Specific Sequence Elements Are Required for the Expression of Functional Tumor Necrosis Factor-α-converting Enzyme (TACE)*

(Received for publication, May 28, 1999, and in revised form, August 19, 1999)

Marcos E. Milla, M. Anthony Leesnitzer, Marcia L. Moss, William C. Clay, H. Luke Carter, Ann B. Miller, Jui-Lan Su, Millard H. Lambert, Derril H. Willard, Douglas M. Sheeley, Thomas A. Kost, William Burkhardt, Mary Moyer, R. Kevin Blackburn, Gregory L. Pahel, Justin L. Mitchell, Christine R. Hoffman, and J. David Becherer

From the Department of Biochemistry and Biophysics and Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 and the Divisions of Biochemistry and Chemistry, Glaxo Wellcome Research and Development, Research Triangle Park, North Carolina 27709

The tumor necrosis factor-α-converting enzyme (TACE) is a membrane-anchored zinc metalloprotease involved in precursor tumor necrosis factor-α secretion. We designed a series of constructs containing full-length human TACE and several truncate forms for overexpression in insect cells. Here, we demonstrate that full-length TACE is expressed in insect cells inefficiently: only minor amounts of this enzyme are converted from an inactive precursor to the mature, functional form. Removal of the cytoplasmic and transmembrane domains resulted in the efficient secretion of mature, active TACE. Further removal of the cysteine-rich domain located between the catalytic and transmembrane domains resulted in the secretion of mature catalytic domain in association with the precursor (pro) domain. This complex was inactive and function was only restored after dissociation of the complex by dilution or treatment with 4-aminophenylmercuric acetate. Thereafter, the pro domain of TACE is an inhibitor of the catalytic domain, and the cysteine-rich domain appears to play a role in the release of the pro domain. Insect cells failed to secrete a deletion mutant encoding the catalytic domain but lacking the inhibitory pro domain. This truncate was inactive and extensively degraded intracellularly, suggesting that the pro domain is required for the secretion of functional TACE.

TNFα is a potent cytokine that is secreted by activated monocytes and macrophages in a tightly regulated manner (1). Upon release, TNFα mediates the recruitment and activation of inflammatory cells to injured or infected tissues (2). Elevated levels of circulating TNFα have been demonstrated in several acute and chronic pathological states, such as lipo polymyositis, rheumatoid arthritis, Crohn’s disease, and inflammatory bowel disease (3). TNFα is synthesized as a pro, membrane-anchored form facing the lumenal/extracellular side of the secretory pathway. Our group and others have shown that proTNFα is released from cells after endoproteolytic cleavage at positions Ala76-Val77, mediated by a zinc metalloprotease sensitive to hydroxamic acid inhibitors (4–6). Because neutralization of TNFα activity has been demonstrated in the clinic, this enzyme constitutes a potential target for drug discovery.

The TNFα-converting enzyme (TACE) was purified to homogeneity and cloned (7, 8). Analysis of its amino acid sequence demonstrates a multidomain protein closely resembling members of the disintegrin family of metalloproteases, also commonly referred to as ADAMs or metalloprotease and disintegrin-containing proteins (9). Starting at the N terminus, TACE exhibits a classical signal peptide followed by a ~200-residue pro domain that includes a consensus cysteine switch motif (PKVCGY186), which can act as an inhibitor by ligating the zinc ion in the catalytic site (10, 32). The catalytic domain starts downstream from a consensus furin cleavage site (RVKRR215) and contains a canonical zinc binding site and a MYP loop involved in formation of the P1’ pocket (11). A ~200-amino acid cysteine-rich domain follows, including a 100-amino acid disintegrin-like region. A single transmembrane domain defines the end of the catalytic domain of TACE and is followed by a ~150-residue cytosolic tail that contains consensus sequences for binding to proteins containing Src homology 2 and Src homology 3 domains. Little is known at this point about the role these different domains play in regulating catalytic activity and physiological substrate recognition. TACE, ADAM 9, and ADAM 10 are the only members of this family for which proteolytic activity on a specific substrate has been demonstrated. TACE and Kuzbanian (an ADAM metalloprotease involved in processing of the Drosophila protein Notch and neuronal development (12)) are the only two members of this family for which the physiological substrates are known.

We report here the construction, overexpression, and characterization of full-length recombinant TACE and a series of truncates nested around the predicted catalytic domain sequence using baculoviral expression vectors. A comparison is presented of the activity of the different truncates against a synthetic peptide substrate containing the cleavage site of this cytokine. Such recombinant forms of TACE will be of use in establishing relationships between the domain architecture of TACE and the function and regulation of this enzyme.

