Loss of Ectodomain Shedding Due to Mutations in the Metalloprotease and Cysteine-rich/Disintegrin Domains of the Tumor Necrosis Factor-α Converting Enzyme (TACE)*

Xiaojin Li and Huizhou Fan‡
From the Department of Physiology and Biophysics, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Tumor necrosis factor-α converting enzyme (TACE), a multidomain protease essential for development and disease, releases the ectodomains from many transmembrane proteins in a regulated fashion. To understand the mechanism underlying the regulation of TACE activity, we sought to identify the cause of ectodomain shedding deficiencies in two mutated CHO sublines designated M1 and M2. Transfection of expression vectors for human and mouse TACE restored ectodomain shedding of TNF-α and TGF-α, suggesting that defects in the TACE gene contribute to the phenotype of M1 and M2 cells. The overall levels of endogenous TACE forms in M1 cells were significantly lower than those found in their parental cells, whereas only TACE zymogen, but not its mature form, was detectable in M2 cells. Molecular analyses suggested that M1 cells contained only one expressible TACE allele encoding an M435I point mutation in the catalytic center of the protease, and M2 cells produced two TACE variants with distinct point mutations in the catalytic domain (C225Y) and the cysteine-rich/disintegrin domain (C600Y). Overexpression of the C225Y and C600Y TACE by transient transfection largely compensated for maturation defects in the variants but failed to restore TNF-α and TGF-α release in the shedding-defective CHO cell lines and fibroblasts derived from TACE-null mouse embryo. Further mutagenesis and functional analyses demonstrated that Cys600 was absolutely essential for ectodomain shedding, suggesting that Cys800, similar to Cys225, participates in disulfide bonding, which is critical for both the processing and catalysis of TACE.

Protease-mediated protein ectodomain shedding regulates the biological activities of many transmembrane molecules (1–3). TACE, initially identified as the sheddase for the inflammatory cytokine TNF-α (4, 5), is now known to cleave numerous other proteins, including the mitogenic TGF-α and family members (6–11), the tissue adhesins L-selectin (6, 12–14), and vascular cell adhesion molecule-1 (15), the chemokine fractalkine (16, 17), the ectoprotease meprin β (18), a number of growth factor/cytokine receptors (6, 19–29), and various miscellaneous molecules such as Notch (30), mucin (31), β-amyloid protein precursor (32–34), and the prion protein PrP (35). Whereas the function of TACE is essential for development (6, 36–38), excessive TACE activity plays a critical role in the pathogenesis of inflammatory diseases (39–43) and cancer development (44). Therefore, anti-TACE therapies have been actively sought for these pathologies (39–43).

As a member of a disintegrin and metalloprotease family, TACE is also named ADAM17 and is biosynthesized as a zymogen that contains an inhibitory N-terminal prodomain (4, 5). TACE zymogen is a type 1 membrane protein, which becomes N-glycosylated in the endoplasmic reticulum. After being further modified at N-linked sugars in the medial Golgi, full-length TACE is transported to the trans-Golgi where it is cleaved by furin or a furin-like protease to release the prodomain (45). It was estimated that only a small proportion of the biosynthesized TACE is processed to the cell surface (45).

There are several other non-catalytic domains in TACE in addition to the prodomain: a cysteine-rich/disintegrin domain, a transmembrane domain, and a cytoplasmic domain (4, 5). The transmembrane domain serves to anchor the protease to the plasma membrane where the substrates of the enzyme are located (19, 47). The cysteine-rich/disintegrin domain of TACE, unlike that in most other ADAM family members, does not have a noticeable integrin binding motif (48). Also, the cysteine-rich/disintegrin domain of TACE can be substituted by that of ADAM10 for the cleavage of most substrates (19).

Ectodomain cleavage by TACE is induced within minutes following treatment of cells with stimuli (49, 50). This rapid and efficient activation occurs through post-translational mechanisms, because it is not affected by compounds targeting RNA or protein synthesis (49). Whereas the removal of the prodomain is essential for TACE function, it does not seem to be sufficient to confer a proteolytic activity in the cellular context because the prodomain is cleaved constitutively, and the amount of mature TACE does not change in response to shedding inducers (4, 51).

Various stimuli induce TACE-mediated shedding by activating intracellular signaling pathways mediated by Erk and p38
TACE Mutated in Shedding-defective CHO Cells

EXPERIMENTAL PROCEDURES

Reagents—Concanavalin A (Con A)-conjugated Sepharose, [35S]cysteine/methionine labeling, immunoprecipitation of proteins from medium and cell extract, and visualization of the precipitated proteins by autoradiography or quantitation of released growth factor/cytokine by scintillation counting have been described previously (49, 61).

We have also developed a flow cytometry assay to report TACE shedding by various TACE constructs in M1-14 cells. Approximately 20 h after transfection with either the TACE expression plasmids or the control RK5 vector, PMA was added to culture medium (final concentration: 100 nM). After a 20-min incubation at 37 °C, cell culture plates were placed on ice, and the medium was replaced with 0.2 ml of cold PBS supplemented with 0.1% NaN3, 1 μM 1,10-phenanthroline, 5 mM EDTA, and 2% bovine serum albumin (PEB). Cells were scraped off the plastic with a Cell Lifter (Corning Inc., Corning, NY), collected, and centrifuged at 1000 rpm, 4 °C for 5 min. Following the removal of supernatant, cells were resuspended in 50 μl of PEB containing 200 ng of monoclonal anti-TACE, incubated on ice for 30 min with gentle agitation, and washed twice with 300 μl of 0.2% NaN3, 1 μM 1,10-phenanthroline, 5 mM EDTA, and 2% bovine serum albumin (PEB). Cells were resuspended in 50 μl of 1% paraformaldehyde (prepared in PEB), and analyzed by flow cytometry using a Coulter Epic XL-MCL flow cytometer (Beckman Coulter). For each sample, 100,000 cells were analyzed.

