volume of 100 μL. The reaction components were incubated at 37 °C for 3 h, and the products were subjected to N-terminal sequencing after separation by SDS−PAGE and electroelution onto PVDF membranes.

*Specificity Constants against ProTNF*α. Matrilysin (MMP 7), microsomal TACE, and recombinant TACE were assayed for TNFα converting activity using FLAG-tagged proTNFα as a substrate. Briefly, 53 μL of a 26.9 μg/mL solution of affinity-purified proTNFα was incubated with enzyme for 0−5 h at 37 °C in buffer B, which contained 10 mM HEPES, pH 7.4, and 0.25 M sucrose, and the protease inhibitors leupeptin (5 μM), pepstatin (0.5 μM), phosphoramidon (5 μM), E-64 (5 μM), AEBSF (0.5 mM), diprotinins A and B (5 μM), and amstatin (5 μM). Reactions were carried out in the presence or absence of 10 μM GW 9471 or 20 mM EDTA. Digestion products were separated and analyzed by SDS−PAGE and Western blotting using an anti-TNF monoclonal antibody. Alternatively, products were separated by dilution of 10 μL of the reaction mixture into 990 μL of buffer B containing 10 μL of M2 antibody resin. Intact substrate bound to the resin and the unbound fraction containing mature product were quantified by an L-929 killing assay (*34*) or with a TNFα ELISA kit from R&D Systems. The NH_2-terminal sequence of the 17 kDa fragment

generated from this digestion was determined by transferring the SDS−PAGE separated products to PVDF filters as previously described (*35*).

Determination of ProTNFα and TNFα Concentrations. A small aliquot of proTNFα was reacted with matrilysin as described above, except that the incubation time was long enough to convert all of the substrate to the product, mature TNFα. The concentration of TNFα was then determined by using an L-929 killing assay (*34*). A standard curve was run with known concentrations of TNFα (purchased from Cistron).

Preparation of Truncated MMP 9. Total RNA was isolated from PMA-stimulated U937 cells (1×10^8) using a total RNA isolation kit (Stratagene). Poly(A) mRNA was purified from the total RNA preparation as directed by the manufacturer using a poly(A) RNA isolation kit (U.S. Biochemicals). cDNA was prepared from mRNA using a cDNA synthesis kit (U.S. Biochemicals). Polymerase chain reactions were performed in a Perkin-Elmer thermal cycler using the GeneAmp PCR reagent kit with either AmpliTaq DNA polymerase (Perkin-Elmer) or Vent polymerase (New England Biolabs). All oligonucleotide primers were synthesized by Oligos, Etc., Seattle, WA.

Initially, PCR primers were designed to obtain the MMP 9 cDNA in two halves ("A" and "B") to circumvent problems of secondary structure encountered during PCR. The A primer set was 5′-TTT ACT AGT AGA TCT ATG AGC CTC TGG CAG CCC-3′ and the antisense strand 5′-AAA GAA TTC GTT AAC GTC GTC CTT ATG CAA GGG-3′. The B primer set was 5′-TTT CTC GAG GTT AAC GGC ATC CGG CAC CTC TAT G-3′ and the antisense primer 5′-AAA GGT ACC AGA TCT CTA GTC CTC AGG GCA CTG-3′. The MMP 9 cDNA in its entirety was cloned into pBluescript SK+ (Stratagene). The truncated 50 kDa MMP 9 was constructed by using a pair of PCR primers that would create a version of MMP 9 terminating at the end of the catalytic domain. The primers 5′-TTT ACT AGT AGA TCT ATG AGC CTC TGG CAG CCC-3′ and 5′-AAA GAA TTC AGA TCT TCA GCG AGG ACC ATA GAG GTG-3′ were used to PCR out the truncated MMP 9. The DNA fragment was cloned into pBluescript SK+, sequenced, and then subcloned into the baculovirus expression vector pAcUW51 (Pharmingen). The resulting DNA utilizing the polyhedrin promoter was then used to express the truncated MMP 9.

Purification of Truncated 92 kDa Gelatinase. Truncated MMP 9 (50 kDa) was purified from the supernatant of baculovirus-infected Sf-9 cells. All procedures were performed at 4 °C. A 10 mL bed of gelatin−agarose (Sigma) was equilibrated with 10 column volumes of 50 mM Tris, pH 7.5, 200 mM NaCl, 10 μM $ZnSO_4$, 5 mM $CaCl_2$, and 0.005% Brij 35 (buffer A). Supernatants containing gelatinase B were loaded onto the gelatin−agarose column at a flow rate of 2 mL/min. The material was eluted with 50 mL of buffer A containing 1 M NaCl and 7.5% DMSO using a flow rate of 0.5 mL/min. Fractions were collected across the protein peak and assayed for activity by zymography (Novex 10% zymogram gels). The two most active fractions were pooled (10 mL), divided into 1 mL aliquots, and stored at −80 °C. For assays using peptide substrates, the enzyme was activated with 1 mM 4-aminophenylmercuric acetate (APMA) for 4 h at 37 °C. The APMA was removed by desalting through Bio-Rad P6 Bio-Spin columns equilibrated with buffer A.