MATERIALS AND METHODS

Recombinant TACE Virus Production—The full-length cloning of human TACE cDNA has been previously described (7). This insert was subcloned into pFastBac1 by using engineered Ncol and BamHI sites at the 5’ and 3’ ends of the clone, respectively. Construction of Met1 to Arg261 has been reported elsewhere (7). The following truncates were also made by expression cassette polymerase chain reaction (Fig. 1):
Domain Dissection of TACE

Protein chemistry and purification were performed as described previously (7). The purification consists of the combined use of Q-Sepharose, concanavalin A-Sepharose, and Superdex S75 gel permeation chromatography to purify the recombinant protein to homogeneity from concentrated insect cell culture media. Arg473 was purified in a way similar to Arg651, with the difference that 10 µM 4-aminophenylmercuric acetate (APMA) was added to the initial concentrate and all subsequent chromatography buffers (see under “Results”). The deletion mutant R473Δpro (see under “Results”) was purified as follows. T. ni cells were harvested 48 h postinfection by centrifugation at 2000 rpm in a Sorvall H6000-A rotor (1, 164 g) for 30 min. The supernatant was discarded, and the pellet was immediately resuspended in 100 mM sodium phosphate, pH 8.0, 10 mM Tris-Cl, pH 8.0, 1× Complete protease inhibitors mixture (Roche Molecular Biochemicals) and 6 mM guanidine hydrochloride (Buffer A), at a ratio of 100 ml of this buffer per liter of original culture. After stirring for 1 h on ice, the lysate was centrifuged at 13,000 rpm in a Sorvall GSA rotor (27,500 × g) for 45 min. The supernatant was immediately loaded onto a nickel chelate-agarose column (Qiagen, 10 ml of bead volume per liter of original culture), preequilibrated with Buffer A. The column was washed with 20 bed volumes of Buffer A, followed by elution with a 0–400 mM linear gradient of imidazole in Buffer A. The presence of R473Δpro was monitored by Coomassie stain of fractions after SDS-PAGE and by Western blotting using an antibody against the catalytic domain of TACE. Fraction elution from this chromatography step was monitored by absorbance at 280 nm, 12% denaturing SDS-polyacrylamide gels. In each case, nonspecific absorption of residuals, the lowest obtainable m/z 30564.

Expression of TACE Truncates—Logarithmically growing Trichoplusia ni cells were infected with TACE baculovirus at a multiplicity of infection of 1. Cultures were harvested at 24, 48, and 72 h postinfection. The cells were separated from the media by low speed centrifugation, and samples were frozen at −85 °C for further analysis. At the end of the experiment, cells and media were mixed with Laemmli sample buffer and subjected to SDS-PAGE analysis after visualization with Coomassie Brilliant Blue R250. Identical sets of samples were blotted onto nitrocellulose filters and probed with a mouse monoclonal antibody that recognizes the catalytic domain of TACE. Blots were developed using the Amersham Pharmacia Biotech ECL kit after incubation with a sheep anti-mouse horseradish peroxidase conjugate.

Purification of TACE Truncates—Arg651 was purified as described previously (7). The purification consists of the combined use of Q-Sepharose, concanavalin A-Sepharose, and Superdex S75 gel permeation chromatography to purify the recombinant protein to homogeneity from concentrated insect cell culture media. Arg473 was purified in a way similar to Arg651, with the difference that 10 µM 4-aminophenylmercuric acetate (APMA) was added to the initial concentrate and all subsequent chromatography buffers (see under “Results”). The deletion mutant R473Δpro (see under “Results”) was purified as follows. T. ni cells were harvested 48 h postinfection by centrifugation at 2000 rpm in a Sorvall H6000-A rotor (1, 164 g) for 30 min. The supernatant was discarded, and the pellet was immediately resuspended in 100 mM sodium phosphate, pH 8.0, 10 mM Tris-Cl, pH 8.0, 1× Complete protease inhibitors mixture (Roche Molecular Biochemicals) and 6 mM guanidine hydrochloride (Buffer A), at a ratio of 100 ml of this buffer per liter of original culture. After stirring for 1 h on ice, the lysate was centrifuged at 13,000 rpm in a Sorvall GSA rotor (27,500 × g) for 45 min. The supernatant was immediately loaded onto a nickel chelate-agarose column (Qiagen, 10 ml of bead volume per liter of original culture), preequilibrated with Buffer A. The column was washed with 20 bed volumes of Buffer A, followed by elution with a 0–400 mM linear gradient of imidazole in Buffer A. The presence of R473Δpro was monitored by Coomassie stain of fractions after SDS-PAGE and by Western blotting using an antibody against the catalytic domain of TACE. Fraction elution from this chromatography step was monitored by absorbance at 280 nm, 12% denaturing SDS-polyacrylamide gels. In each case, nonspecific absorption of residuals, the lowest obtainable m/z 30564.