Cell Lines, Culture, and Transfection Conditions—The shedding-defective M1 and M2 cell lines along with their parental CHOT cell line (57, 58) were kindly provided by Dr. J. Massague (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York). Although CHOT cells were stably transfected with HA-tagged rat transmembrane TACE, the expression of this protein in CHOT cells, as well as M1 and M2 cells, was not detectable by an antibody raised against human TACE. We have derived an M1 subclone designated M1α-14 that stably expresses human transmembrane TACE. The EC2 cell line that lacks the expression of catalytically active TACE as a result of targeted gene inactivation, i.e. the tacε528α mutation (4), was kindly provided by Drs. R. Blakesley and J. Peschon (Emgen). All cell lines were maintained in DMEM supplemented with 8% FBS. Transfection of plasmid DNA into cells was achieved by using LipofectAMINE 2000 (Invitrogen).

MAP kinases (49, 52). Therefore, the cytoplasmic domain of TACE was thought to serve as a signal transducer that regulates the cleavage by the (extracellular) protease domain (49, 52–54). Surprisingly, even though the cytoplasmic domain binds other proteins and is phosphorylated in response to shedding inducers (19, 26, 54–56), its deletion has had no detectable effects on the cleavage of TACE substrates so far tested, which include TNF-α, the p75 TNF-α receptor, the type II interleukin-1 receptor, and transmembrane TGF-α (19, 55). Therefore, the mechanism by which intracellular signaling events lead to changes of TACE activity remains unknown.

Two mutant cell lines, the M1 and M2 cell lines, may be particularly useful to elucidating the mechanisms controlling ectodomain shedding by TACE. These cell lines were isolated from ethyl methane sulfonate-treated CHO cells stably transfected with transmembrane TGF-α, i.e. CHOT cells. Compared with CHOT cells, M1 and M2 cells no longer released soluble TGF-α when stimulated by the shedding inducer PMA (57, 58). Though M1 and M2 were obtained from independently mutagenized CHOT stocks, results of cell fusion experiments suggest that a same recessive gene defect accounts for the loss of TGF-α shedding in these cell lines (57, 58). Further studies revealed that M2 cells are also defective in TNF-α ectodomain shedding (59), whereas M1 cells are unable to cleave L-selectin (60). These characteristics exhibited by M1 and M2 cells strikingly resemble those of embryonic fibroblasts, i.e. EC2 cells, isolated from TACE-null mouse (6). Interestingly, overexpression of TACE reportedly failed to restore TNF-α release in M2 cells. Nevertheless, fusion of M2 cells to EC2 cells led to recovery of TNF-α shedding (59). Therefore, it appears that a cellular component that is essential for the functioning of TACE is defective in M1 and M2 cells. We were motivated to identify this hypothetical TACE regulator. However, our findings point to mutations in TACE as the direct causes of shedding deficiencies in these cells. One of these TACE variants had a single point mutation in the cysteine-rich/disintegrin domain, suggesting that this non-catalytic domain may regulate TACE activity.

Sample preparation for analyzing TACE—Western Blotting of TACE—Western blotting of TACE was performed as previously described with slight modifications. The procedures for transfection, [35S]cysteine/methionine labeling, immunoprecipitation of proteins from medium and cell extract, and visualization of the precipitated proteins by autoradiography or quantitation of released growth factor/cytokine by scintillation counting have been described previously (49, 61).
examined to ensure the accuracy of data interpretation. The coding sequences of wild-type and mutated hamster TACE cDNA were cloned into the mammalian expression vector pRK5 for expression of untagged or FLAG epitope-tagged proteins. The sequence integrity of inserts in all resulting vectors was verified by automated sequencing.

**Additional Mammalian Expression Vectors**—Murine TACE was originally cloned by Amour et al. (62) and we obtained a plasmid containing its cDNA from Dr. C. Blobel (Memorial Sloan-Kettering Cancer Center). The murine TACE open reading frame was transferred to pRK5 where subsequent site-directed mutagenesis was performed using polymerase chain reaction (PCR)-based methodology. The expression vector we used for human TACE has been described previously (55), as has the human transmembrane TGF-α expression vector pRK5α (63). A previously described expression vector for transmembrane TGF-α (49) was modified to express the type II membrane protein with an extracellular FLAG epitope tag at the C terminus.

**RESULTS**

**Lack of Basal and PMA-stimulated TGF-α and TNF-α Ectodomain Shedding in M1 and M2 Cells**—The M1 and M2 cell lines were isolated on the basis of loss of TGF-α shedding in response to PMA stimulation (57, 58). We verified the lack of TGF-α shedding in these cells with pulse-chase experiments comparing TGF-α release in M1 and M2 along with parental CHOT cells following transfection with an expression plasmid for transmembrane TGF-α. In resting CHOT cells, substantial proportions of radiolabeled transmembrane TGF-α forms on the plasma membrane (Fig. 1A, top panel) were converted into free soluble TGF-α forms with or without the glycosylated prosequence (Fig. 1A, bottom panel). Treatment of cells with PMA further stimulated this conversion (Fig. 1A). In strong contrast, M1 and M2 cells retained significantly higher levels of cell surface TGF-α forms (Fig. 1A, top panel); no free TGF-α forms were detectable in the chase medium for the mutant cells (Fig. 1A, bottom panel). The lack of TGF-α shedding in M1 and M2 cells remained unchanged even after they were treated with PMA.

