Epidermal growth factor (EGF) family ligands are derived by proteolytic cleavage of the ectodomains of integral membrane precursors. Previously, we established that tumor necrosis factor-α-converting enzyme (TACE/ADAM17) is a physiologic transforming growth factor-α (TGF-α) sheddase, and we also demonstrated enhanced shedding of amphiregulin (AR) and heparin-binding growth factor (HB-EGF) upon restoration of TACE activity in TACE-deficient EC-2 fibroblasts. Here we extended these results by showing that purified soluble TACE cleaved single sites in the juxtamembrane stalks of mouse pro-HB-EGF and pro-AR ectodomains in vitro. For pro-HB-EGF, this site matched the C terminus of the purified human growth factor, and we speculate that the AR cleavage site is also physiologically relevant. In contrast, ADAM9 and -10, both implicated in HB-EGF shedding, failed to cleave the ectodomain or cleaved at a nonphysiologic site, respectively. Contransfection of TACE in EC-2 cells enhanced phosphor myristate acetate-induced but not constitutive shedding of epiregulin and had no effect on betacellulin processing. Additionally, soluble TACE did not cleave the juxtamembrane stalks of either pro-BTC or pro-epiregulin ectodomains in vitro. Substitution of the shorter pro-BTC juxtamembrane stalk or truncation of the pro-TGF-α stalk to match the pro-BTC length reduced TGF-α shedding from transfected cells to background levels, whereas substitution of the pro-BTC P2-P2′ sequence reduced TGF-α shedding less dramatically. Conversely, substitution of the pro-TGF-α stalk or shortening of the pro-BTC stalk, especially when combined with substitution of the pro-TGF-α P2-P2′ sequence, markedly increased BTC shedding. These results indicate that efficient TACE cleavage is determined by a combination of stalk length and scissile bond sequence.

The epidermal growth factor (EGF) family includes seven structurally related proteins as follows: EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding epidermal growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen (reviewed in Ref. 1). Members of this family share a distinctive disulfide-bonded 3-loop domain, the EGF-like motif, which is required for high affinity binding to the EGF receptor (EGFR). Soluble receptor ligands containing this EGF-like sequence are in each case released from integral membrane precursor proteins. The critical “C-terminal” cleavage events that are required for release occur within short juxtamembrane stalks that link the EGF-like motifs to the transmembrane (TM) domains. Additionally, variable cleavage at membrane distal sites (N-terminal cleavage) gives rise to larger soluble forms and could modulate the function of EGF family members. Whether both C- and N-terminal processing events are mediated by the same or different enzymes is presently unclear.

Although both integral membrane and soluble growth factors have been shown to activate EGFR (2–5) with potentially different consequences (6–9), the observed phenotypes of EGF family knockouts are consistent with paracrine roles for these growth factors (10–14), and some biological responses may be impaired by preventing release of soluble growth factor (7, 15–18). Moreover, regulated shedding of EGF ligands has been linked recently to G-protein-coupled receptor-mediated EGFR transactivation (1, 19–24). Thus, identifying the mechanisms that govern EGF family shedding is critical to an understanding of the regulation of EGF family/EGFR action.

Several lines of evidence have implicated the disintegrin and metalloprotease TACE/ADAM17 (25, 26) in the release of soluble TGF-α, a well studied model of ectodomain shedding. Mice homozygous for a mutant TACE gene (TACE<sup>−/−</sup>) encoding an inactive enzyme displayed the same open eye at birth and wavy
TACE-dependent Shedding of EGF Family

whisker (27) phenotype as TGF-α (10, 11) and EGF-α-/- (28–30) mice. Fibroblasts, as well as primary keratinocytes, derived from these TACE-deficient mice, were dramatically impaired in their ability to shed TGF-α, whereas restoration of functional TACE enhanced processing (27, 31). Moreover, solubilizable TACE correctly cleaved the pro-TGF-α ectodomain at both N- and C-terminal processing sites in vitro (31). Collectively, these results established TACE as a major TGF-α sheddase, although TACE-deficient cells retain reduced ability to shed TGF-α, apparently because of the minor action of one or more other metalloproteinases (32, 33).