Activity Assays—The activity of full-length TACE and its variants was determined in an assay employing the synthetic peptide Dnp-Ala-Pro-Pro-Lys-Arg-Pro (Peptide Institute) as substrate. Its sequence corresponds to the cleavage site of TACE on proTNFα. The ratio of product versus substrate was determined by measuring the absorbance at 370 nm of the correspondent peaks after HPLC separation of the reaction mixture using a C18 column (13). Activity was also measured against recombinant proTNFα by a gel-shift assay based on the substantial difference in mobility of the substrate (26 kDa) and products (17 and 9 kDa) in 12% denaturing SDS-polyacrylamide gels. In each case, nonspecific activity was determined by measuring product formation in the presence of 10 µM GW9471, an hydroxamic acid competitive inhibitor that totally blocks TACE activity at that concentration (4).

Association Studies—TACE Arg473 association with its pro domain was monitored by size exclusion chromatography using an HPLC silica-based 60-cm TSK column at a flow of 0.5 ml/min. Runs were done in the presence or absence of 10 µM APMA. Sedimentation equilibrium analytical ultracentrifugation was performed on TACE truncates Arg473 and Arg651 using a Beckman XL-A (Palo Alto, CA) centrifuge with either two-sector or six-sector 12-mm charcoal-filled epon centerpieces. TACE samples were centrifuged at 1 µM protein concentrations. Samples were studied in 20, 10, and 50 mM NaCl. Data were recorded at 17,500, 20,000, and 25,000 rpm for all samples at 4 °C with scans taken at 280 nm at 1-h intervals. Equilibrium was judged to be achieved by the absence of change between plots of several successive scans after approximately 20 h. 100 µl of each sample was centrifuged against 125 µl of the equivalent buffer blank. Solvent density was determined empirically at 4 °C using a Mettler DA-110 density/species gravity meter calibrated against water. The partial specific volume of each protein was calculated using the method of Cohn and Edsall (14). Temperature was incorporated using the appropriate equation (15) modified from values of each amino acid at 25 °C (16) and further modified as necessary for glycosylation content of the domains. Raw data were analyzed using the Beckman/Microcal Origin nonlinear regression software package using multiple iterations of the Marquardt-Levenberg algorithm for parameter estimation. Best fits were judged by the random distribution of residuals, the lowest obtainable χ² value, and the usefulness of the derived model.

Proteolysis and LC-MS Mapping—TACE samples were dissolved in 50 m urea/200 mM ammonium bicarbonate. Disulfide bonds were reduced by addition of dithiothreitol to a concentration of 4 mM and incubation at 50 °C for 15 min. After addition of iodoacetamide to a concentration of 8 mM, the sample was incubated at room temperature for an additional 15 min in the dark. Following reduction and alkylation, the samples were diluted to a final urea concentration of 2 mM and digested overnight (16 h) at 37 °C with trypsin (Promega, Madison, WI) with a 1:25 (w/w) ratio of enzyme to substrate. LC-MS was performed using a Hewlett-Packard (Palo Alto, CA) model 1090 HPLC system equipped with a Rheodyne (Cotati, CA) model 9125–080 injector, 50-µl loop, and a tunable UV absorbance detector. For all HPLC a capillary C18 column (LC Packings, San Francisco, CA) (0.320 × 150 mm, 300 Å) was used. Peptides were eluted using a two-part linear water/acetonitrile gradient from 1.6 to 64% organic over 100 min. Both buffers contained 0.06% trifluoroacetic acid. UV chromatograms were acquired at a wavelength of 214 nm. The column eluent was delivered to the IonSpray® (electrospray) source of an API III triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada). The mass spectrometer (Q1) was scanned from m/z 100 to 2350, stepping the orifice potential from 140 (m/z 100–450) to 60 V (m/z 450–2350), using a 0.5-Da step size and a dwell time of 0.5 ms.
Domain Dissection of TACE

RESULTS

Insect Cells Inefficiently Express Full-length, Functional TACE—A series of constructs was made to determine what the minimal primary sequence requirements are for the expression of functional TACE in a soluble, secreted form. For this, we used insect cells infected with several recombinant baculoviruses harboring different truncations in the TACE cDNA (Fig. 1): (a) full-length TACE, (b) Arg473d, comprising the signal peptide, pro, catalytic, and cysteine-rich domains; (c) Arg473pro, containing the signal peptide, pro, and catalytic domains; (d) R473pro, containing the signal peptide fused to the catalytic domain (pro domain deleted); and (e) pro domain.

Full-length TACE was expressed at low levels, and it remained cell-associated. This form of TACE was not evident on Coomassie-stained SDS-PAGE gels from T. ni cells lysed 48 h postinfection (not shown), but Western blots showed an intense band of size consistent with immature TACE containing an uncleaved pro domain (Fig. 2, lane 2). There are also several minor bands. For some of them, the size range is consistent with the mature form lacking the pro domain and with mature TACE truncated before the transmembrane domain (similar to Arg651). This latter form appeared to be generated with time in the cell extracts.