A previous report showed that M2 cells had also lost the ability to convert transmembrane TNF-α to soluble TNF-α. By transient transfection of an expression plasmid encoding C-terminally (i.e. extracellularly) FLAG-tagged transmembrane TNF-α and pulse-chase analysis of the amounts of free FLAG-tagged TNF-α in the medium and the transmembrane precursor in cell extracts, we now show that both M1 and M2 cells, in the presence or absence of PMA, are defective in TNF-α ectodomain shedding (Fig. 1B). Taken together, these results demonstrate that both basal and stimulated ectodomain shedding of TGF-α and TNF-α is severely impaired in M1 and M2 cells.

**Preservation of Erk and p38 MAP Kinase Signaling Activities in M1 and M2 Cells**—A previous study reported that M2 cells maintained their inability to release TNF-α when they were transfected to overexpress TACE. However, fusion of M2 cells to EC2 cells (in which TACE was inactivated by gene targeting) restored TNF-α shedding (59). It therefore appeared that the loss of shedding in these cells was caused by the defect of a regulatory molecule essential for the functioning of TACE. We and others (49, 52, 64) have demonstrated that the signaling activities of Erk and p38 MAP kinases are critical for ectodomain shedding of many transmembrane proteins. Specifically, basal shedding of TGF-α and TNF-α requires p38 activity, whereas PMA induces shedding through an Erk-dependent mechanism. We also showed that expression of activated Erk2 or the p38α activator MKK6 was sufficient to activate ectodomain shedding in the absence of any exogenous shedding inducer (49). To investigate whether there is a defect in Erk and/or p38 signaling that may account for loss of ectodomain shedding in M1 and M2 cells, we analyzed the activation of Erk2 and p38α MAP kinases in response to serum stimulation and UV irradiation, respectively (65, 66). Stimulation with 20% serum for 20 min resulted in ~5–7-fold induction of Erk2 activity as assessed by measuring the levels of myelin basic protein that was phosphorylated by immunoprecipitated Erk2 in serum-starved CHOT and the shedding defective M1 and M2 cell lines (Fig. 2A). Exposure to 50 J/cm² UV strongly stimulated p38α phosphorylation in all the three CHO-derived lines (Fig. 2B). These results indicate that M1 and M2 cells retained full capacity for Erk2 and p38α activation and suggest that the
molecular defect of ectodomain shedding likely resides downstream of these MAP kinases.

Decreased Endogenous TACE Forms in M1 and Lack of Mature TACE in M2—Similar to most other metalloproteases, TACE is biosynthesized as a zymogen that remains inactive because the catalytic center is blocked by the prodomain (67). During the maturation process the prodomain is removed (45). Interestingly, it was very recently reported that only the zymogen, but not the mature form of TACE was endogenously expressed in M1 and M2 cells (68). Using the same techniques that had been employed by others for analyzing TACE maturation, we were also unable to detect the 100-kDa mature TACE in M2 cells (Fig. 3). However, we observed two forms of glycosylated TACE in M1 cells, the zymogen and mature TACE (Fig. 3). Noticeably, the amounts of both TACE forms in M1 cells were significantly lower than those in CHOT cells (Fig. 3). Also, the TACE zymogen in M1 cells was at a significantly lower level than that in M2 cells (Fig. 3).

Similar Expression and Processing of Transfected TACE in CHOT and the Shedding-defective Mutants—We reasoned that changes of TACE expression observed in M1 and M2 cells (Fig. 3) might have been caused by mutation(s) in TACE itself or, as suggested previously, by defects with cellular machineries that process TACE (68). To distinguish between these two possibilities, we analyzed the expression of transfected TACE. As shown in Fig. 4, after the transfection of an expression vector for human TACE, no difference in TACE expression among M1, M2, and CHOT cells could be observed. Accordingly, the amounts of TACE in M1 cells were comparable to those in CHOT cells; M2 cells also produced the mature form in addition to the zymogen (Fig. 4). Therefore, the overall decrease in expression in M1 cells and lack of the mature form in M2 cells of endogenous TACE are likely caused by mutations in the TACE gene.

Restoration of Ectodomain Shedding in M1 and M2 Cells by Transfection of TACE—The recovery of TACE expression in M1 cells and particularly the reexpression of mature TACE in M2 cells following transfection with a TACE expression plasmid led us to reexamine whether transfected TACE would restore the shedding deficiency in M1 and M2 cells. Although it was previously reported that overexpression of TACE had no effect on the lack of shedding in M2 cells (59), we observed efficient recovery of TNF-α ectodomain shedding in both M1 and M2 cell lines following transfection with an expression vector for mouse TACE as judged by increased amounts of free TNF-α in chase medium and decreased amounts of the transmembrane precursor in cell extracts (Fig. 5A). As expected, a catalytically inactive TACE construct with the E406A point mutation (55) failed to restore the production of free TNF-α (Fig. 5B). Consistent with data obtained with mouse TACE (Fig. 5, A and B), expression of human TACE also restored TGF-α ectodomain shedding in M1 and M2 cells as demonstrated by increased quantities of free TGF-α released into chase medium (Fig. 5C).