Substrate Assays. The TNFα substrate, Dnp-SPLAQAVRSSSR-NH_2, 50 μM, was incubated with the standard Dnp-Ser (20 μM) in buffer containing 0.0035%−0.0075% Brij 35, 10 mM HEPES, pH 7.5, and the protease inhibitors, E-64 (10 μM), pepstatin (1 μM), leupeptin (10 μM), and phosphoramidon (10 μM), in a final reaction volume of 100 μL. Microsomal TACE, recombinant TACE, MMP 1, MMP 9, MMP 7, or MMP 3 was added, and the samples were incubated at 22 or 37 °C for 10 min to 4 h. Samples were quenched by addition of an equal volume of 1% heptafluorobutyric acid (HFBA). Separation of substrate and product was performed on a C-18 reverse phase column (Vydac, 150 mm length), using a 22−35% water/acetonitrile gradient. Both the water and acetonitrile contained 0.1% HFBA. All separations were performed with a HP 1090 liquid chromatographer from Hewlett Packard.

For peptide substrates corresponding to the cleavage sites of TNFR-I (Dnp-LPQLENVKGTED-NH_2) and -II (Dnp-SMAPGAVHLPQP-NH_2), APP (Dnp-EVHHQKLVFFAE-NH_2), KL-1 (Dnp-LPPVAASSLRND-NH_2), Notch (Dnp-PYKIEAVKSEPV-NH_2), HER4 (Dnp-HGLSLPVEN-RLYTYDH-NH_2), IL-6R (Dnp-TSLPVQDSSSVP-NH_2), and RANKL (Dnp-IVGPQRFSGAPA-NH_2), the same conditions were used, at a substrate concentration of 20 μM. Reactions were also performed with the TNFα substrate Dnp-SPLAQAVRSSSR-NH_2 at that same concentration for comparative purposes.

The substrate Dnp-PChaGC(Me)HK(NMA)-NH_2 was diluted from a DMSO stock solution to a concentration of 30 μM in buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 10 μM $ZnSO_4$, and 0.005% Brij 35 (buffer A). A 60 μL aliquot was removed and added to a black Polyfiltronics 96-well plate. TACE, MMP 1, MMP 9, MMP 7, or MMP 3 was diluted from stock solutions into buffer A. The enzyme (60 μL) was added, and fluorescence was monitored at an excitation of 343 nm and emission of 450 nm. Reactions were quenched by addition of 30 μL of 0.5 M EDTA. LC/MS analysis was performed to establish the identity of the products.

For the substrate NBD-RPKPLANvaWK(DMC)-NH_2, microsomal TACE was added to 10−20 μM substrate in buffer containing 10 mM HEPES, pH 7.5, and the protease inhibitors E-64 (10 μM), pepstatin (1 μM), leupeptin (10 μM), phosphoramidon (10 μM), and diprotinin A (10 μM). The final volume of the reaction was 200 μL. For reactions with rTACE, protease inhibitors were excluded. Fluorescence was monitored at an excitation wavelength of 343 nm and emission of 450 nm using a SLT Fluostar fluorescence plate reader.

Titration of TACE. In a 10 μL assay volume, TACE and inhibitor GW 9471 (0−1 μM) were directly added to 10 μL of buffer containing 100 μM Dnp-SPLAQAVRSSSR-NH_2, 20 μM Dnp-Ser (standard), 10 mM HEPES, pH 7.5, and the protease inhibitors E-64 (10 μM), pepstatin (1 μM), leupeptin (10 μM), and phosphoramidon (10 μM). Reactions were run for 1−2 min at 22 °C and then quenched with a 50 μL volume of 1% HFBA. Samples were resolved on a C18 Vydac column as described above. Initial velocities calculated from the percent product formation were plotted

TNFα	SPLAQA	*	VRSSSR
APP	EVHHQK	*	LVFFAE
TNFR-I	LPQLEN	*	VKGTED
TNFR-II	SMAPGA	*	VHLPQP
Il-6R	TSLPVQ	*	DSSSVP
HER4	HGLSLPVENRLYTYDH[a]		
KL-1	LPPVAA	*	SSLRND
Notch	PYKIEA	*	VKSEPV
TRANCE	IVGPQR	*	FSGAPA

FIGURE 1: Cleavage sites of TNFα and other reported TACE substrates analyzed in this study. An asterisk denotes the scissile bond. The superscript a indicates that the physiological cleavage site has not been established conclusively.

as a function of inhibitor concentration, and the curve was fit to eqs 1 and 2 (*36*):

$$v = \{v_0[(E_0 - I_0 - K_{i\,app})^2 + 4K_{i\,app}E_0]^{1/2} + (E_0 - I_0 - K_{i\,app})\}/2E_0 \quad (1)$$

$$K_{i\,app} = K_i(1 + [S]/K_m) \quad (2)$$

The inhibition constant was determined independently (K_i = 8 nM) and was used as an initial estimate. The K_m and substrate concentrations were fixed parameters (K_m = 270 μM; [S] = 50 μM).

Titration of MMPs. The enzyme concentration of MMP 1, MMP 3, and MMP 9 was determined by active site titration with GW 9471. Several titrations were performed at two to six different enzyme concentrations. The inhibitor was diluted 3-fold from 100 to 0.002 nM for MMP 1 and MMP 9 and from 200 to 0.1 nM for MMP 3. The assays were run in buffer A with 10 μM fluorogenic substrate Dnp-PChaGC(Me)HAK(NMA)-NH_2 (*37*). Product formation was followed with a SLT Fluostar fluorescence detector at an excitation wavelength of 343 nm and an emission of 450 nm. Values were determined at enzyme concentrations 5−10 fold above the K_i for the inhibitor. Inhibition constants for the MMPs were determined independently and were 1.5, 3, and 1 nM for MMP 1, MMP 3, and MMP 9, respectively.