Significant levels of activity were measured in these extracts, when assayed for cleavage of the synthetic peptide substrate (640 versus 0 pmol/min · μg of protein for wild type virus). This indicates that, although small, the fraction of mature full-length TACE was functional. Examination of the cDNA encoding TACE reveals the consensus furin cleavage site RVKVRR215 where Arg215 constitutes the new N terminus of the mature polypeptide. Because it has been previously shown that insect cells seem to be deficient in furin-mediated processing of certain heterologous proteins (17, 18), in addition to infecting cells with full-length TACE virus, we also did co-infection experiments with a virus harboring a full-length cDNA encoding furin (19). Co-infection with furin resulted in a dramatic decrease in the expression of TACE. However, there seems to be less immature TACE relative to the putative mature species in this sample (Fig. 2, lane 3). Consistent with this, the specific activity is comparable or slightly higher than that observed without furin co-expression (746 pmol/min · μg of protein). Such results suggest that a furin-like enzyme or an unrelated protease already present in insect cells must be responsible for cleaving the precursor form of TACE to its mature form. The low ratio of conversion of full-length TACE to the mature form in the absence of furin could be due to inefficient proteolytic processing or, alternatively, to the lack of a factor needed to secrete TACE efficiently to the compartment where cleavage of the pro domain actually occurs.

The Cytosolic and Transmembrane Domains of TACE Are Not Essential for Catalytic Activity—A shorter construct, lacking the cytosolic and transmembrane domains of TACE, Arg651, was efficiently processed and secreted from infected cells in a soluble form (Fig. 3, A and B, lanes 1 and 2). This was expected, because the only membrane-spanning domain of TACE comprises residues Ile672 to Val694. Therefore, the TACE Arg651 construct encodes the entire luminal/extracellular domain of TACE. This material was active against the synthetic substrate (Table I), with an affinity and turnover rate similar to those of native TACE purified from human MonoMac 6 cells (this previously reported finding is shown here for comparative purposes) (7). Arg651 was purified as described before (7) and is also shown here for comparison (Fig. 4A). Interestingly, N-terminal microsequencing analysis of this secreted form (Table II) showed that the N terminus is Arg215. This result is consistent with utilization of the putative consensus cleavage site for furin at positions 211–215 (RVKRR215). Immature TACE Arg651 was only detectable in Western blots (Fig. 3B, lane 2), in the cell-associated material. As mentioned above, we do not know whether this processing event is exerted by an insect cell furin-like activity or by a nonspecific endopeptidase competent at cleaving the Arg214–Arg215 bond.

The Pro Domain Inhibits TACE Activity—A construct similar to Arg651 except for a deletion removing the cysteine-rich region, Arg473, was also expressed and secreted in a soluble form. This truncate was processed efficiently in insect cells to its mature form and was secreted into the culture media (Fig. 3A, lanes 3 and 4). Western blots indicated that immature Arg473 was mostly retained inside insect cells (Fig. 3B, lanes 3 and 4). Unexpectedly, even though Arg473 levels of expression were even higher when compared with Arg651, medium extracts containing Arg473 had much lower specific proteolytic activity against the synthetic peptide substrate when compared with Arg651. However, the specific activity increased upon dilution (not shown). This suggested that an inhibitor-protease complex existed in the Arg473 preparations.

Several lower molecular mass species between 15 and 20 kDa co-purified with Arg473 throughout the entire purification procedure, including the last step (gel filtration, Fig. 4B), indi-

| TABLE I |
| --- |
| TACE form | A<sup>c</sup> | B<sup>c</sup> | C<sup>c</sup> | Inhibition 10 μM GW9471 |
| --- | --- | --- | --- | --- |
| Arg651 | 1.8 × 10<sup>5</sup> | 1.3 × 10<sup>5</sup> | Inactive | 100 |
| Arg473 | 1.8 × 10<sup>5</sup> | 1.3 × 10<sup>5</sup> | Not applicable | 100 |
| R473pro | | | | |
| Native | 2.8 × 10<sup>5</sup> | | | 100 |
cating formation of a complex. N-terminal microsequencing and mass spectroscopy analyses revealed that these contaminants were TACE pro domain fragments frayed at the C terminus, the longest one ending with Arg214 (Table II). Activity assays of those fractions showed that TACE activity was almost equally distributed between two peaks: the first one corresponded to the pro-Arg473 complex and accounted for nearly all of the protein. The second one corresponded to trace amounts of free Arg473 (Fig. 4, B and C). These results demonstrate that the pro domain of TACE is capable of forming an inactive complex with the catalytic domain. The pro domain was tightly bound to Arg473 and significant dissociation occurred only at urea concentrations in excess of 3–4 M, consistent with substantial unfolding of one or both members of the complex.