TACE-deficient mice die soon after birth, displaying epithelial defects in lung, small intestine, stomach, thyroid, parathyroid, and salivary glands (27). These defects are broadly similar to those observed in EGFR null mice, prompting speculation that TACE is a Pan-EGF family sheddase (27, 34). Supporting instructions. TACE-deficient mice and cell lysates were harvested. For PMA experiments, cells were switched to complete media after 24 h. After 24 h, cells were washed with serum-free media and incubated in serum-free media in the presence and absence of 100 ng/ml TACE (Sigma) for 2 h (33) before conditioned media and cell lysates were harvested. For each PMA experiment sample, media or lysate from three transfected 60-mm dishes were pooled and treated together. All media was concentrated on Sep-Pak C-18 cartridges (Waters) and lyophilized (44). To collect lysates, cells were washed in 1× phosphate-buffered saline (PBS) and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100 with protease inhibitors (10 μg/ml leupeptin, 20 μg/ml aprotonin, 1 μM phenylmethylsulfonyl fluoride (PMSF), and 1 μM EDTA). Protein concentrations were determined by using the BCA Protein Assay (Pierce). Conditioned media and cell lysates (75 μg) were analyzed by Western blot (31) as indicated.

Sandwich ELISA—To quantitate nanogram levels of epitope-tagged growth factors, we developed a sandwich ELISA for TGF-α and BTC similarly to published protocols (45). Briefly, 96-well enzyme immunoassay/radioimmunoassay high binding plates (Corning Glass) were incubated with 10–15 μg/ml anti-HA antibody overnight at room temperature. After each incubation, wells were washed three times with wash buffer (1× PBS, 0.05% Tween 20). Wells were blocked in 2% BSA, 1× PBS, 0.05% Tween 20 for 1 h at room temperature. Media and lysate samples or standards (ligand ectodomains) were diluted in dilution buffer (2% PBS, 1× PBS, 0.05% Tween 20), added to wells in triplicate, and incubated for 1.5 h at room temperature. Anti-growth factor antibodies were added at 100 μg/ml (anti-TGF-α) or 1,200 μg/ml (anti-BTC) in dilution buffer. Antibodies were eluted by using 1 mg of HA peptide in equilibration buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1% Triton X-100) for 1.5 h at room temperature. The ELISA was read on a plate reader at 450 nm.

Production of Ectodomain Ligands—Pro-TGF-ecto was generated previously (31). Forward primers used for mutagenesis are as follows: ProARecto, 5′-GGAGGATCAAGAGGACACATCTCAAGGACGACGACGACGACGATGAC-3′; Pro-HB-EGFecto, 5′-CCATATGACCACACTGATTACAAGGACGACGATGAC-3′; Pro-HB-EGFecto, 5′-CTGTTAGCTACGTTCACATTTCATCAAGGAGGACGACGATGAC-3′; Pro-BTCecto, 5′-GCGAGGATGACAAGGACCTATCCAAGGATTACAACTTTTGTACACGCTTTCTTGCGTTGACAGTGATTCTCATTTTCGATTACAAGGACGACG-3′. To produce ectodomain proteins, cDNAs were transfected into COS-1 cells using FuGENE 6 (Roche Applied Science). After 24 h, cells were harvested in complete media (DME, 10% FBS) and cultured for an additional 24 h before conditioned media were collected. For pro-BT-Cecto and pro-EPRecto, conditioned media were concentrated as described (31). For pro-ARepto and pro-HB-EGFecto, media were immunoprecipitated by using anti-HA antibody resin at 4 °C with tumbling for 24–48 h. Beads were washed with 20 bed volumes of wash buffer (20 mM Tris, pH 7.5, 0.1 M NaCl, 0.05% Tween 20). The sample was eluted by using 1 mg of HA peptide in equilibration buffer (20 mM Tris, pH 7.5, 0.1 M NaCl, 0.1 M EDTA) at 37 °C with tumbling. To remove excess HA peptide, proteins were dialyzed against 10 mM Tris, pH 8.0, at 4 °C for 16–18 h using Spectra-Por membrane (10,000 molecular weight cut-off) Spectrum Laboratories Inc., Rancho Dominguez, CA. The protein concentration was assayed using the BCA Protein Assay (Pierce) and normalized for ectodomain proteins by comparison with Western blots. In Vitro Digests—Aliquots (1–10 μg) of ectodomain proteins were incubated with 150–300 μg/ml recombinant human TACE or ADAM9 or ADAM10 (R & D Systems) or 10 mM trypsin, pH 8.0, at 37 °C (31), or 24 h. Reactions were stopped by adding 2× SDS-PAGE sample buffer, and products were separated by SDS-PAGE, transferred to Immobilon polyvinylidene difluoride, and probed with the indicated antibodies. For determination of N- and C-terminal cleavage sites, reactions were stopped by adding 10 mM EDTA, and products were