Identification of TACE Mutations in M1 and M2 Cells—The restoration of ectodomain shedding in M1 and M2 cells by expression of wild-type TACE but not the catalytically inactive E406A mutant further implies that the function of TACE in the shedding-defective cell lines is likely abolished by mutation(s) in this protein. Therefore, we compared the sequences of TACE cDNA in the parental and shedding-defective cell lines. From CHOT cells, we obtained the sequence of the entire open reading frame of hamster TACE as well as its 5′- and 3′-untranslated regions (see footnote for GenBank™ accession number). The open reading frame encodes a peptide of 827 amino acids, which is the same size as mouse and rat TACE, but three amino acids longer than the human protease. As predicted from sequences from other species, hamster TACE has a highly conserved sequence that is 91% identical to those from human, rat, and mouse (data not shown).

Direct sequencing of RACE products suggested that there were three point mutations in the TACE cDNAs from M1 and M2. Compared with the sequencing data of wild-type TACE cDNA isolated from CHOT cells (Fig. 6A), the total TACE cDNA from M1 cells showed a guanosine → adenosine substitution at base 1305 (of the TACE open reading frame) (Fig. 6B). As expected, the cloned cDNA had the same base substitution, which results in an isoleucine in place of Met345 in the metalloprotease domain (Fig. 6, B and D). For the sake of clarity, the cloned variant is named M435I(M1mp) (Fig. 6D).

Sequencing data obtained with uncloned total TACE cDNA from M2 cells exhibited two dual base peaks; an additional adenosine was found together with guanosine in positions 674 and 1799. These guanosine → adenosine substitutions, if existing, would translate to C225Y and C600Y mutations at the
protein level (Fig. 6 C). Cloning and subsequent sequencing confirmed that these two mutations are carried by different alleles (Fig. 6, E and F). Of six cloned plasmids containing the M2 TACE cDNA inserts, three showed the C225Y mutation, and the other three had the C600Y substitution (data not shown). Because Cys225 and Cys600 are located in the metalloprotease domain and the cysteine-rich/disintegrin domain, respectively, the M2-derived mutants were named C225Y(M2mp) (Fig. 6 E) and C600Y(M2crd) (Fig. 6 F).

Expression and Maturation of Cloned Hamster TACE—cDNA sequencing and cloning described above suggest that there is only one expressible TACE allele in M1, which explains why an overall decreased TACE level was found in this cell line (Fig. 3). Because M1 cells express both the zymogen and mature form of TACE, we concluded that the M435I mutation does not affect the biosynthesis and processing of the protein. However, because two mutated mRNA sequences were detected in M2 cells, the results in Fig. 3 do not differentiate whether the endogenous protein detected were translated from only one or both TACE gene transcripts. We therefore analyzed the expression of C225Y(M2mp) and C600Y(M2crd) in COS cells that have been previously used to study TACE processing (45) as well as M2 cells. As expected, transfection of wild-type hamster TACE and M435I(M1mp) resulted in overexpression of both the zymogen and mature form of TACE in COS cells (Fig. 7A, left panel). Surprisingly, cells transfected with C225YM2mp and C600Y(M2crd) also yielded mature TACE (Fig. 7A, left panel), even though no mature TACE was detected endogenously from M2 cells (Fig. 3). Similar observations were made when the TACE expression plasmids were transfected into M2 cells (Fig. 7A, right panel).

We next sought to verify the maturation of TACE variants by cell surface staining, because mature TACE but not the zymogen is located on the cell surface (45). Anti-TACE ectodomain antibodies currently available were raised against the human sequence; they do not cross-react with the protein from other species, such as mouse and hamster.2 Because an anti-ectodomain antibody for the hamster enzyme was not available, we constructed extracellular tagged TACE in which a FLAG epitope was placed near the N terminus of the metalloprotease domain (between Ala216 and Asp217). The resulting eFLAG (extracellular FLAG)-tagged constructs were transiently transfected into COS cells. Con A precipitation and Western blotting were performed to verify the expression of the resulting eFLAG-tagged constructs. eFLAG-tagged wild-type TACE, expression plasmids were transfected into M2 cells (Fig. 7A, right panel).

2 H. Fan, unpublished data.

Fig. 5. Restoration of ectodomain shedding in M1 and M2 cells by transfected TACE. A and B, cells were transfected with the FLAG-tagged transmembrane TNF-α expression vector plus an expression vector for wild-type mouse TACE or a vector for a catalytically inactive TACE (Cat−) (55) or the control RK5 vector. The amounts of soluble TNF-α and transmembrane TNF-α were determined as described in the legend to Fig. 1. C, cells were transfected with an expression vector for human transmembrane TGF-α plus an expression vector for wild-type human TACE or the control RK5 vector. The amounts of soluble TGF-α released into chase medium were quantified by scintillation counting following immunoprecipitation (49). Values represent averages ± S.E. of duplicate samples.