Several reagents were examined to affect the release of TACE Arg473 from the pro domain and recovery of TACE activity (Fig. 5, A–D). Interestingly, the thiol-modifying agent APMA activated TACE. This reagent, known to activate matrix metalloproteinases (20), was effective at concentrations of 10–20 μM (Fig. 5A). Total dissociation of the pro-catalytic domain complex was observed under those conditions, as determined by analytical gel permeation (Fig. 6, A and B). Remarkably, APMA inhibited TACE completely at concentrations used for the activation of several matrix metalloproteinases (Fig. 5A and data not shown). This is most likely due to the presence of disulfide bonds in the catalytic domain of TACE, but not in the matrix metalloproteinases.

This result suggests that the pro domain binds the catalytic zinc in Arg473 via a cysteine in the context of the consensus sequence PKVCGY186. This has been demonstrated for several members of the matrix metalloproteinase family (10, 21). Purified, pro-free Arg473 exhibits the same kinetic properties against synthetic substrate as Arg651 (Table I), indicating that the cysteine-rich domain is not essential for catalytic activity. However, our results also indicate that the cysteine-rich domain may play a key role in displacement of the pro domain from the catalytic domain upon cleavage of the proenzyme at position 214–215, because we did not detect any complexes between pro and the Arg651 construct that contains the cysteine-rich domain.

The Pro Domain Is Essential for the Secretion of Active TACE—Given that the pro domain acts as an inhibitor of the catalytic domain of TACE, we explored whether this domain was dispensable for the expression of active enzyme. A baculovirus was made from an expression construct containing a deletion including codons 25–223 by taking advantage of the presence of two blunt-end restriction enzyme sites that do not change the reading frame of the cDNA once joined together: SmaI (position 77 of the open reading frame) and PmlI (position 677). Such deletion entirely removes the pro domain, fusing the signal peptide to the catalytic domain. Cells infected with this recombinant virus failed to express soluble, secreted catalytic domain (Fig. 3A, lanes 5 and 6). Western blot analysis revealed low levels of cell-associated material, as well as lower molecular bands, suggesting intracellular proteolysis (Fig. 3B,
Attempts to purify this truncate were unsuccessful: it was readily extracted with the nonionic detergent Nonidet P-40 (indicating intrinsic attachment to the membrane presumably via the signal peptide), but it was extensively proteolyzed in the detergent extract, even in the presence of comprehensive mixtures of protease inhibitors. A variant of this form containing a hexahistidine extension at the C-terminal end introduced by polymerase chain reaction was also extremely susceptible to proteolysis. Microsequencing analysis done on this variant purified under denaturing conditions (6 M guanidine hydrochloride added to the lysis and metal chelate chromatography buffers; Fig. 4D) revealed the presence of two species at about equal molar amounts. The first one was processed by the signal peptidase, and the second one still contained the signal peptide (Table II). These results taken together indicate that the pro domain of TACE is needed for appropriate secretion and processing. Interference with these processes appears to make this form a target for intracellular degradation. Therefore, the pro domain probably not only acts as an inhibitor but also serves in the folding and/or secretion of the catalytic domain. Interestingly, expression of the pro domain in isolation by making a baculovirus from a construct containing a stop after codon 214 was not detectable, and we were unable to purify it even after the addition of a hexahistidine tail. This further suggests the existence of significant surface complementation between the pro and catalytic domains of TACE, which seems to be essential for correct folding and secretion.

TACE Is Active as a Monomer—Given the ternary nature of proTNFα and TNFα (22, 23), we decided to examine whether TACE itself followed a trimeric architecture. Sedimentation analyses were performed on purified Arg473 and Arg651 at different sodium chloride concentrations. They yielded molecular mass values corresponding to the appropriate monomer for each sample. The single species ideal model produced the best fits. Self-association models were also applied to the data, and fits were obtained. The inclusion of higher order terms resulted in poorer fits as judged by randomness of residuals and χ² minimization. Additionally, the association constants of higher order species calculated when using the self-associating models were insignificant, indicating that monomer was the predominant species in all cases. Similar results were obtained by size exclusion chromatography. This indicates that soluble TACE is most likely active as a monomer, because concentrations used for these experiments (1 μM) were well above the ones used in our assays with intact proTNFα and synthetic substrate. This also suggests that the Cys domain is not involved in oligomer formation. In fact, the recombinant cysteine-rich domain purified from infected insect cell media also sedimented as a monomer. Ionic strength did not influence the results.

Surprisingly, while testing the activity of the samples subjected to sedimentation, we found that sodium chloride has a dramatic inhibitory effect on the activity of TACE, with an apparent inhibition constant close to 5 mM. This effect is observed equally with Arg473 and Arg651 (Fig. 7A) and does not seem to be a result of displacement of the zinc ion from the catalytic site: essentially the same inhibition curves were obtained when ZnCl₂ at several concentrations, ranging from 1 to 50 μM, was added (Fig. 7B).

