Tumor Necrosis Factor-α Converting Enzyme (TACE) Regulates Epidermal Growth Factor Receptor Ligand Availability*

Received for publication, December 18, 2001, and in revised form, January 28, 2002
Published, JBC Papers in Press, January 31, 2002, DOI 10.1074/jbc.M112050200

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We previously implicated tumor necrosis factor-α converting enzyme (TACE/ADAM17) in the processing of the integral membrane precursor to soluble transforming growth factor-α (TGF-α), pro-TGF-α. Here we examined TGF-α processing in a physiologically relevant cell model, primary keratinocytes, showing that cells lacking TACE activity shed dramatically less TGF-α as compared with wild-type cultures and that TGF-α cleavage was partially restored by infection of TACE-deficient cells with TACE-encoding adenovirus. Moreover, cotransfection of TACE-deficient fibroblasts with pro-TGF-α and TACE cDNAs increased shedding of mature TGF-α with concomitant conversion of cell-associated pro-TGF-α to a processed form. Purified TACE accurately cleaved pro-TGF-α in vitro at the N-terminal site and also cleaved a soluble form of pro-TGF-α containing only the ectodomain at the C-terminal site. In vitro, TACE accurately cleaved peptides corresponding to cleavage sites of several epidermal growth factor (EGF) family members, and transfection of TACE into TACE-deficient cells increased the shedding of amphiregulin and heparin-binding EGF (HB-EGF) proteins. Consistent with the hypothesis that TACE regulates EGF receptor (EGFR) ligand availability in vivo, mice heterozygous for Tace and homozygous for an impaired EGFR allele (wa-2) were born with open eyes significantly more often than Tace⁺/+EGFr⁺/+wa⁺/+ counterparts. Collectively, these data support a broad role for TACE in the regulated shedding of EGFR ligands.

The proteolytic processing of growth factors and cytokines is a key regulatory mechanism controlling receptor-mediated signaling. For example, proteases activate latent forms of transforming growth factor-β (1) and hepatocyte growth factor (2), regulate interactions between insulin-like growth factors and their binding proteins (3), and mediate the release of numerous soluble growth factors from their membrane-anchored precursors (4). In the case of membrane-anchored growth factors, the proteolytic processing has been proposed to regulate the availability of active, soluble forms, switch receptor signaling from autocrine or juxtacrine modes to paracrine or endocrine mechanisms, and/or influence the nature (e.g. duration) of the signaling event. Growth factors and cytokines that are released from membrane-anchored forms include members of the EGF superfamily, colony-stimulating factor-1, TNF-α, and the Kit ligand (4).

The EGF superfamily includes two structurally related subfamilies: the EGF-like growth factors and the neuregulins (5). The EGF subfamily also includes TGF-α, amphiregulin (AR), HB-EGF, betacellulin, and epiiregulin as well as the recently described epigen (5, 6). Soluble EGF family growth factors are all derived by proteolytic cleavage of the ectodomains of integral membrane precursors. However, the precursors display no significant homology outside of the 40–50-amino acid EGF-like motif that is the shared bioactive and structural feature of this family. Proteolytic cleavage at the C terminus of the EGF-like sequence, required to release soluble forms, generally occurs within 15 amino acids of the transmembrane domain despite the absence of a consensus cleavage site. In contrast, cleavage at the N terminus of the EGF motif is variable. Many cell types release varying levels of larger bioactive forms of TGF-α in addition to the mature, 50-amino acid growth factor due to cleavage at the C-terminal site only (7, 8). On the other hand, the EGF-like domains of HB-EGF and AR are typically released with N-terminal extensions rich in basic residues that confer heparin binding ability (9, 10). In both cases, multiple cleavage sites that are N-terminal to the basic domains have been observed (11, 12).

The proteolytic processing of EGF family members shares several characteristics with the general phenomenon of ectodomain shedding of cell surface proteins. It is a regulated event that can be rapidly induced upon exposure of cells to phorbol esters, calcium ionophores, serum factors, and phosphatase inhibitors (13–15), and it is sensitive to inhibitors of metalloproteases (16–19). However, until recently, the identity of the processing enzymes remained obscure. A major advance was the identification of a novel protease, tumor necrosis factor-α converting enzyme (TACE/ADAM17), responsible for converting membrane-anchored TNF-α to its soluble form (20, 21).