Fig. 6. Mutated TACE sequences in M1 and M2 cells. A–C, uncloned TACE cDNA amplified from total cellular RNA samples from CHOT, M1, and M2 cells were sequenced. The corresponding amino acids coded by the TACE open reading frame are shown. The areas of wild-type TACE (from CHOT cells), corresponding to mutations found in M1 and M2 cells are shown in A. The adenosine that substitutes for guanosine in codon 435 of the TACE cDNA from M1 cells (B), and the adenosine/guanosine dual peak signals in the cDNA from M2 cells (C), are marked with stars and triangles, respectively. D, sequencing of cloned M435I(M1mp) TACE cDNA showed the same mutation as total cDNA in Fig. 1B. E and F, two TACE variants, C225Y and C600Y, were cloned from M2 cells as revealed by DNA sequencing. The substituted bases in the mutants are marked with stars.
M435I(M1<sub>mp</sub>), and C600Y(M2<sub>crd</sub>) were synthesized and processed to the glycosylated zymogen and mature form (Fig. 7B), similar to the untagged versions (Fig. 7A). However, eFLAG-tagged C225Y(M2<sub>mp</sub>) was not expressible (Fig. 7B), presumably because the combination of C225Y mutation and the insertion of the FLAG epitope nearby renders the protein overly sensitive to degradation (whereas either alone was not sufficient to trigger this event). To determine whether TACE constructs yielded mature TACE at the cell surface, binding of FLAG antibody to unfixed live cells was performed. Consistent with results from Western blot analyses (Fig. 7, A and B), M2 cells transfected with eFLAG-tagged wild-type TACE, M435I(M1<sub>mp</sub>), and C600Y(M2<sub>crd</sub>) all stained positive (Fig. 7C). As expected, no positively stained cells were observed in the control RK5 vector-transfected cells (Fig. 7C). Taken together, these data confirm that the M435I(M1<sub>mp</sub>) mutation does not affect the biosynthesis and processing of TACE. They also strongly suggest that C600Y(M2<sub>crd</sub>) can be processed to the mature form upon overexpression and that the maturation of the endogenous protein is inefficient and thus undetectable. As predicted from Western blotting data presented in Fig. 7B, eFLAG-tagged C225Y(M2<sub>mp</sub>)-transfected cells were negatively stained (data not shown). Thus, the maturation of C225Y(M2<sub>mp</sub>) was suggested by Western blotting alone.

Lack of Enzymatic Activity in Cloned TACE Variants—Although no mature TACE was detectable endogenously in M2 cells, the two M2-derived TACE variants C225Y and C600Y both yielded the mature protein upon overexpression (Fig. 7); epitope tagging and immunostaining of the C600Y variant also suggested that it resides at the cell surface (Fig. 7). This therefore raises the question of whether these variants can cleave substrates under overexpressed conditions. As shown in Fig. 8, A–C, only wild-type hamster TACE was capable of mediating TNF-α and TGF-α release in M1, M2, and EC2 cells; all hamster TACE variants released essentially the same amounts of cytokine/growth factor as the control empty RK5 plasmid, indicating a lack of proteolytic activity in the variants.

Cys<sup>600</sup> Is Irreplaceable in Ectodomain Shedding—Met<sup>435</sup> is strictly conserved among all metzincins and has been revealed by x-ray crystallography to be part of the catalytic center of TACE (69). The crystal structure of TACE catalytic domain also shows that Cys<sup>333</sup> forms a disulfide bond with Cys<sup>333</sup>, and this disulfide bridge contributes to the maintenance of the tertiary structure required for catalysis (69). It is therefore not surprising that the M435I(M1<sub>mp</sub>) and C225Y TACE(M2<sub>mp</sub>) variants are proteolytically inactive. However, no structural role for Cys<sup>600</sup> in TACE has been assigned. Given the large number of additional cysteine residues in the cysteine-rich/disintegrin domain, one possibility is that Cys<sup>600</sup> also participates in disulfide bonding. We reason that Cys<sup>600</sup> will be essential for ectodomain shedding if this hypothetical disulfide bond is critical for TACE processing and/or catalysis; otherwise, substitution by amino acids that are structurally similar

---

**Fig. 7. Expression of cloned hamster TACE variants.** A, COS and M2 cells were transiently transfected with expression vectors for the indicated TACE constructs or the control RK5 plasmid. Glycosylated TACE forms (zymogen (Z) or mature form (M)) were detected by Western blotting as described in the legend to Fig. 3. WT TACE, wild-type hamster TACE. B, COS cells were transiently transfected with expression vectors for wild-type hamster TACE or M1- and M2-encoded TACE tagged with eFLAG (i.e., the FLAG epitope inserted near the N terminus of the catalytic domain). Glycosylated eFLAG-tagged TACE proteins were detected by Western blotting with the anti-FLAG antibody. Note the eFLAG-C225YM2<sub>mp</sub>) was not expressible. C, M2 cells were transiently transfected with eFLAG-tagged wild-type or mutated TACE constructs shown in B; TACE proteins on the cell surface were stained with the anti-FLAG antibody before cells were fixed. Photographs were taken using the same exposure times.
to cysteine may be tolerated. We therefore pursued extensive mutagenesis at Cys600 and functional analyses.

To analyze TACE activity more efficiently, we have developed a flow cytometry assay that measures TGF-β levels on the surface of an M1 subclone, M1/H9251-14, that was engineered to stably express human transmembrane TGF-β. Compared with the control RK5 vector-transfected cells, the majority of M1/H9251-14 cells transfected with wild-type hamster TACE displayed significantly decreased transmembrane TGF-β levels on the cell surface (Fig. 9A). Consistent with results from pulse-chase experiments (Fig. 8), C600Y(M2crd)-transfected cells displayed the same levels of transmembrane TGF-β on the cell surface as the control RK5 vector-transfected cells (Fig. 9A), indicating a lack of shedding activity in this variant.