TACE Arg473 and Arg651 Are Differentially Glycosylated—Both Arg651 and Arg473 were characterized by proteolysis and LC-MS mapping. Arg651 contains six conventional consensus sites for N-linked glycosylation (Asn²⁶⁴, Asn⁴⁹₂, Asn⁴⁹⁸, Asn⁵₃⁹, and Asn⁵⁵¹, Asn⁵⁹⁴). It also contains two NXC sites that can potentially be glycosylated in some circumstances (Asn⁷²³ and Asn⁵⁹⁸). These were of interest because of their proximity to the catalytic domain of the molecule. Peptides containing each potential site of N-glycosylation except Asn²²³ were identified in the LC-MS map. It was determined that sites Asn²⁶⁴, Asn⁴⁹₈, and Asn⁵⁵¹ are glycosylated, whereas no evidence for glycosylated forms of the other peptides was found. The data appeared to indicate a conventional distribution of high mannose glycoforms present in each case.

Arg⁷²³ contains two potential sites of N-linked glycosylation, one of which is occupied in the Arg⁷²⁵–Arg⁸¹⁵ version of the protein. Asn⁴⁹₂, which is not occupied in the Arg⁶⁵¹ variant is glycosylated in this case. The molecular weights detected indicate the attachment of a series of low molecular weight, highly processed high mannose chains, of the structure Manₙ(Fuc)GlcNAC₂, where n = 2 or 3. Fucosylated and unfucosylated species are present. This site may be glycosylated in this case because it is more exposed than in Arg⁶⁵¹, making it more accessible to glycosyltransferases. Interestingly, the Asn⁴⁹₂ site, previously characterized as carrying high mannose species including ManₙGlcNAC₂ where n = 5–8, has been processed much more extensively in this preparation, to a series in which the only major species is Man₇GlcNAC₂, with far less abundant glycoforms extending from Man₄ to Man₇.

**DISCUSSION**

Our goal is to understand the relationship among the domain architecture, function, and regulation of TACE. The studies reported here show that only certain recombinant forms of TACE can be overexpressed in insect cells in a soluble, secreted form, fully active as compared with native TACE purified from a human monocytic cell line.

Insect cells expressed mature, functional full-length TACE at very low levels. Most of the protease remained in its immature form, with the pro domain intact, as judged from Western blots of cell extracts. This result is particularly perplexing because truncates Arg⁷²³ (catalytic domain) and Arg⁶⁵¹ (catalytic and cysteine-rich domains) are matured at the predicted furin cleavage site in insect cells. Full-length TACE contains a C-terminal extension of Arg⁶⁵¹, including a transmembrane domain and a cytoplasmic tail. We hypothesize that this cytoplasmic domain contains a negative signal that must be reversed to allow transport of TACE to the secretory compartment where activation occurs. It is intriguing that this domain contains a potential tyrosine phosphorylation site in the con-

---

2 M. Milla, unpublished work.
text of a Src homology 2 domain binding site, KKLD-KQYESL, as well as a potential Src homology 3 domain binding site, PAPQTPGR. In fact, the tyrosine kinase domain of the epidermal growth factor receptor can convert the recombinant TACE cytoplasmic tail (produced in E. coli) to its phosphotyrosine form.

Pradines-Figuere and Raetz (24) have shown that TNFα secretion from MonoMac 6 cells occurs only after stimulation of these cells with both bacterial lipopolysaccharide and phorbol-12-myristate-13-acetate. Although proTNFα biosynthesis is enhanced after lipopolysaccharide stimulation, release does not occur to significant levels in the absence of phorbol 12-myristate 13-acetate (13). Potentially, a phorbol 12-myristate 13-acetate-initiated phosphorylation or dephosphorylation event at the cytosolic tail of TACE precedes TACE activation, either via a conformational change that exposes the cleavage site to the processing protease(s) or by directing transport of TACE to the compartment where activation takes place. Remarkably, it has been shown that mutation of an equivalent tyrosine residue to phenylalanine in the cytoplasmic tail of furin results in failure of this enzyme to localize properly inside the cell (25, 26). Therefore, it is possible that insect cells contain a TACE maturing activity but lack the cellular factors responsible for making TACE available to such protease.

Maturation by proteolytic cleavage is essential in order to generate active TACE. The pro domain strongly inhibits TACE, probably through complexing the catalytic zinc in the active site via an unpaired cysteine residue in the putative cysteine switch box. Our observation that the mercurial compound APMA efficiently mediates dissociation of the pro-catalytic domain complex supports this hypothesis. Additionally, peptides from the putative Cys-switch region do inhibit TACE activity (32).