EXPERIMENTAL PROCEDURES

Materials—The antibodies used are as follows: monoclonal anti-HA.11 (Covance Research Products, Denver, PA), monoclonal anti-FLAG M2 (Sigma), polyclonal anti-human TGF-α (Sigma), and polyclonal anti-mouse BTC (R & D Systems, Minneapolis, MN). Peroxidase-conjugated secondary antibodies were from Roche Applied Science or Santa Cruz Biotechnology. For PMA experiments, cells were switched to complete media after 24 h. After 24 h, cells were washed with serum-free media and incubated in serum-free media in the presence and absence of 50 μM TAPI-2 (Peptides International, Louisville, KY) after 24 h. After 24 h, conditioned media and cell lysates were harvested. For PMA experiments, cells were switched to complete media after 24 h. After 24 h, cells were washed with serum-free media and incubated in serum-free media in the presence and absence of 100 ng/ml TGF-α (Sigma) for 2 h (33) before conditioned media and cell lysates were harvested. For each PMA experiment sample, media or lysate from three transfected 60-mm dishes were pooled and treated together. All media was concentrated on Sep-Pak C-18 cartridges (Waters) and lyophilized (44). To collect lysates, cells were washed in 1× phosphate-buffered saline (PBS) and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100 with protease inhibitors (10 μg/ml leupeptin, 20 μg/ml aprotonin, 1 μM phenylmethylsulfonyl fluoride (PMSF), and 1 μM EDTA). Protein concentrations were determined by using the BCA Protein Assay (Pierce). Conditioned media and cell lysates (75 μg) were analyzed by Western blot (31) as indicated.

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immunoprecipitated by using M2 anti-FLAG affinity gel at 4°C for 16–18 h. Beads were washed five times with fresh 50 mM ammonium bicarbonate. Molecular weights of cleavage products were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) using a Bruker Reflex III mass spectrometer (Bruker Instruments Co., Billerica, MA) optimized in the linear mode (46, 47), and recrystallized α-cyano-4-hydroxycinnamic acid (Aldrich) was used as the matrix. The α-cyano-4-hydroxycinnamic acid solvent was 50:50:0.1 acetonitrile/water/trifluoroacetic acid and used as a saturated solution. A 0.5-μL aliquot of the settled beads was spotted on the target, followed by 0.5 μL of matrix solution, and the solution was allowed to dry at room temperature.

**Construction of Juxtamembrane Domain Mutants—** Epitope-tagged pro-TGF-α (41) and pro-BTC cDNAs were used to construct juxtamembrane domain mutants. For the BTC-JM_{V12-LAVV}{sup}–2 mutant, sequential rounds of mutagenesis were performed using the BTC-JM_{V12-LAVV}{sup} and BTC-JM–2 primers. Forward primers used are as follows: TGF-JMBC, 5′-GGTCTGCTTGATAGTGG-3′; BTC-JM–2, 5′-GTACTGGTACGTGGTGTGAC-3′; and BTC-JM–2 mutant, 5′-GGTCTGCTTGATAGTGG-3′.

**Residual tail** (TM)/cytoplasmic domains of pro-TGF-α were isolated on Protein A-Sepharose beads (Figure 2B) and subjected to 16% polyacrylamide gel electrophoresis (PAGE) and Western blotting with anti-HA (41) and anti-FLAG (data not shown). These likely correspond to the residual TM/cytoplasmic domains (50). Media from cells transfected with TGF-α and TACE as compared with cells transfected with TGF-α alone (data not shown).

**Transfection of pro-BTC cDNA** into EC-2 fibroblasts produced several forms of cell-associated BTC, including prominent species of 36 and 42 kDa that were recognized by anti-HA (Figure 2A) and anti-FLAG (data not shown). These likely correspond to variably glycosylated full-length forms, because murine BTC has three N-glycosylation sites (49). An 18-kDa protein recognized only by anti-FLAG (Figure 2A) likely corresponds to the residual TM/cytoplasmic domains (50). Media from cells transfected with TGF-α and TACE as compared with cells transfected with TGF-α alone (data not shown).

**Statistical Analysis**—Statistical analysis was performed by the University of North Carolina Lineberger Comprehensive Cancer Center Biostatistics Facility using SAS statistical software, version 8.2 (SAS Institute Inc., Cary, NC), and the Wilcoxon rank sum test for pairwise group comparisons. p values are exact and have been adjusted by using the Bonferroni method to allow multiple comparisons.