For convenience we used a murine TACE construct named /H9004Cm as the template to derive additional mutants. In /H9004Cm, the cytoplasmic domain covering amino acids 698–827 were replaced with the 9E10 human Myc epitope sequence. We (55) and others (19) have shown that deletion of the cytoplasmic domain from human TACE has no effect on shedding of TGF-β and other membrane proteins. This was also true for mouse TACE, because ΔCm efficiently down-regulated the transmembrane TGF-α levels on M1α-14 cells (Fig. 9B). However, TGF-α shedding was disabled when Cys600 was replaced with tyrosine in the ΔCm construct (Fig. 9B). Thus, the shedding-defective phenotype of the C600Y mutation was not unique to hamster TACE, but was also reproduced with the mouse enzyme. In further analyses, none of the other 18 amino acids was able to replace Cys600 in TGF-α ectodomain shedding (Fig. 9C). Con A precipitation coupled with Western blotting showed that all the shedding-defective constructs, similar to the enzymatically active ΔCm construct, were processed to mature form in the cell (Fig. 9D). Taken together, these results indicate that Cys600 is essential for the proteolytic activity of TACE.

**DISCUSSION**

M1 and M2 cell lines have provided a wealth of information on the process of ectodomain shedding (44, 57, 58, 70, 71). An especially noteworthy observation made from these mutants is that numerous transmembrane proteins depend on metalloprotease activity for shedding (58). Nevertheless, the molecular defects in these cells remain unknown. In this study we have demonstrated that inactivating mutations in TACE are the cause of ectodomain shedding deficiencies in both M1 and M2 cells. Accordingly, we identified TACE mutants from these two cell lines and further showed that the mutants are enzymatically inactive in the cellular context using TNF-α and TGF-β release as readouts. Thus, like EC2 cells, the M1 and M2 cell lines should be valuable reagents for identifying additional TACE substrates and for structure-function analyses of TACE.

M1 and M2 cell lines were isolated from independently mutagenized CHO stocks (58, 60). The TACE mutations identified from these cell lines confirm that they are indeed distinct clones. Thus, M1 carries only one expressible TACE allele with...
the M435I mutation, whereas M2 contains two mutated alleles, encoding the C225Y and C600Y TACE proteins, respectively. The M435I(M1\text{mp}) mutant could not restore ectodomain shedding in M2 cells; the C225Y(M2\text{mp}) and C600Y(M2\text{crd}) variants also failed to recover shedding in M1 cells. These data are consistent with a previous study, which found no complementation between M1 and M2 cell lines upon cell fusion (58).

However, our data also contrast with several published findings that include a lack of TNF-α shedding in TACE-transfected M2 cells (59). Because we reproducibly restored shedding of both TNF-α and TGF-α in M1 and M2 by transfection with expression plasmids for wild-type human, mouse, and hamster TACE, the reason for this discrepancy remains unknown.

We have shown that the TACE variants isolated from M1 and M2 cells failed to restore shedding in EC cells. These results were consistent with our prediction because both TACE alleles in EC2 cells were replaced with the ΔZn mutant, which is functionally identical to the variants isolated from M1 and M2 cells. Therefore, it was rather surprising that cells created through EC2-M2 fusion regained TNF-α shedding activity (59).

A recently published study analyzed the sequence of nearly the entire open reading frame of hamster TACE, and found no mutation in the cDNA from M2 cells (68). We have sequenced the TACE cDNAs of both M1 and M2, and have identified mutations within the previously sequenced region. To minimize the possibility of obtaining mutations created in vitro, we...
Fig. 10. Alignment of human ADAM-17 (TACE) and ADAM10 cysteine-rich/disintegrin domains. Residues identical in both sequences are marked with an asterisk, and those structurally conserved are marked with a colon. Cys600 of TACE and the putative counterpart in ADAM10 are bolded and underlined.

directly sequenced uncloned total TACE cDNA. To ensure that no mutations were overlooked by the computer program that interprets the automated sequencing data, we reviewed the sequencing wave forms manually. In fact, the C225Y and C600Y mutations were only recognized by manual examination of the wave forms. In comparison, the M435I mutation from M1 cells was correctly reported by the program because it is the only species of TACE mRNA expressed in this cell line.

Met435 is strictly conserved in all metzincins (72). Our data revealed that endogenously expressed M435I(M1mp) was efficiently processed to mature form, suggesting that Met435 is only required for the catalysis, but not for the processing of TACE. Significantly, the importance of Met435 in the catalysis of TACE has also been highlighted by x-ray crystallography revealing this residue as a constituent of the catalytic center of the enzyme (69). Interestingly, a newly published study showed the structure and catalysis of matrix metalloprotease 2 (gelatinase A) is not affected when the absolutely conserved methionine was replaced with leucine or serine (73). Future mutagenesis studies should reveal whether or not Met435 is essential for the catalytic activity of TACE.