Analysis of Kinetic Parameters and Enzyme Concentrations. K_m values were calculated by nonlinear least-squares fitting of a plot of initial velocities vs substrate concentraton to the Michaelis−Menten equation. Specificity constants were calculated from initial velocities. Conditions of k_{cat}/K_m were verified by running reactions at more than one substrate concentration. Enzyme concentrations for TACE were calculated by fitting plots of initial velocities vs inhibitor concentration to the Morrison equation (*36*) using the program KineTic v 1.34 from Biokin Ltd.

RESULTS

Processing of TACE Substrates. Since the isolation of TACE and cloning of its cDNA, several groups have reported that cells derived from mice that bear a partial deletion in TACE's gene (*1*) fail to process a host of ectodomains. This increasing list includes APP, L-selectin, TNFα receptors I and II, TGFα, HER4, IL-6R, fractalkine, M-CSFR, CD30, GHR, the Notch 1 receptor, and PrP^c. Alignment of the cleavage sites for these substrates indicates no obvious consensus sequence elements to explain this broad specificity (Figure 1, partial list). Although the knockout gene was constructed by eliminating a short exon encoding the zinc binding motif of TACE, the possibility of a dominant negative effect seemed unlikely on the basis of control experiments (*14*). Therefore, TACE has been proposed to act as a "lawnmower" sheddase that will cleave an ectodomain as long as the distance of the segment between the membrane plane and the globular portion of the substrate ectodomain allows for accessibility to the cleavage site. Therefore, sequence specificity is deemed as not important by this model.

For TACE to be an effective sheddase in vivo, its processivity for alternate substrates must be comparable to the one reported for TNFα. We decided to directly address this hypothesis by testing TACE's specificity constant for proteolytic processing of synthetic dinitrophenyl-labeled dodecapeptides comprising the cleavage sites of TNFα, KL-1, TNFR-I and -II, HER4, IL-6R, and Notch. We also added to this series APP and the receptor activator of nuclear factor-κB ligand (RANKL). APP processing shows a TACE component and an additional component that appears to correspond to ADAM 10, a TACE homologue (*38*, *39*). RANKL appears to be converted by the membrane-type 1 matrix metalloproteinase (MT1-MMP; *40*).

Under conditions used to detect TNFα substrate cleavage (enzyme concentration in the subnanomolar range), recombinant forms of TACE processed only the TNFα substrate itself, with a k_{cat}/K_m of (1.2−1.7) × 10^5 M^{-1} s^{-1} (Figure 2 and Table 1). Cleavage occurred at the expected site, as confirmed by mass spectrometry (Table 1). At much higher enzyme concentrations (0.5−1 μM), slow cleavage of APP and RANKL was also detected, resulting in modest k_{cat}/K_m values relative to those for TNFα [(0.5−3) × 10^3 M^{-1} s^{-1}, Table 1]. In all of these cases, correct cleavage was verified by mass spectrometric analysis of expected products (Table 1). We also observed cleavage of KL-1, TNFR-II, and HER4 at high enzyme ratios, but incorrect products were detected in addition to the predicted one (Figure 2 and Table 1). The k_{cat}/K_m was also low [(0.3−1.2) × 10^3 M^{-1} s^{-1}, Table 1]. Finally, no turnover was observed for the substrates TNFR-I, IL-6R, and Notch, even at high enzyme ratios. These results obtained with two different enzyme preparations indicate that TACE has distinct substrate preferences. Even under conditions when the concentration of the substrate is saturating, this enzyme will display drastically different processivity depending on the specific sequence of each substrate.

Cleavage assays were repeated using a subcellular fraction from MonoMac 6 human macrophages highly enriched in TACE activity (*33*). This was done in order to compare our results obtained with soluble, recombinant forms of TACE against the full-length, native TACE form embedded in membranes of the secretory pathway of natural TNFα-secreting cells. Results were essentially the same: only the TNFα peptide substrate was turned over, at a rate of 2.06 $nmol^{-1}$ (mg of protein)$^{-1}$ min^{-1}. All other substrates were either not turned over at all or were processed incorrectly at low rates.

Processing of Precursor TNFα by TACE and MMPs. We compared the kinetics of TACE-mediated cleavage of precursor TNFα relative to members of the MMP family. Previous studies have indicated that TACE and MMPs process precursor TNFα to generate bioactive TNFα (*1*, *2*, *28*). The specificity constants for TACE, but not for the MMPs, were recently reported (*2*). It is therefore difficult