* This work was supported by Grants CA85410 and CA43793 (to D. C. L.) and Grant DK53804 (to W. E. R.) from the National Institutes of Health and by Training Grant CA11941 from the National Institutes of Health (to C. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α; TACE, TNF-α converting enzyme; ADAM, A disintegrin and metalloprotease; AR, amphiregulin; LC/MS, liquid chromatography/mass spectrometry; MALDI/TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RLEC, rat liver epithelial cells; RIA, radioimmunoassay; FBS, fetal bovine serum; HA, hemagglutinin; IC-3, Immunex compound 3; Ad, adenovirus; PMA, phorbol 12-myristate 13-acetate.

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TACE is a member of a family of integral membrane proteins termed ADAMs (A Disintegrin and Metalloproteinase) that contain multiple conserved domains, including a catalytic domain with homology to the adamalysin family of metzincin proteases, an extracellular disintegrin sequence, and a cytoplasmic domain with putative signaling motifs (reviewed in Ref. 22).

The potential importance of TACE in EGF family shedding was demonstrated through a germ line mutation in mice that eliminated the zinc-binding domain (Tace<sup>Zn<sup>−</sup>−</sup>), thereby inactivating the protease (23). Tace<sup>Zn<sup>−</sup>−</sup>/Zn<sup>−</sup> mice exhibited wavy hair and skin defects identical to those observed in mice lacking transforming growth factor-α (TGF-α) (24, 25), and immortalized fibroblasts derived from Tace<sup>Zn<sup>−</sup>−</sup>/Zn<sup>−</sup> mice shed reduced levels of TGF-α into the medium (23). Moreover, TACE-deficient mice perinatally, exhibiting widespread epithelial defects reminiscent of EGFR-deficient mice (26–28). These observations suggested that TACE might regulate the availability of EGF family ligands (23, 29).

In this report, we provide several lines of evidence derived from <i>in vitro</i>, cell-based, and whole animal studies that establish TACE as a major pro-TGF-α convertase. We also provide evidence consistent with a role for TACE in mediating the release of other EGF family members, especially AR and HB-EGF. Finally, we show that limiting the gene dosage of TACE exacerbates the phenotype of a weak EGFR allele (waved-2) <i>in vivo</i>. Collectively, these results indicate a broad role for TACE in regulating EGFR activity.

**Experimental Procedures**

**Materials—**Monoclonal anti-FLAG M2 was obtained from Sigma, anti-HA was obtained from Covance (Berkeley, CA), and anti-Myc 9E10 was purchased from Sigma (St. Louis, MO). All cDNAs used in this study were expressed from pcDNA3 (Invitrogen) and, after 24 h, returned to complete media containing 100 ng/ml EGF. Conditioned media and lysates were collected after 48 h and concentrated using Sep-Pak C-18 reverse phase beads were spotted directly onto a target, and the molecular weights of resulting bands were identified by SDS-PAGE/Western blot as described above. Equivalent amounts of protein (50–100 μg) were separated by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride (Millipore). Membranes were stained with Coomassie Blue according to the manufacturer’s instructions. Pro-TGF-α was eluted with FLAG peptide, and eluates were pooled and concentrated using Centricon-10 concentrators (Millipore). Concentrated pro-TGF-α was incubated with ~200 units of partially purified native TACE (through the Mono-Q step in Ref. 20) from Chinese hamster ovary cells or with buffer only (1 unit of TACE cleaves 0.42 fmol/min of TGF-β peptide at 37°C, pH 7.5). Resulting products were identified by SDS-PAGE/Western blot as described above. For sequencing of TACE cleavage products, lysates from the same cells were pooled and enriched for pro-TGF-α using a monoclonal antibody to FLAG. Cells were transfected as described above. For N-terminal cleavage, lysate from a rodent cell line stably expressing epitope-tagged TGF-α (34) was cotransfected with a negative control (beads only) and a synthetic peptide, and a peptide from a negative control (beads only) and a synthetic peptide, VVASSKKQQKDYKDDDDKVV, matching the sequence of the expected product.