The C225Y(M2mp) and C600Y(M2mp) TACE variants can be overexpressed by transfection, suggesting that both are synthesised endogenously. How these mutations affect TACE function is very intriguing. Clearly, they had a very strong inhibitory effect on TACE maturation, as the endogenously expressed variants were not detectably processed to the mature form. However, their negative impact on maturation could be compensated by overexpression as shown by Western blot analyses, and in the case of C600Y(M2mp), also by cell surface immunostaining. As a matter of fact, the amounts of the mature variant proteins in transfected cells far exceeded that of the endogenous mature TACE in wild-type cells. Yet, the C225Y(M2mp) and C600Y(M2mp) variants failed to cleave TNF-α and TGF-α under these conditions. Therefore, Cys225 and Cys600 appear to be required not only for efficient maturation, but also for the catalysis of this enzyme.

M2 cells are defective in shedding of numerous proteins in addition to TGF-α and TNF-α (58). This underscores the general importance of Cys225 and Cys600 in ectodomain cleavage by TACE. As revealed by x-ray crystallography, the disulfide bridge formed by the Cys225 and Cys600 residues is critical for the maintenance of the conformation required for TACE activity (69). Because our mutagenesis studies showed that Cys600 is absolutely essential for ectodomain shedding, it suggests that as with Cys225, Cys600 may also participate in disulfide bond formation that could contribute to the maintenance of the tertiary structure of the cysteine-rich/disintegrin domain. A conformational change in this domain caused by the C600Y mutation may have one or more of the following adverse effects on ectodomain shedding. First, it may prevent the protease domain from binding substrates. Second, it could directly affect the function of the catalytic domain independent of substrate binding, because the cysteine-rich/disintegrin likely interfaces the protease domain (69). Finally, it might alter the association of TACE with other regulatory molecules. For example, the tissue inhibitor of metalloprotease (TIMP)-3 (the only TIMP that is effective on TACE) is known to interact with the cysteine-rich/disintegrin domain of TACE (74, 75).

Reddy et al. (19) showed that the cysteine-rich/disintegrin domain of TACE can be substituted by that of ADAM10 for the shedding of a number of membrane proteins. Alignment of these two sequences revealed a significant level of homology and predicted Cys594 of ADAM10 as the structural equivalence to Cys460 of TACE (Fig. 10). This suggests that the conserved cysteine may be required for the function of ADAM10 as well.

After our release of the mutant sequences on September 2, 2004, Villanueva et al. (76) later published a report documenting the existence of the same mutations in M1 and M2 cell lines, while this article was under review. Although their work demonstrated that all the mutant alleles are defective in TGF-α release, there are several important discrepancies between this earlier report and our findings. First, in addition to an inhibitory impact on maturation, our data suggest that the C225Y and C600Y mutations have a detrimental effect on the catalytic activity of TACE. However, the possibility of the direct effect of the mutations on TACE catalysis was not considered by Villanueva et al. Second, our data clearly demonstrate that both the endogenous and overexpressed M435I(M1mp) matures efficiently. Conversely, Villanueva et al. suggested that this mutant, similar to C225Y(M2mp) and C600Y(M2crd), is defective in maturation. Therefore, our study uncovered an alternative mechanism for a lack of shedding activity in all the TACE variants encoded by the M1 and M2 cell lines.

In addition, there are several technical discrepancies between our paper and the report of Villanueva et al. (76). We show that transient expression of TACE cloned from human, mouse and hamster efficiently recover the shedding deficiency in the M1 and M2 cell lines. However, Villanueva et al. reported that shedding in these cell lines was not observed unless they were stably transfected. Our results are consistent with studies published by several other groups demonstrating that transient transfection efficiently restored shedding in the EC2 cells (e.g. Refs. 7, 11, and 19). The ability to restore shedding by transient transfection without having to rely on time-consuming stable transfection would make the M1 and M2 cells ex-
tremely useful for identifying additional TACE substrates and structure-function studies of TACE. Finally, Villanueva et al. (76) reported the ability to immunostain murine TACE using an antibody raised against the ectodomain of human TACE. However, we demonstrated that this same antibody has limited utility after testing by several distinct methods (including the same immunostaining technique reported by Villanueva et al., Ref. 55). More specifically, we were not able to detect endogenously or overexpressed TACE of mouse and hamster origin by Western blotting, immunoprecipitation, or immunostaining. Therefore, we suggest that generation of additional antibodies for this domain or epitope-tagging would be useful for future experiments on the ectodomain of mouse and hamster TACE.

In summary, we have demonstrated that mutations in TACE cause the loss of ectodomain shedding in M1 and M2 cells. The characteristics exhibited by the M2 cell line and its encoded C600T TACE variant indicate that the cysteine-rich/disintegrin domain plays a critical role in the processing and proteolytic activity of this important enzyme. The identification of the molecular defects in these cell lines makes them invaluable reagents for future research of functional regulation of numerous cellular surface proteins critical for development, immunity, cellular diseases, and carcinogenesis.

Acknowledgments—We thank Dr. J. Massague for providing the CHO cell line, Drs. A. J. Docherty, G. Murphy, and C. Blobel for making the mouse TACE expression vector available, Drs. R. Black and J. Peschon for supplying EC2 cells, Drs. N. C. Partridge and J. Lenard for useful discussions, and Drs. R. Derynck and N. Ywchoy for critical reading of the article. We also thank Dr. C. Tsai for generously sharing his flyover microscope, M. J. Wang for the help with densitometry, and Mr. A. Balakrishnan for performing radiation safety.