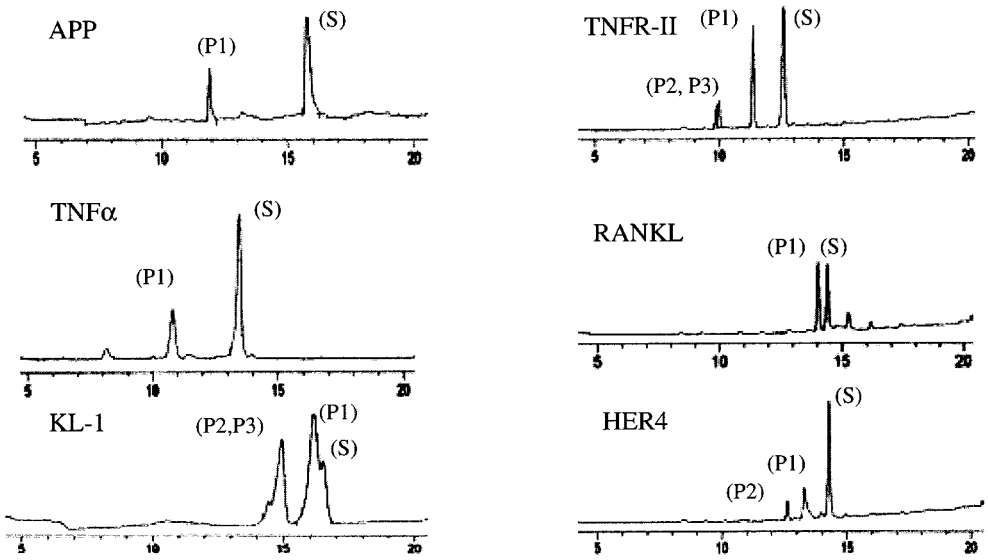

FIGURE 2: HPLC chromatograms of reaction products after incubation of TACE with substrates at a concentration of 20 μM for 30 min at 37 °C. S: substrate peak. P: product peak. Other peaks not noted correspond to minor impurities present in the substrate stock.

Table 1: Cleavage of Reported TACE Substrates by Recombinant Forms of TACE

TACE substrate	Dnp-peptide sequence	Dnp-peptide products	k_{cat}/K_m 473[b] ($M^{-1}s^{-1}$)	k_{cat}/K_m 651[c] ($M^{-1}s^{-1}$)
TNFα	SPLAQA*VRSSSR	SPLAQA (P1)	1.7×10^5	1.2×10^5
APP	EVHHQK*LVFFAE	EVHHQK (P1)	1.8×10^3	0.9×10^3
HER4	HGLSLPVENRLYTYDH[a]	HGLSPVE (P1)	1.2×10^3	0.8×10^3
		HGLSLP (P2)	1.1×10^3	0.3×10^3
c-KLR	LPPVAA*SSLRND	LPPVAA (P1)	0.5×10^3	0.3×10^3
		LPPVAAS (P2)	2.0×10^3	0.3×10^3
		LPPVAASS (P3)	0.7×10^3	2.0×10^3
RANKL	IVGPQR*FSGAPA	IVGPQR (P1)	2.7×10^3	0.5×10^3
TNFR-II	SMAPGA*VHLPQP	SMAPGAVH (P1)	3.0×10^3	0.8×10^3
		SMAPGA (P2)	1.0×10^3	0.5×10^3
		SMAPG (P3)	0.6×10^3	0.1×10^3
TNFR-I	LPQLEN*VKGTED	LPQLEN (P1)	$<1 \times 10^2$ [d]	$<1 \times 10^2$ [d]
IL-6R	TSLPVQ*DSSSVP	TSLPVQ (P1)	$<1 \times 10^2$ [d]	$<1 \times 10^2$ [d]
NOTCH	PYKIEA*VKSEPV	PYKIEA (P1)	$<1 \times 10^2$ [d]	$<1 \times 10^2$ [d]

[a] The physiological cleavage site has not been established conclusively. [b] 473: recombinant TACE, mature catalytic domain. [c] 651: recombinant TACE, mature ectodomain. [d] No detectable cleavage. Errors are within 30% of reported values.

Table 2: Cleavage Products from ProTNFα

enzyme	MMP 1	MMP 9	MMP 7	MMP 3	TACE
cleaved bond	A74/Q75	A74/Q75		NR[a]	
	A76/V77		A76/V77	NR	A76/V77

[a] NR: no conversion observed.

to estimate the relative importance that each of these enzymes has in processing precursor TNFα in vivo. Therefore, substrate specificities were determined for native and recombinant forms of TACE and were compared to several MMPs.

We first investigated whether the MMPs and TACE were able to process precursor TNFα at the correct cleavage sequence. MMP 1, MMP 3, MMP 7, MMP 9,and TACE were incubated with precursor TNFα, and the mature TNFα generated from the reaction was subjected to N-terminal sequencing. The results are indicated in Table 2. Whereas TACE and MMP 7 cleaved this substrate solely between Ala76 and Val77, MMP 1 processed the precursor TNFα polypeptide between Ala74 and Gln75 as well as between Ala76 and Val77 (Table 2). In addition, MMP 9 processed the natural substrate solely between Ala74 and Gln75. Interestingly, MMP 3 did not react at all with precursor TNFα, even at high concentrations of enzyme (>100 nM). At comparable enzyme concentrations (see Materials and Methods section), MMP 1 was more efficient than MMP 3 at processing the natural substrate. In fact, MMP 3 did not process the precursor TNFα at all under reaction conditions of 100 nM enzyme. In contrast, MMP 7 and MMP 9 were capable of converting precursor TNFα to a 17 kDa band at comparable levels.

Specificity constants were obtained for the conversion of precursor TNFα by TACE and MMP 7, at pH 7.5 and 22 °C, by directly measuring product formation using an L-929 killing assay or ELISA (see Materials and Methods section). The results are shown in Table 3. Microsomal and recombinant TACE processed the natural substrate with a k_{cat}/K_m of $(1.7 \pm 0.2) \times 10^4$ M^{-1} s^{-1} and $(1.0 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, respectively. The specificity constant for TACE was 30-fold higher than the one for MMP 7. However, the k_{cat}/K_m for cleavage of the synthetic substrate, Dnp-SPLAQAVRSSSR-NH_2, by microsomal TACE or recombinant TACE, was 10-fold higher than for the cleavage reaction with precursor TNFα (Table 3).