Interestingly, APMA-mediated dissociation of the pro-catalytic domain complex occurs at concentrations well below the ones used for activating matrix metalloproteinases. At higher concentrations, APMA acts as an inhibitor of TACE. We do not know the mechanism of this inactivation effect. APMA may react with one or more cysteine residues in the catalytic domain that are essential for keeping TACE in its functional form. Release of the cleaved pro domain appears not to be a simple diffusion-driven process, because pro remained tightly associated to the catalytic domain through several purification steps and even in the presence of substantial concentrations of the chemical denaturant urea. This suggests a role for the cysteine-rich domain in freeing the catalytic domain from the pro domain as indicated by the apparent absence of complex when Arg651 was overexpressed and purified. The cysteine-rich domain may have an interacting surface with the catalytic domain that overlaps the one used by the pro domain. Given the remarkable sensitivity of TACE pro domain to proteolysis, it is likely that once it is detached from the catalytic domain, its degradation prevents reassociation with the catalytic domain. It might also be possible that the cysteine-rich domain induces changes in conformation that dramatically reduce the affinity of the pro domain for the catalytic domain. The observed differences in glycosylation sites between Arg473 and Arg651, as well as the observed differences in oligosaccharide processing, may also have a role in stabilizing/destabilizing the pro-catalytic domain complex. It is less likely, however, that the cysteine-rich domain acts as an exchange factor, accepting pro after TACE activation, because of the apparent absence of Arg651-pro complexes.

The pro domain is not only an inhibitor of the catalytic
domain, but also appears to have at least some of the properties observed in chaperones, as it seems to facilitate either secretion or folding or both. When expressed separately, both domains failed to be secreted and appeared to be extremely sensitive to proteolysis. It seems unlikely that this result is due to inability of the endoplasmic reticulum signal peptidase to access its cleavage site: about half of the purified R473\textsubscript{D} protein did have Ala\textsubscript{17} as its N terminus, consistent with signal peptidase processing. In addition, the R473\textsubscript{D} construct contains a signal peptide-catalytic domain fusion starting well downstream (after Pro\textsubscript{24}) from the signal peptidase cleavage site. Furthermore, a similar fusion of the signal peptide to the cysteine-rich domain (Fig. 1) is efficiently secreted by insect cells, and is recognized by a monoclonal antibody specific for the native form of this domain.\textsuperscript{3} This dependence is not surprising; it has been previously reported for several proteases, including zinc metalloproteases, that the pro domain is essential for proper secretion (27, 28). Similar results have been described for other members of the ADAMs family, namely metalloprotease and disintegrin-containing proteins 9 and 15 (32). For at least two proteases, the \(\alpha\)-lytic protease and subtilisin, the pro domain seems to control the kinetics of the folding reaction, supporting an intramolecular chaperone role (29, 30).

How does TACE quaternary structure relate to its function? Both proTNF\(\alpha\) and TNF\(\alpha\) are homotrimers. As the release of proTNF\(\alpha\) from cellular membranes requires cleaving three stems, we investigated the oligomerization state of these recombinant forms by equilibrium sedimentation analysis to address whether TACE itself is a trimer. Both Arg\textsuperscript{473} and Arg\textsuperscript{651} were monomeric at concentrations well above the ones used to demonstrate cleavage against the synthetic peptide substrate. Bode and co-workers (11) published the three-dimensional structure of the catalytic domain of TACE (31). They propose a model in which the “right side” of the catalytic domain forms such interactions with the base of the proTNF\(\alpha\) trimer. A role for the transmembrane or cytoplasmic domains in substrate recognition or oligomerization remains to be investigated.

The extreme salt sensitivity of TACE is intriguing. Concentrations of NaCl that completely inhibit its activity (100 mM) do not seem to have an effect on its oligomeric state or solubility. Examination of electrostatic surface potential models of TACE (11) reveals that the relatively flat left-hand side of the active-site cleft is positively charged. Therefore, disruption of favorable electrostatic interactions with the substrate peptide by chloride ions is formally possible. The physiological meaning of this effect remains to be established.

We believe that some of these truncates will be useful in studies aimed at understanding how TACE interacts with its natural substrate, proTNF\(\alpha\), as well as to dissect the levels of regulation of its biogenesis and activity. In turn, this should allow us to explore new avenues for targeting this key enzyme for therapeutic intervention in inflammatory diseases, cancer, and AIDS.