REFERENCES

1. Blobel, C. P. (2000) Curr. Opin. Cell Biol. 12, 606–612
2. Kheradmand, F., and Werb, Z. (2000) Bioscience 24, 8–12
3. Arribas, J., and Merlos-Suarez, A. (2003) Curr. Top. Dev. Biol. 54, 125–144
4. Black, R. A., Rauch, C. T., Papadopoulos, E., Murphy, G. A., and Blobel for making the mouse TACE expression vector available, Drs. R. Black and J. Peschon for supplying EC2 cells, Drs. N. C. Partridge and J. Lenard for useful discussions, and Drs. R. Derynck and N. Ywchoy for critical reading of the article. We also thank Dr. C. Tsai for generously sharing his flyover microscope, M. J. Wang for the help with densitometry, and Mr. A. Balakrishnan for performing radiation safety-related procedures.

1. Blobel, C. P. (2000) Curr. Opin. Cell Biol. 12, 606–612
2. Kheradmand, F., and Werb, Z. (2000) Bioscience 24, 8–12
3. Arribas, J., and Merlos-Suarez, A. (2003) Curr. Top. Dev. Biol. 54, 125–144
4. Black, R. A., Rauch, C. T., Papadopoulos, E., Murphy, G. A., and Blobel for making the mouse TACE expression vector available, Drs. R. Black and J. Peschon for supplying EC2 cells, Drs. N. C. Partridge and J. Lenard for useful discussions, and Drs. R. Derynck and N. Ywchoy for critical reading of the article. We also thank Dr. C. Tsai for generously sharing his flyover microscope, M. J. Wang for the help with densitometry, and Mr. A. Balakrishnan for performing radiation safety-related procedures.

1. Blobel, C. P. (2000) Curr. Opin. Cell Biol. 12, 606–612
2. Kheradmand, F., and Werb, Z. (2000) Bioscience 24, 8–12
3. Arribas, J., and Merlos-Suarez, A. (2003) Curr. Top. Dev. Biol. 54, 125–144
4. Black, R. A., Rauch, C. T., Papadopoulos, E., Murphy, G. A., and Blobel for making the mouse TACE expression vector available, Drs. R. Black and J. Peschon for supplying EC2 cells, Drs. N. C. Partridge and J. Lenard for useful discussions, and Drs. R. Derynck and N. Ywchoy for critical reading of the article. We also thank Dr. C. Tsai for generously sharing his flyover microscope, M. J. Wang for the help with densitometry, and Mr. A. Balakrishnan for performing radiation safety-related procedures.
TACE Mutated in Shedding-defective CHO Cells

63. Shum, L., Turek, C. W., and Derynck, R. (1996) *J. Biol. Chem.* 271, 28502–28508
64. Montero, J. C., Yuste, L., Diaz-Rodriguez, E., Esparris-Ogando, A., and Pandiella, A. (2002) *Biochem. J.* 363, 211–221
65. Alessi, D. R., Cohen, P., Ashworth, A., Cowley, S., Leevers, S. J., and Marshall, C. J. (1995) *Methods Enzymol.* 255, 279–290
66. Selvamurugan, N., Fung, Z., and Partridge, N. C. (2002) *FEBS Lett.* 532, 31–35
67. 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
68. Borroto, A., Ruiz-Paz, S., de la Torre, T. V., Borrell-Pages, M., Merlos-Suarez, A., Pandiella, A., Blobel, C. P., Baselga, J., and Arribas, J. (2003) *J. Biol. Chem.* 278, 25933–25939
69. Maskos, K., Fernandez-Catalan, C., Huber, R., Bourenkov, G. P., Bartunik, H., Ellestad, G. A., Reddy, P., Wolfson, M. F., Rauch, C. T., Castner, B. J., Davis, R., Clarke, H. R., Petersen, M., Fitzner, J. N., 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
70. Codony-Servat, J., Albanell, J., Lopez-Talavera, J. C., Arribas, J., and Baselga, J. (1999) *Cancer Res.* 59, 1196–1201
71. Merlos-Suarez, A., Ruiz-Paz, S., Baselga, J., and Arribas, J. (2001) *J. Biol. Chem.* 276, 48510–48517
72. Stecker, W., Grams, F., Baumann, U., Reinem, P., Gomis-Ruth, F. X., McKay, D. B., and Bede, W. (1995) *Protein Sci.* 4, 823–840
73. Butler, G. S., Tam, E. M., and Overall, C. M. (2004) *J. Biol. Chem.* 279, 15615–15620
74. Lee, M. H., Verma, V., Maskos, K., Becherer, J. D., Knauper, V., Dodds, P., Amour, A., and Murphy, G. (2002) *FEBS Lett.* 520, 102–106
75. Lee, M. H., Dodds, P., Verma, V., Maskos, K., Knauper, V., and Murphy, G. (2003) *Biochem. J.* 371, 369–376
76. Villanueva de la Torre, T., Bech-Serra, J. J., Ruiz-Paz, S., Baselga, J., and Arribas, J. (2004) *Biochem. Biophys. Res. Commun.* 314, 1028–1035
77. Bringman, T. S., Lindquist, P. B., and Derynck, R. (1987) *Cell* 48, 429–440
Loss of Ectodomain Shedding Due to Mutations in the Metalloprotease and Cysteine-rich/Disintegrin Domains of the Tumor Necrosis Factor-α Converting Enzyme (TACE)

Xiaojin Li and Huizhou Fan

J. Biol. Chem. 2004, 279:27365-27375.
doi: 10.1074/jbc.M401690200 originally published online April 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401690200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 76 references, 49 of which can be accessed free at http://www.jbc.org/content/279/26/27365.full.html#ref-list-1