**RESULTS**

**TACE Does Not Enhance Shedding of Betacellulin or Epi-regulin from Cells**—We showed previously that cotransfection of TACE with TGF-α, AR, or HB-EGF enhanced shedding of these growth factors from TACE-deficient (EC-2) fibroblasts (31). To assess the role of TACE in the shedding of two additional EGF family members, BTC and EPR, we generated epitope-tagged constructs encoding the precursors (Figure 1A). As with pro-TGF-α (41), the HA epitope was inserted into the N-terminal portion of the EGF-like sequence, either three (BTC) or two (EPR) amino acids upstream of the first cysteine. In both cases, the FLAG epitope was inserted into the cytoplasmic domain of the precursor immediately preceding the penultimate amino acid. Tagged pro-BTC or pro-EPR were transfected into EC-2 fibroblasts, which lack functional TACE (43), in the presence or absence of TACE cDNA, and lysates and conditioned media were analyzed by Western blot with anti-HA and FLAG antibodies. Pro-TGF-α and empty pcDNA3 vector were transfected as positive and negative controls.

As observed previously (31), lysates of TGF-α-transfected EC-2 cells (43) contained 36- and 20–25-kDa species that were recognized by both anti-HA (Figure 2A) and anti-FLAG (data not shown); the 36-kDa form is the fully glycosylated precursor, and the 20–25-kDa forms are immature glycoprotein precursors (41). FLAG, but not HA antibody, also recognized a minor 16-kDa species corresponding to the residual transmembrane (TM)/cytoplasmic domains of pro-TGF-α (Figure 2A). Media from the transfected cells contained low levels of soluble HA-reactive 6- and 25-kDa proteins corresponding to mature TGF-α and larger fully glycosylated forms cleaved only at the C-terminal site (48). High speed centrifugation of conditioned media confirmed that the 25-kDa protein was soluble and not membrane-associated (data not shown). In some experiments, the species appeared as closely spaced doublets. Inclusion of the metalloprotease inhibitor, TAPI-2, in the culture increased levels of the precursor forms (Figure 2A), decreased the 16-kDa tail, and shifted media forms to higher molecular weight, implicating metalloproteases in residual pro-TGF-α processing in EC-2 cells. As observed previously (31), cotransfection of TACE decreased the 36-kDa form, dramatically increased levels of the 16-kDa, FLAG-reactive residual cytoplasmic tail (Figure 2A), and increased levels of both the soluble 6- and 25-kDa growth factors. An ELISA confirmed the large (~17-fold) TAPI-inhibitable increase in media TGF-α seen in Figure 2A when cells were cotransfected with TGF-α and TACE as compared with cells transfected with TGF-α alone (data not shown).

Transfection of the pro-BTC cDNA into EC-2 fibroblasts produced several forms of cell-associated BTC, including prominent species of ~36 and 42 kDa that were recognized by anti-HA (Figure 2A) and anti-FLAG (data not shown). These likely correspond to variably glycosylated full-length forms, because murine BTC has three N-glycosylation sites (49). An 18-kDa protein recognized only by anti-FLAG (Figure 2A) likely corresponds to the residual TM/cytoplasmic domains (50). Media from cells transfected with TGF-α and TACE as compared with cells transfected with TGF-α alone (data not shown).

**Transfection of pro-EPR cDNA** into EC-2 fibroblasts produced several forms of cell-associated BTC, including prominent species of ~36 and 42 kDa that were recognized by anti-HA (Figure 2A) and anti-FLAG (data not shown). These likely correspond to variably glycosylated full-length forms, because murine BTC has three N-glycosylation sites (49). An 18-kDa protein recognized only by anti-FLAG (Figure 2A) likely corresponds to the residual TM/cytoplasmic domains (50). Media from cells transfected with TGF-α and TACE as compared with cells transfected with TGF-α alone (data not shown).

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HA-reactive, ~6-kDa protein that is consistent in size with the purified mouse growth factor (51). TAPI-2 treatment reduced the levels of this soluble protein (Fig. 2A). Most important, cotransfection of TACE reduced the levels of intact pro-EPR species but had no effect on levels of the 20- or 17-kDa cell-associated or 6-kDa soluble products (Fig. 2A).

Collectively, these results implicate a metalloprotease other than TACE in constitutive C-terminal BTC and EPR cleavage, but leave open the possibility that TACE contributes to the N-terminal processing of the respective precursors.