**Transfection and Western Blot Analysis—**Wild-type and Tace<sup>Zn<sup>−</sup>−</sup>/Zn<sup>−</sup> fibroblasts (clones EC-4 and EC-2 (35)) were grown in Dulbecco’s modified Eagle’s medium-H, 10% FBS. Cells were transfected using the expression vectors kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University). AdTACE<sup>−</sup> virus was created by subcloning a TACE cDNA from pAdTrackCMV, a primary epitoderm keratinocyte from Tace<sup>Zn<sup>−</sup>−</sup>/Zn<sup>−</sup> mice were grown to 70% confluence, washed twice with 1× phosphate-buffered saline, and shifted to 1% FBS medium containing 100 ng/ml EGF. Conditioned media and lysates were collected after 48 h and concentrated using Sep-Pak C-18 reverse phase.
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**RESULTS**

Efficient TGF-α Shredding Requires TACE—We showed previously that polyclonal Ras/Myc-transformed Taceα/ΔZn/EV embrionic fibroblasts shed about 20-fold less TGF-α into the medium as compared with wild-type counterparts (23). Here we compared TACE α/ΔZn/EV sheds primary epidermal keratinocytes. RIA analysis showed that newborn Taceα/ΔZn/EV keratinocytes released 12-fold less TGF-α as compared with Taceα/ΔZn/EV counterparts (Fig. 1). Northern blots revealed equivalent TGF-α mRNA levels in the two cell populations (not shown) excluding differences in TGF-α expression as an explanation for the difference in shedding. Both genotypes also exhibited similar amounts of keratin 5 (not shown), a marker of basal keratinocytes, indicating that differences in shedding were not accounted for by alterations in Taceα/ΔZn/EV keratinocyte differentiation. Consistent with a previous report that induced shedding of transfected TGF-α requires metalloprotease activity (16), both TACE-dependent as well as the residual release of endogenous TGF-α in Taceα/ΔZn/EV keratinocytes were sensitive to the hydroxamic acid compound, IC-3 (20). Shedding by Taceα/ΔZn/EV was inhibited 10-fold in the presence of IC-3, whereas release from Taceα/ΔZn/EV keratinocytes was inhibited >4-fold (Fig. 1).

To determine whether reintroduction of TACE restores pro-TGF-α processing, we infected Taceα/ΔZn/EV keratinocytes with either control adenovirus (Ad) (33) or virus-expressing mouse TACE cDNA (AdTACE). Staining for virus-encoded green fluorescent protein confirmed comparable infection by the two vectors. AdTACE infection of Taceα/ΔZn/EV cells increased TGF-α shedding about 4-fold as compared with cells infected with Ad (Fig. 1). The failure to fully restore TGF-α shedding may be due to incomplete infection of the cell population. It is unlikely that the incomplete restoration of TGF-α shedding reflects a dominant negative effect of the Taceα/ΔZn/EV allele; the mutant TACE protein encoded by the Taceα/ΔZn/EV allele does not function as a dominant negative since TNF and L-selectin were released at wild-type levels from Taceα/ΔZn/EV cells (23).

**Pro-TGF-α Is Converted to Mature Growth Factor upon Reintroduction of TACE—**Besides mature, 50-amino acid TGF-α, many cell types also release larger forms of the growth factor due to cleavage at the C-terminal site only (7, 8). To determine whether reintroduction of TACE into Taceα/ΔZn/EV cells reconstituted complete processing of the TGF-α precursor, we transiently transfected these cells with pro-TGF-α containing HA and FLAG epitopes (Fig. 2A). We previously used this construct to establish a pro-TGF-α processing scheme in rat liver epithelial cells (RLEC) in which an immature glycoprotein precursor of 22-25 kDa was converted to a mature, cell surface glycoprotein of 36 kDa; the latter was then cleaved to produce soluble TGF-α and a stable, cell-associated 16-kDa species corresponding to the residual tail of the precursor (34). Others have described similar pro-TGF-α processing schemes (43-45).

For these experiments, we utilized a clonal Ras/Myc-immortalized fibroblast cell line, EC-2 (35). EC-2 (Taceα/ΔZn/EV) cells released about 20-fold less endogenous TGF-α into the medium as compared with corresponding Taceα/ΔZn/EV cells (EC-4; Fig. 2B), confirming that TACE is required for TGF-α shedding by this cell type. Anti-FLAG recognized a prominent pro-TGF-α species of 25 kDa together with less abundant precursor proteins of 22 and 36 kDa in lysates of EC-2 cells transfected with TGF-α expression vector only (Fig. 2C). In contrast, anti-FLAG recognized a prominent 16-kDa protein in lysates of EC-2 cells transfected with both TGF-α and TACE expression vectors. Unlike the larger immunoreactive bands, the 16-kDa protein was not recognized by anti-HA (not shown); thus, it corresponds to the residual precursor tail resulting from cleavage of the ectodomain at the C-terminal site. Analysis of TGF-α species present in media supported this conclusion. In the absence of transfected TACE, two HA-reactive species of ~6 kDa were present, replaced by a more prominent band of intermediate size in the presence of TACE.