Table 3: Cleavage of TACE Substrates[a]

enzyme	k_{cat}/K_m ($M^{-1}\,s^{-1}$) product Dnp-SPLAQA	k_{cat}/K_m ($M^{-1}\,s^{-1}$) product TNFα
MMP 7	1×10^3	7×10^2
mTACE	2×10^5	2×10^4
rTACE	2×10^5	1×10^4

[a] MMP 7 and mTACE reactions performed at 22 °C. rTACE reactions performed at 37 °C. Errors were within 30% of reported values.

Table 4: Cleavage of the Substrate Dnp-SPLAQAVRSSSR−NH_2[a]

enzyme	k_{cat}/K_m ($M^{-1}\,s^{-1}$) product Dnp-SPLAQA	k_{cat}/K_m ($M^{-1}\,s^{-1}$) product Dnp-SPLA
MMP 1	$7 \times 10^{2\,b}$	$3 \times 10^{2\,b}$
	$1 \times 10^{3\,c}$	$2 \times 10^{3\,c}$
MMP 9	$<8 \times 10^{2\,c}$	$7 \times 10^{3\,c}$
	$7 \times 10^{3\,c}$	$6 \times 10^{4\,c}$
MMP 3	$9 \times 10^{2\,b}$	$1 \times 10^{2\,b}$
MMP 7	$1 \times 10^{3\,c}$	not observed
mTACE	$2 \times 10^{5\,b}$	not observed
	$3 \times 10^{5\,c}$	
rTACE	$2 \times 10^{5\,c}$	not observed

[a] Errors were within 20% of reported values. [b] Reaction performed at pH 7.5 and 22 °C. [c] Reaction performed at pH 7.5 and 37 °C.

Specificity Constants for the TACE-Specific Substrate. The specificity constants were obtained for the cleavage of the synthetic substrate Dnp-SPLAQAVRSSSR-NH_2 by the MMPs to compare them to the k_{cat}/K_m for cleavage by TACE. The MMPs and TACE were reacted at 22 and/or 37 °C and pH 7.5 with this synthetic TACE substrate. Reaction products were separated by reverse-phase HPLC, and k_{cat}/K_m values were calculated for each enzyme. The results are shown in Table 4. Whereas TACE processed the synthetic substrate between Ala and Val, MMP 1 and MMP 3 cleaved Dnp-SPLAQAVRSSSR-NH_2 between Ala-Val and Ala-Gln with almost equivalent k_{cat}/K_m values (Table 4). The specificity constants for reactions with more than one product generated were calculated from initial velocities measured under conditions of low percent turnover (<10%) so that the substrate concentration did not change significantly. Furthermore, full progress curves were run to ensure that the products were not generated processively but, rather, independently from one another. MMP 9 cleaved predominantly between Ala and Gln whereas MMP 7 processed the substrate solely between Ala and Val. Microsomal TACE also cleaved Dnp-SPLAQAVRSSSR-NH_2 between Ala and Val but did so with a specificity constant at pH 7.5 and 37 °C of $(2.7 \pm 0.3) \times 10^5$ M^{-1} s^{-1}. The k_{cat}/K_m for the recombinant form of TACE was similar (Table 4). The TACE rate constants were 300−1000-fold faster than any of the k_{cat}/K_m values reported for the MMPs (Table 4).

Specificity Constants for the Substrates Dnp-PChaGC-(Me)HK(NMA)NH_2 and NBD-RPKPLANvaWK(DMC)-NH_2. The MMP fluorescent substrate Dnp-PChaGC(Me)HK-(NMA)-NH_2 was described previously as an efficient substrate for MMP 1 and MMP 9 but not MMP 3 (*37*). TACE and MMP 1, MMP 9, MMP 3, and MMP 7 were incubated with the fluorescent substrate, and k_{cat}/K_m values were calculated. MMP 9 processed this substrate with the highest k_{cat}/K_m (3×10^4 M^{-1} s^{-1}, Table 5). MMP 1 and MMP 7 were 10- and 30-fold worse than MMP 9 with specificity constants of 3×10^4 M^{-1} s^{-1} and 10^4 M^{-1} s^{-1}, respectively. MMP 3 had the lowest k_{cat}/K_m value for this substrate (Table 5). In contrast, TACE did not turn over the MMP substrate even at high concentrations of enzyme (>20 nM). Therefore, the k_{cat}/K_m was estimated to be less than 10^3 M^{-1} s^{-1}.

Table 5: Cleavage of the Substrate Dnp-PChaGC(Me)HK(NMA)-NH_2

enzyme	product Dnp-PChaG k_{cat}/K_m ($M^{-1}\,s^{-1}$)	temp (°C)
MMP 1	3×10^4	37
MMP 9	3×10^5	37
MMP 3	5×10^2	37
MMP 7	1×10^4	22
mTACE	NR[a]	37
rTACE	NR[b]	37

[a] The k_{cat}/K_m for this reaction has an upper limit of 1×10^3 M^{-1} s^{-1} since no turnover was seen at the highest achievable concentrations of mTACE (20 nM). [b] The k_{cat}/K_m for this reaction has an upper limit of 1×10^2 M^{-1} s^{-1} since no turnover was seen at concentrations of rTACE up to 200 nM.

NBD-RPKPLANvaWK(DMC)-NH_2 was reported to be an excellent substrate for MMP 3 (*41*) with a k_{cat}/K_m of 3×10^4 M^{-1} s^{-1}. Recombinant TACE was reacted with NBD-RPKPLANvaWK(DMC)-NH_2 to determine whether this substrate could be turned over. TACE was able to process this substrate with a k_{cat}/K_m that was close to what was reported for MMP 3 (7.5×10^3 M^{-1} s^{-1}). The microsomal enzyme turned over this same substrate faster, indicating that there may have been cleavage by a contaminating proteolytic activity (data not shown). Mass spectrometric analyses of both reaction mixtures were performed to determine the site of cleavage. Both MMP 3 and TACE processed this synthetic substrate between Ala and Nva.