Acknowledgments—We thank Dr. Robert Fuller for generously sharing a baculovirus strain encoding full-length furin. We also thank Mike Luther, Tom Consler, Fred Kull, Blaine Knight, Jerry McGeehan,

\textsuperscript{3} A. Miller and M. Milla, unpublished work.
REFERENCES

1. Vasalli, P. (1992) Annu. Rev. Immunol. 10, 411–452
2. Old, L. J. (1985) Science 230, 630–632
3. Beutler, B., and Cerami, A. (1988) Annu. Rev. Biochem. 57, 505–518
4. McGeehan, G. M., Becherer, J. D., Bast, R. C., Jr., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Purdon, P., Karp, S., Kidao, S., McElreys, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Vergheze, M., Warner, J., and Ways, J. P. (1994) Nature 370, 558–561
5. Mahler, K. M., Steath, R. P., 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
6. Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Gallaway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Patel, S., Thomas, W., Wells, G., Wood, L. M., and Woolley, K. (1994) Nature 370, 555–557
7. Moss, M. L., Jin, S.-L. C., Mills, M. E., Burkhardt, W., Carter, H. L., Chen, W.-J., Clay, W. C., Dubsbury, J. R., Hassler, D., Hoffmann, C. R., Koet, 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
8. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfsim, 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
9. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfsim, 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
10. Van Wart, H. E., and Birkedal-Hansen, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5578–5581
11. Mackos, K., Fegi, S., Hoder-Catalan, C., Huber, R., Bouronov, G. P., Bartunik, H., Ellestad, G. A., Reddy, P., Wolfsim, M. F., Rauch, C. T., Castner, B. J., Davis, R., Clarke, H. G. R., Petersen, M., Fitzner, J. M., Cerretti, D. P., March, C. J., Paxton, R. J., Black, R. A., and Bode, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3408–3412
12. Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakin, P., Xu, T., and Artavanis-Tsakonas, S. (1999) Science 283, 91–94
13. McGeehan, G. M., Becherer, J. D., Mills, M. E., Pahel, G., Lambert, M., Andrews, H., Frye, S., Haffner, C., Cowan, D., Maloney, P., Dixon, E. P., Jansen, M., Mitchell, J. L., Leesnitzer, T., Warner, J., Conway, J., Bickett, D. M., Bird, M., Priest, R., Reinhard, J., and Lin, P. (1998) in Metalloproteinases as Targets for Anti-inflammatory Drugs (Bradshaw, D., Nixon, J. S., and Bottomley, K., eds) pp. 187–204, Birkhauser Publishers, Basel
14. Cohn, E. J., and Edsall, J. T. (1943) in Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, p. 157, Reinhold, New York
15. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) p. 102, The Royal Society of Chemistry, Cambridge, United Kingdom
16. Durschlag, H. (1986) in Thermodynamic Data for Biochemistry and Bio/Technology (Hinz, H. J., ed) p. 45, Springer-Verlag, New York
17. Yamanakou, G. V., Ritter, G. D., Vey, M., and Companos, R. W. (1995) Virology 214, 50–58
18. Fensouillet, E., and Jones, I. M. (1995) J. Gen. Virol. 76, 1509–1514
19. Bravo, D. A., Gleason, J. R., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) J. Biol. Chem. 269, 25830–25837
20. Galazka, G., Windsor, L. J., Birkedal-Hansen, H., and Engler, J. A. (1996) Biochemistry 35, 11221–11227
21. Gramps, F., Huber, R., Kress, L. F., Moroder, L., and Bode, W. (1993) FEBS Lett. 335, 76–80
22. Tang, P., Hung, M.-C., and Klostergaard, J. (1996) Biochemistry 35, 8216–8225
23. Tang, P., Hung, M.-C., and Klostergaard, J. (1996) Biochemistry 35, 8226–8233
24. Pradines-Figueres, A., and Raetz, C. R. H. (1992) J. Biol. Chem. 267, 23261–23265
25. Schafer, W., Stroh, A., Beherifer, S., Seiler, J., Vey, M., Kruse, M.-L., Kern, H. F., Klenk, H.-D., and Garten, W. (1995) EMBO J. 14, 2424–2435
26. Takahashi, S., Nakagawa, T., Banno, T., Watanabe, T., Murakami, K., and Nakayama, K. (1995) J. Biol. Chem. 270, 29397–29401
27. Wetmore, D. R., and Hardman, D. K. (1996) Biochemistry 35, 6549–6558
28. Becker, J. W., Marcy, A. I., Klostergaard, J., Axel, M. G., Barber, J. J., Fitzgerald, P. M., Cameron, P. M., Esco, C. K., Hagmann, W. K., Hermes, J. D. (1995) Protein Sci. 4, 1966–1976
29. Baker, D., Sohl, J. L., and Agard, D. A. (1992) Nature 356, 263–265
30. Wang, L., Ruan, B., Ruvinov, S., and Bryan, P. N. (1998) Biochemistry 37, 3165–3171
31. Clarke, H. R., Wolfsim, M. F., Rauch, C. T., Castner, B. J., Huang, C. P., Gerhart, M. J., Johnson, R. S., Cerretti, D. P., Paxton, R. J., Price, V. L., and Black, R. A. (1998) Protein Expression Purif. 13, 104–110
32. Boghain, M., Becherer, J. D., Moss, M. L., Atherton, B. R., Erdjumen-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempest, P., and Blobel, C. P. (1999) J. Biol. Chem. 274, 3531–3540