**TACE Cleaves Pro-TGF-α in Vitro—**To assess the ability of TACE to directly cleave pro-TGF-α, lysates of RLEC stably expressing HA/FLAG-tagged pro-TGF-α (34) were incubated in the absence or presence of native TACE. Consistent with previous observations (34), anti-HA recognized prominent pro-TGF-α proteins of about 25 and 36 kDa in Western blots of reaction mixtures incubated in the absence of TACE (Fig. 3A, left). In the presence of TACE, a prominent new species of about 20 kDa was detected with corresponding decreases in larger pro-TGF-α species, especially the 25-kDa form. This 20-kDa product was readily detected after a 15-min incubation with exogenous TACE but was present at higher levels after 60 min. Its appearance was inhibited in the presence of IC-3. Minor amounts of the 20-kDa product were also detected when reactions were performed for 60 min in the absence of TACE, presumably due to endogenous TACE in RLEC.4,5

Importantly, the novel, TACE-dependent 20-kDa product

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3 C. S. Raska, C. E. Parker, S. W. Sunnarborg, D. C. Lee, G. L. Glish, R. M. Pope, and C. H. Borchers, unpublished observations.

4 S. W. Sunnarborg and D. C. Lee, unpublished observations.

5 C. L. Hinkle, M. J. Mohan, M. E. Mills, P. Lin, W. E. Russell, M. Stevenson, D. C. Lee, and M. L. Moss, unpublished observations.
was also recognized by anti-FLAG (Fig. 3A), indicating that TACE cleaved only the N-terminal processing site of pro-TGF-α \textit{in vitro} and not the C-terminal site. Although we occasionally observed modest increases in the levels of 16-kDa tail following incubation with TACE (Fig. 3A, 15-min time point), anti-HA did not detect mature TGF-α in these reactions, which is consistent with limited or absent cleavage of the C-terminal site.

To confirm accurate cleavage by TACE, pro-TGF-α was partially purified by anti-FLAG affinity chromatography and incubated with recombinant human TACE ectodomain (36). The N-terminal sequence of the 20-kDa protein identified after SDS-PAGE was determined to be VVSHY, confirming cleavage at the site corresponding to the N terminus of mature TGF-α (46). As part of these analyses, we also determined N-terminal sequences of endogenous TGF-α proteins to confirm their identity. The sequence of the 16-kDa species was VVAAS, confirming its identity as the precursor tail derived by accurate cleavage at the C-terminal site of pro-TGF-α. The N-terminal sequences of the FLAG-reactive 25- and 36-kDa pro-TGF-α species were identical: LE\textit{X} (the expected N residue at +3 was not identified, presumably due to glycosylation), confirming these as forms of intact pro-TGF-α lacking only the signal peptide.

The failure of TACE to cleave the C-terminal site of pro-
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Fig. 3. TACE cleaves pro-TGF-α in vitro. A, lysates from cells stably expressing epitope-tagged pro-TGF-α were incubated with TACE buffer or ~200 units of native TACE for the indicated times at 37 °C and products detected by Western blot using anti-HA or anti-FLAG. Where shown, the hydroxamic acid inhibitor, IC-3, was included at 50 μM. Note that the 20-kDa product arising from incubation of pro-TGF-α with TACE is detected by both antibodies. B, concentrated media from cells transfected with pro-TGFecto were incubated with ~1 μg of recombinant human TACE for the indicated times at 37 °C. Where indicated, IC-3 was included at 50 μM. The resulting products were separated by SDS-PAGE and detected by Western blot using anti-HA or anti-FLAG. C, MALDI/TOF-MS analysis of anti-FLAG-reactive products from pro-TGFecto cleavage by TACE. Top panel, anti-FLAG M2 affinity gel control; middle panel, products of TACE reaction; lower panel, synthetic peptide representing cleavage at the C-terminal Ala-Val site.