DISCUSSION

Despite profuse genetic evidence for TACE involvement in shedding of more than a dozen cellular ectodomains, little information is available on the relative efficiency displayed by this enzyme against their seemingly different cleavage sites (Figure 1). By using a series of model dinitrophenyl-labeled dodecapeptide substrates, here we provide a direct, side-by-side comparison of TACE's processivity against each of them. Surprisingly, our results show that only the TNFα substrate is processed, as previously reported (*1*, *2*, *31*). This enzyme exhibits very poor or no processivity at all against all other substrates. At least some of the values obtained for them may represent nonspecific cleavage at high TACE concentrations, since they are similar to the ones obtained for incorrect products and for a negative control substrate, RANKL (Table 1). This ligand appears to be processed by the membrane-bound matrix metalloproteinase MT1-MMP (*40*).

The fact that essentially the same results were obtained with vesicles containing native TACE, derived from human macrophages, and with recombinant soluble forms of TACE indicates that this lack of processivity is not due to the lack of a native membrane environment, neither is it due to differences in the catalytic properties of TACE's recombinant forms relative to the native enzyme.

TGFα release also seems to be mediated by TACE: $TACE^{-/-}$ fibroblasts showed decreased shedding of this

ectodomain (*14*). Such observation was confirmed recently using TACE$^{-/-}$ keratinocytes, a more physiological cell type (*42*). It is important to point out that significant residual shedding was observed. Even though biochemical experiments with isolated TACE and model peptide substrates were performed (*14*, *42*), quantitative information was lacking for either the cleavage between TGFα and its N-terminal pro domain (site 1) or the cleavage between TGFα and its transmembrane domain (site 2). Recent evidence does suggest that additional activities are involved. Merlos-Suarez and co-workers have conclusively showed metalloprotease-dependent TGFα shedding in the absence of TACE (*39*). Consistent with this, Moss and collaborators found evidence for an additional enzymatic activity that processes TGFα shedding with an inhibition profile inconsistent with TACE (C. L. Hinkle, M. J. Mohan, M. E. Milla, P. Lin, W. E. Russell, M. Stevenson, D. C. Lee, and M. L. Moss, in preparation). Finally, this latter study also provides for the first time quantitative evidence demonstrating that TACE processes efficiently TGFα's site 1 (the site for maturation) but not site 2 (the site for shedding).

It can be argued that these model peptides do not really reflect the way that intact substrates are presented to TACE's active site cleft. Therefore, the observed k_{cat}/K_m values do not approximate true enzyme processivity in vivo. This seems highly unlikely, since we have also shown that, on the contrary, cleavage of the complete precursor TNFα substrate exhibits a specificity constant about an order of magnitude slower than for cleavage of Dnp-SPLAQAVRSSSR-NH_2 (Table 3). This discrepancy most likely arises from differences in the diffusion limit, which is faster for small synthetic peptides, and is also due to steric constraints associated with the docking of a complete substrate as opposed to a small, soluble peptide. Therefore, substrate presentation and membrane topology considerations cannot account for the differential behavior of TACE against different substrates relative to the TNFα peptide. On the other hand, colocalization of both TACE and substrates to specific regions of the plasma membrane such as lipid rafts or caveolae (*43*, *44*) may increase their concentrations, making those shedding events viable in vivo. Substrate processivity may also be increased via interactions different from the ones formed between the catalytic cleft and the substrate's cleavage site. Such appears to be the case for ADAM 10 cleavage of ephrin A-2 (*45*).

One way of reconciling our results with previous observations involving TACE$^{-/-}$ cells is to argue for the existence of adaptor proteins that increase TACE's affinity for substrates different from TNFα. Those adaptor proteins may act as "substrate loaders". The existence and identity of such factors remain to be proven, but it may allow reinterpretation of available genetic data. Studies with ADAM 10 and ADAM 13 mutants have revealed dominant negative phenotypes (*38*, *46*). TACE (ADAM 17), ADAM 10, and ADAM 13 mutants were constructed by deleting a short exon encoding the zinc binding motive. This allows for the expression of the ADAM$^{\Delta Zn/\Delta Zn}$ polypeptide. It may be possible that TACE is able to cleave precursor TNFα or TGFα's site 1 by itself but needs to associate with other factors in order to process all of the other reported substrate ectodomains. Should that be the case, the TACE $^{-/-}$ knockout phenotype would be recessive for TNFα/TGFα shedding but dominant negative for all other substrates tested.

TACE is a unique metalloprotease even though certain hydroxamic acid inhibitors can inhibit both TACE and the MMPs (*9*−*11*). In fact, it has been shown that TACE's inhibition by TIMP (tissue inhibitor of metalloproteinases) proteins is markedly different relative to MMPs: TACE is inhibited by TIMP-3 but not by TIMP-1, -2, or -4 (*47*). Previous reports have investigated the processing of precursor TNFα by the MMPs. However, the complete cleavage sequences were not presented nor were the specificity constants for the reactions given. We emphasize here the importance of determining sites of cleavage and specificity constants in order to elucidate which enzyme is responsible for the in vivo processing activity of precursor TNFα. Besides TACE, only MMP 7 cleaves ProTNFα as well as the synthetic substrate, Dnp-SPLAQAVRSSSR-NH_2, solely at the correct cleavage site. Since mature TNFα begins with Val77 at the N-terminus, MMP 1 and MMP 9 are ruled out as processing enzymes for precursor TNFα in vivo since they cleave both the natural substrate and synthetic substrates between either Ala and Gln or Ala and Val or both. In addition, MMP 3 is also ruled out because it did not turn over precursor TNFα even under conditions of high enzyme concentration. Interestingly, genetic evidence further supports a role for MMP 7 in nonsystemic TNFα release from macrophages (*28*).