TGF-α could be due to interference by the hydrophobic transmembrane domain in vitro. To address this possibility, we created a soluble ectodomain construct (Fig. 2A), pro-TGFecto. Pro-TGFecto was expressed in COS-1 cells, and conditioned media were harvested and incubated with recombinant human TACE ectodomain. In the absence of TACE, anti-FLAG and anti-HA both detected several 18–25-kDa precursor forms (Fig. 3B). After a 60-min incubation with TACE, 6- and 8-kDa HA-reactive products appeared with a concomitant decrease in pro-TGFecto species. The 8-kDa product was also FLAG-reactive, indicating that it resulted from N-terminal cleavage. After 4 h, pro-TGFecto was converted to a single product equal in size to fully processed 6-kDa TGF-α and containing the HA but not the FLAG epitope (Fig. 3B). The predicted 2.1-kDa C-terminal, FLAG-reactive product resulting from TACE cleavage at the C-terminal site was not detected on Western blots, presumably due to its small size.

To confirm that conversion to 6-kDa TGF-α resulted from cleavage of the C-terminal site in pro-TGFecto, we used mass spectrometry to characterize the C-terminal, FLAG-reactive product. MALDI/TOF-MS (Fig. 3C) revealed the singly protonated C-terminal product to be 2151 m/z, equal to a synthetic peptide matching the expected product. This confirms TACE cleavage at the physiological AV cleavage site (see Table I for sequence). A second product of 1811 m/z was also detected. MALDI-MS/MS3 revealed that this product resulted from the A–S peptide bond, 4 residues C-terminal to the AV site. TACE also cleaved the synthetic peptide at this site (not shown). However, since the N-terminal sequence (see above) of the endogenous cytoplasmic tail recovered from pro-TGF-α-expressing RLEC was VVAAS, cleavage of the A-S site may be an in vitro artifact.

Peptide Cleavage Assays—Tace<sup>ΔZn/ΔZn</sup> mice exhibit widespread epithelial defects reminiscent of those observed in EGFR<sup>−/−</sup> mice, suggesting that it might have a role in the shedding of multiple EGFR ligands (23, 29). We therefore tested the ability of TACE to cleave peptides representing the published N- and C-terminal cleavage sites of multiple EGF family members (10, 12, 19, 23, 38–42). Recombinant TACE cleaved peptides corresponding to the C-terminal sites of betacellulin, epiregulin, and HB-EGF as well as the N-terminal sites of AR and epiregulin (Table I). Notably, cleavage of all these peptides required 20-fold more TACE than cleavage of either TGF-α peptide. Interestingly, the C-terminal HB-EGF peptide was cleaved by TACE at two positions, both with non-polar residues in the P<sub>1</sub>′ position: a Pro-Val dipeptide corresponding to the site predicted from the sequence of mature HB-EGF released following PMA stimulation (42), whereas the Arg-Leu cleavage site was not reported previously. We did not observe cleavage of the Glu-Asn dipeptide shown previously to be a substrate for matrix metalloproteinase-3 (19). In contrast, TACE did not cleave peptides representing the C-terminal site for AR, the N-terminal site for betacellulin, or either of the mature EGF processing sites.

AR and HB-EGF Transfections—To extend the peptide assays, we tested the ability of transfected TACE to release the ectodomains of cotransfected epitope-tagged AR and HB-EGF in cell culture. For both, the HA tag was inserted in the mature growth factor domain, and a Myc epitope was inserted at the C terminus of the precursor (Fig. 2A). These cDNAs were cotrans-
Peptides representing the cleavage sites of human EGFR family members (arrows denote predicted cleavage sites) were incubated with recombinant TACE (36) at 37 °C for 4 h and products analyzed by LC/MS as previously described (23). Results of cleavage of TGF-α peptides (23) are included for comparison. Observation of cleavage and the site are indicated. Approximate concentration of TACE required for reaction is indicated. Note that cleavage of TGF-α peptides required approximately 10× more TACE than cleavage of a TNF peptide (23).

**TABLE I**

| Ligand | N-terminal peptide | Cleaved \( ^{a} \) | [TACE] | C-terminal peptide | Cleaved \( ^{a} \) | [TACE] |
|--------|-------------------|-----------------|--------|-------------------|-----------------|--------|
| TGF-α  | PVAAS \| VVSHF | Yes | 1.4 μM | ADLAA \| VVAAS | Yes | 1.4 μM |
| AR     | SVRVEQ \| VKFPQ | Yes Q/V | 28 μM | ERCGER \| SMKTHS | No | 28 μM |
| BTC    | RSPETN \| LLCGDE | No | 28 μM | RVDFLY \| LBGDRG | Yes Y/L | 28 μM |
| EPR    | NPRVAQ \| VSITKC | Yes Q/V | 28 μM | CEHFFL \| TVGLPL | Yes N.D.° | 28 μM |
| HB-EGF | N.D.° | No | 28 μM | GLSLP \| VNRILYTDY | Yes P/V R/L | 28 μM |
| EGF    | HHHYSVR \| NSDSEC | No | 28 μM | KWWELR \| HAGHQG | No | 28 μM |

\( ^{a} \) N.D., not determined.

Several lines of evidence unambiguously establish a role for TACE in TGF-α shedding. Although the TACE\( ^{Δ2N/Δ2N} \) mutation is perinatal lethal, survivors are invariably born with open eyes and curly whiskers, and rare mice that survive beyond the first few days display misoriented hair follicles as well as wavy hair (23). These eye, skin, and hair phenotypes are the hallmark of TGF-α-deficient mice (24, 25). In addition, we showed previously that TACE cleaves peptide substrates corresponding to the two processing sites of pro-TGF-α in vitro and that transformed TACE\( ^{Δ2N/Δ2N} \) fibroblasts are deficient in TGF-α shedding (23).

**DISCUSSION**

Several lines of evidence unambiguously establish a role for TACE in TGF-α shedding. Although the TACE\( ^{Δ2N/Δ2N} \) mutation is perinatal lethal, survivors are invariably born with open eyes and curly whiskers, and rare mice that survive beyond the first few days display misoriented hair follicles as well as wavy hair (23). These eye, skin, and hair phenotypes are the hallmark of TGF-α-deficient mice (24, 25). In addition, we showed previously that TACE cleaves peptide substrates corresponding to the two processing sites of pro-TGF-α in vitro and that transformed TACE\( ^{Δ2N/Δ2N} \) fibroblasts are deficient in TGF-α shedding (23).

Here, we additionally show that the release of soluble TGF-α by primary epidermal keratinocytes derived from TACE\( ^{Δ2N/Δ2N} \) mice is dramatically reduced as compared with shedding by corresponding TACE\( ^{+/−} \) cells. This deficiency is at least partly restored in both keratinocytes and fibroblasts upon introduction of active TACE. In addition, we demonstrate that native or recombinant TACE faithfully cleaves both the N- and C-terminal sites of a soluble form of pro-TGF-α in vitro. Cleavage at the N-terminal site by native TACE was observed with full-length pro-TGF-α, whereas the C-terminal site was not cleaved in the intact precursor. We speculate that the absence of cleavage at this site in vitro is an artifact resulting from interference by the hydrophobic transmembrane domain of the precursor. To date, TACE has been implicated in the shedding of a large number of diverse cell surface molecules. However, apart from TNF-α (20, 21), only pro-TGF-α has been shown to be directly cleaved by TACE at the correct sites. Collectively, these findings suggest that TACE functions directly as a major TGF-α convertase, although they do not exclude possible physiological roles for additional or alternative proteases. It is still possible that TACE functions in vivo as part of a cascade that regulates the processing of pro-TGF-α.

Previous studies found that the N-terminal processing site of pro-TGF-α was cleaved rapidly in Chinese hamster ovary cells, whereas the C-terminal site was cleaved much less efficiently unless ectodomain shedding was induced by phorbol esters, calcium ionophores, etc. (13–15, 45). It is interesting, therefore, that TACE cleaved the N-terminal site for the pro-TGFα substrate more efficiently than the C-terminal site. Specifically, two N-terminal cleavage products differing only at the C terminus were observed with short incubations; these were resolved to a fully processed product upon further incubation. Significantly, we never observed a product representing initial
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**Fig. 4.** Reintroduction of TACE results in increased AR and HB-EGF shedding. Tace<sup>−/−</sup>/Zn<sup>−/−</sup> fibroblasts were transfected with epitope-tagged pro-AR or pro-HB-EGF and either pcDNA3 or TACE as shown. Media were conditioned for 24 h, and lysates and media were analyzed as described in the legend for Fig. 2 except that HB-EGF-conditioned media were directly immunoprecipitated with anti-HA prior to Western blot. Indicated molecular sizes are derived from the migration of known standards. Asterisks indicate HB-EGF forms that increased in the media when TACE was present.