The synthetic substrate k_{cat}/K_m data also confirm the observation that TACE is a unique metalloprotease. The k_{cat}/K_m of cleavage of precursor TNFα by TACE is 30-fold higher than for MMP 7. In addition, the k_{cat}/K_m for processing of the synthetic substrate, Dnp-SPLAQAVRSSSR-NH_2, with TACE, is 100−1000-fold higher than with any of the MMPs tested. Major differences are also observed with the MMP substrate Dnp-PChaGC(Me)HK(NMA)-NH_2. The specificity constant for cleavage of this peptide by TACE is estimated to be less than 1×10^3 M^{-1} s^{-1} since the enzyme could not process the substrate under any reaction conditions tested. MMP 9, in contrast, has a k_{cat}/K_m for this substrate of 3×10^5 M^{-1} s^{-1}. MMP 7, the enzyme that most closely resembles TACE in terms of substrate requirements, is at least 10-fold better than TACE at processing Dnp-PChaGC(Me)HK-(NMA)-NH_2. In contrast, the MMP substrate NBD-RPK-PLANvaWK(DMC)-NH_2 is turned over effectively by TACE even though it has a truncated C-terminus relative to Dnp-SPLAQAVRSSSR-NH_2. The cleavage sites of the NBD-RPKPLANvaWK(DMC)-NH_2 substrate with MMP 3 and TACE are identical and are between Ala and Nva for both enzymes. This is not surprising since the substituents surrounding the scissile bond are comparable with Ala, Val, and Arg vs Ala, Nva, and Trp at P_1, P_1', and P_2', respectively.

In summary, we provide evidence indicating that TACE is a selective converting enzyme that efficiently cleaves TNFα only. TACE's biochemical behavior toward other substrates suggests that the presence of additional factors, yet to be discovered, may play a role in alternate substrate presentation. Such high specificity is also evidenced by TACE's distinct substrate preferences compared to that of several MMPs, including MMP 7, which seems to act as another physiological TNFα converting enzyme under specialized situations. Hopefully, such unique properties of TACE will, in the long term, justify it as a target for the design of selective and efficacious drugs for the treatment of acute and chronic inflammatory conditions.

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REFERENCES

1. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) *Nature 385*, 729−733.
2. Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J.-L., Warner, J., Willard, D., and Becherer, J. D. (1997) *Nature 385*, 733−736.
3. Vassali, P. (1992) *Annu. Rev. Immunol. 10*, 411−452.
4. Utsumi, T., Levitan, A., Hung, M.-C., and Klostergaard, J. (1993) *J. Biol. Chem. 268*, 9511−9516.
5. Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., and Kriegler, M. (1990) *Cell 63*, 251−258.
6. Piguet, P. F., Grau, G. E., Allet, B., and Vasalli, P. (1987) *J. Exp. Med. 166*, 1280−1289.
7. van Dullemen, H. M., van Deventer, S. J., Hommes, D. W., Bijl, H. A., Jansen, J., Tytgat, G. N., and Woody, J. (1995) *Gastroenterology 109*, 129−135.
8. Williams, R. O., Feldman, M., and Maini, R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9784−9788.
9. Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Nayee, P., Owen, K., Patel, S., Thomas, W., Wells, G., Wood, L. M., and Woolley, K. (1994) *Nature 370*, 555−557.
10. McGeehan, G. M., Becherer, J. D., Bast, R. C., Jr., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghese, M., Warner, J., and Ways, J. P. (1994) *Nature 370*, 558−561.
11. Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., and Black, R. A. (1994) *Nature 370*, 218−220.
12. Schlondorff, J., and Blobel, C. P. (1999) *J. Cell Sci. 112*, 3603−3617.
13. Buxbaum, J. D., Liu, K. N., Luo, Y., Slack, J. L., Stocking, K. L., Peschon, J. J., Johnson, R. S., Castner, B. J., Cerretti, D. P., and Black, R. A. (1998) *J. Biol. Chem. 273*, 27765−27767.
14. Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnaborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J., and Black, R. A. (1998) *Science 282*, 1279−1280.
15. Solomon, K. A., Pesti, N., Wu, G., and Newton, R. C. (1999) *J. Immunol. 163*, 4105−4108.
16. Rio, C., Buxbaum, J. D., Peschon, J. J., and Corfas, G. (2000) *J. Biol. Chem. 275*, 10379−10387.
17. Althoff, K., Reddy, P., Voltz, N., Rose-John, S., and Mullberg, J. (2000) *Eur. J. Biochem. 267*, 2624−2631.
18. Tsou, C. L., Haskell, C. A., and Charo, I. F. (2001) *J. Biol. Chem. 276*, 44622−44626.
19. Garton, K. J., Gough, P. J., Blobel, C. P., Murphy, G., Greaves, D. R., Dempsey, P. J., and Raines, E. W. (2001) *J. Biol. Chem. 276*, 37933−38001.
20. Rovida, E., Paccagnini, A., Del Rosso, M., Peschon, J., and Dello Sbarba, P. (2001) *J. Immunol. 166*, 1583−1589.
21. Hansen, H. P., Dietrich, S., Kisseleva, T., Mokros, T., Mentlein, R., Lange, H. H., Murphy, G., and Lemke, H. (2000) *J. Immunol. 165*, 6703−6709.
22. Zhang, Y., Jiang, J., Black, R. A., Baumann, G., and Frank, S. J. (2000) *Endocrinology 141*, 4342−4348.
23. Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A., and Israel, A. (2000) *Mol. Cell 5*, 207−216.
24. Vincent, B., Paitel, E., Saftig, P., Frobert, Y., Hartmann, D., De Strooper, B., Grassi, J., Lopez-Perez, E., and Checler, F. (2001) *J. Biol. Chem. 276*, 37743−37746.
25. Roghani, M., Becherer, J. D., Moss, M. L., Atherton, R. E., Erdjument-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempst, P., and Blobel, C. P. (1999) *J. Biol. Chem. 274*, 3531−3540.
26. Pandiella, A., Bosenberg, M. W., Huang, E. J., Besmer, P., and Massague, J. (1992) *J. Biol. Chem. 267*, 24028−24033.
27. Gearing, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J. M., Crimmin, M., Davidson, A. H., Drummond, A. H., Galloway, W. A., and Gilbert, R. (1995) *J. Leukocyte Biol. 57*, 774−777.
28. Haro, H., Crawford H. C., Fingleton, B., Shinomiya, K., Spengler D. M., and Matrisian, L. M. (2000) *J. Clin. Invest. 105*, 143−150.
29. Campion, C., Dickens, J. P., and Crimmin, M. J. (1990) Patent WO 90/05719, British Biotechnology.
30. Becherer, J. D., Howe, A. S., Patel, I., Wisely, B., Levine, H., and McGeehan, G. M. (1991) *J. Cell. Biochem., Suppl. 15G*, 139.
31. Milla, M. E., Leesnitzer, M. A., Moss, M. L., Clay, W. C., Carter, H. L., Miller, A. B., Su, J.-L., Lambert, M. H., Willard, D. H., Sheeley, D. M., Kost, T. A., Burkhart, W., Moyer, M., Blackburn, R. K., Pahel, G. L., Mitchell, J. L., Hoffman, C. R., and Becherer, J. D. (1999) *J. Biol. Chem. 274*, 30563−30570.
32. Ziegler-Heitbrook, H. W., Thiel, E., Futterer, A., Herzog, V., Wirtz, A., and Riethmuller, G. (1988) *Int. J. Cancer 41*, 456−461.
33. Moss, M. L., Becherer, J. D., Conway, J. G., Warner, J. R., Bickett, D. M., Leesnitzer, M. A., Seaton, T. M., Mitchell, J. L., McConnell, R. T., Tippin, T. K., Whitesell, L. G., Rizzolio, M. C., Hedeen, K. M., Beaudet, E. J., Andersen, H. M., Lambert, M. H., Austin, J. R., Stanford, J. B., Bubacz, D. G., Chan, J. H., Schaller, L. T., Gaul, M. G., Cowan, D. J., Boncek, M. H., Rabinowitz, H., Musso, D. L., McDougald, D. L., Kaldor, I., Glennon, K., Wiethe, R. W., Guo, Y., and Andrews, R. C. (2000) *Cytokine Inhibitors* (Ciliberto, G., and Savino, R., Eds.) pp 163−177, Dekker, New York.
34. Matthews, N., and Neale, M. L. (1987) *Lymphokines and Interferons: A Practical Approach* (Clemens, M. J., Morris, A. G., and Gearing, A. J. H., Eds.) pp 221−225, IRL Press, Oxford.
35. Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035−10038.
36. Williams, J. W., and Morrison, J. F. (1979) *Methods Enzymol. 63*, 437−467.
37. Bickett, D. M., Green, M. D., Berman, J., Dezube, M., Howe, A. S., Brown, P. J., Roth, J. T., and McGeehan, G. M., (1993) *Anal. Biochem. 212*, 58−64.
38. Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., and Fahrenholz, F. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3922−3927.
39. Merlos-Suarez, A., Fernandez-Larrea, J., Reddy, P., Baselga, J., and Arribas, J. (1998) *J. Biol. Chem. 273*, 24955−24962.
40. Schlondorff, J., Lum, L., and Blobel, C. P. (2001) *J. Biol. Chem. 276*, 14665−14674.
41. Bickett, D. M., Green, M. D., Wagner, C., Roth, J. T., Berman, J., and McGeehan, G. M. (1994) *Ann. N.Y. Acad. Sci. 732*, 351−355.
42. Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschon, J. J., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A., and Lee, D. C. (2002) *J. Biol. Chem. 277*, 12838−12845.
43. Galbiati, F., Razani, B., and Lisanti, M. P. (2001) *Cell 106*, 403−411.
44. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell Biol. 1*, 31−39.
45. Hattori, M., Osterfield, M., and Flanagan, J. G. (2000) *Science 289*, 1360−1365.
46. Alfandari, D., Cousin, H., Gaultier, A., Smith, K., White, J. M., Darribere, T., and DeSimone, D. W. (2001) *Curr. Biol. 11*, 918−930.
47. Amour, A., Slocombe, P. M., Webster, A., Butler, M., Knight, C. G. Smith, B. J., Stephens, P. E., Shelley, C., Hutton, M., Knauper, V., Docherty, A. J., and Murphy, G. (1998) *FEBS Lett. 435*, 39−44.

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