**Fig. 5.** TACE deficiency exacerbates the open-eye phenotype of Egfr<sub>wa-2/wa-2</sub> mice. Intercrossing of Tace<sup>−/−</sup> females and Egfr<sup>wa-2/wa-2</sup> males produced pups of the indicated genotypes that were scored for open eye at birth. Note that the open-eye phenotype was restricted to Egfr<sup>wa-2/wa-2</sup> animals and was significantly (p = 0.012) more frequent in TACE heterozygotes.

TACE<sup>+/−</sup>Egfr<sup>+/+</sup> X TACE<sup>+/−</sup>Egfr<sup>wa-2/wa-2</sup>

TACE<sup>+/−</sup>Egfr<sup>+/−</sup>/Zn<sup>−/−</sup> X TACE<sup>+/−</sup>Egfr<sup>wa-2/wa-2</sup>/Zn<sup>−/−</sup>

TACE: +/+ +/−Zn +/− +/−Zn

Egfr: +/wa-2 +/−wa-2 wa-2/wa-2 wa-2/wa-2

Pups born: 38 35 25 24

Open-eyed pups: 0 0 3 11

cleavage at the C-terminal site. These results are consistent with TACE cleaving both sites albeit with different efficiency. Since the sequences surrounding the two cleavage sites (AA ↓ VV and LA ↓ VV) are highly similar, other pro-TGF-α sequence or structural motifs likely contribute to substrate recognition.

Several lines of evidence also indicate a role for TACE in processing additional EGF family members. These include in vitro peptide cleavage assays, cotransfection experiments, and in vivo genetic evidence. Peptides corresponding to the processing sites of several EGF family members, including AR and HB-EGF, were cleaved by recombinant TACE. Despite the failure of TACE to cleave the C-terminal peptide of AR in vitro, restoration of TACE expression in Tace<sup>−/−</sup>/Zn<sup>−/−</sup> cells resulted in a significant increase in the release of both AR and HB-EGF into the media. A role for TACE in pro-HB-EGF processing is further supported by the recent observation that Tace<sup>−/−</sup>/Zn<sup>−/−</sup> cells are deficient in the shedding of transfected human HB-EGF (55).

ADAM9 (MDC9/meltrin-γ), ADAM10, and ADAM12 have also recently been implicated in HB-EGF shedding (56–58), suggesting that distinct enzymes mediate the shedding of a single substrate. In fact, recent studies, including one based on inhibitor data and membrane fractionation, have pointed to the existence of one or more additional pro-TGF-α convertase activities (55). This may explain the residual, metalloproteinase-dependent TGF-α release we observed from Tace<sup>−/−</sup>/Zn<sup>−/−</sup> primary keratinocytes and the limited TACE-independent processing of pro-TGF-α observed in transfection experiments. It seems likely that different proteases catalyze pro-growth factor processing in different cell types and in response to distinct stimuli, as has been suggested for other cell surface targets (59).

Significantly, phenotypic similarities between Tace<sup>−/−</sup>/Zn<sup>−/−</sup> mice and EGFR ligand knockouts suggest that soluble forms of the EGF family members are critical for normal development despite the apparent bioactivity of their membrane-anchored precursors (54, 60–63). Consistent with a role for TACE in regulating EGFR ligand availability, we observed a dramatic increase in the frequency of the open-eye phenotype of Egfr<sub>wa-2/wa-2</sub> mice with a reduction in TACE (Tace<sup>+/−</sup>/Zn<sup>−/−</sup>) gene dosage. This indicates that the TACE activity derived from a single wild-type allele did not mediate sufficient release of soluble ligand to overcome the impaired kinase activity of the waved-2 receptor. (We previously observed waved-2 receptor activation to wild-type levels in the presence of sufficient ligand (49).) This raises the intriguing possibility that TACE might be an important therapeutic target for treatment of hyperproliferative diseases, including cancer, that are characterized by excessive production of EGFR ligands. The therapeutic potential of TACE, as well as its demonstrated role in normal development, underscores the necessity of a thorough understanding of its role in the shedding of this important family of bioregulatory molecules.

Acknowledgments—We thank Nolan Yeung and Aileen Chang for technical assistance. We are also grateful for assistance from Christoph Borchers, Carol Parker, and the UNC Proteomics Core Facility with mass spectrometry and for advice from Marcia Moss on in vitro TACE experiments. We also thank Leslie Jackson and Kelly Troyer for helpful comments on the manuscript.