PMA-induced EPR Shedding Is TACE-dependent—Although TACE did not affect constitutive shedding of BTC or EPR from EC-2 cells, it could have a role in the regulated shedding of these growth factors. To assess this possibility, transfected EC-2 fibroblasts were incubated with 100 ng/ml PMA for 2 h prior to harvesting media and cell lysates. PMA increased media TGF-α levels in cultures cotransfected with TGF-α and TACE as compared with untreated cultures (Fig. 2B). In contrast, PMA did not affect the levels of media BTC shed from cells cotransfected with TACE (Fig. 2B), which is consistent with a previous report (50) that TACE did not stimulate BTC processing. However, PMA markedly increased the levels of media EPR shed from cells cotransfected with TACE (Fig. 2B), indicating that TACE may have a role in PMA-induced shedding of EPR.

TACE Cleaves HB-EGF and AR Ectodomains in Vitro—We confirmed previously that TACE correctly cleaved both the N- and C-terminal processing sites of pro-TGF-α in vitro (31). Here we examined the ability of recombinant, soluble TACE to cleave directly the ectodomains of other EGF family precursors. As with pro-TGF-ecto (31), the constructs used encoded ectodomains truncated immediately prior to the transmembrane domain (Fig. 1B). HA epitopes were incorporated into the mature growth factor sequences as with the full-length precursors (31, 41), whereas FLAG epitopes were incorporated at the new C termini. These constructs were transiently transfected into COS-1 cells, and the soluble ectodomains were harvested from conditioned media as described under “Experimental Procedures.” Correct folding of the mature EGF-like sequence was confirmed for each ectodomain by demonstrating that conditioned media activated EGFR in a dose-dependent fashion when added to receptor-positive cells (52) (data not shown).

To assess cleavage in vitro, ectodomains were incubated in buffer or buffer containing TACE at concentrations of either 150 (AR, HB-EGF) or 300 μg/ml (BTC, EPR) from 0 to 24 h at 37 °C. Products were then resolved by SDS-PAGE and probed with anti-HA or anti-FLAG (Fig. 3). In the absence of TACE, pro-ARecto migrated as a heterogeneous 31-kDa species. Addition of TACE rapidly converted this otherwise stable protein to persistent species of 17–20 kDa that were recognized by both anti-HA and anti-FLAG and therefore corresponded to N-terminal cleavage products (Fig. 3). In contrast, a novel species of ~12 kDa accumulated more slowly and was recognized only by anti-HA; this product likely corresponded to a C-terminal cleavage event (Fig. 3). These results suggest that TACE is capable of cleaving both N- and C-terminal sites in the pro-AR ectodomain.

To identify the critical site of C-terminal cleavage, we incubated the reaction products with anti-FLAG beads to capture the short C-terminal product generated by TACE cleavage (not
Two alternative human N-terminal cleavage sites have been identified, YIVDD and VVIKPK. Direct MALDI-TOF analysis of 24-h HB-EGF digestion products bound to anti-FLAG beads by MALDI-TOF/MS and identified a single ADAM10 reactive 9-kDa product in the presence of 300 µg/ml TACE (data not shown), and no C-terminal cleavage products were observed. Because of the low levels of the ~9-kDa BTC and EPR products, we were unable to identify the corresponding N-terminal cleavage sites by MALDI-TOF/MS (Fig. 4 and data not shown).

TACE-dependent Cleavage Is Determined by the Juxtamembrane Domain—Our results indicate that TACE is not an efficient C-terminal convertase for pro-BTC or pro-EPR either in cell culture or in vitro. To gain insights into the primary sequence features that determine which EGF family precursors are efficient TACE substrates, we aligned the juxtamembrane domains of the EGF ligands. In addition to sequence differences at the scissile bond, the juxtamembrane stalks of pro-BTC and pro-EPR are shorter when compared with pro-TGF-

To assess whether these differences in sequence and stalk length affect TACE cleavage, we created a series of pro-TGF-α mutants differing with respect to stalk features, and we compared their processing to wild-type TGF-α in transfected TACE-positive (25) CHO cells. As shown in Fig. 6A, the native stalk of pro-TGF-α was replaced with the counterpart sequence from BTC in TGF-JMBTC. For TGF-JMΔ2, the two terminal amino acids were truncated from the pro-TGF-α stalk to model the BTC stalk length. For TGF-JM154-LAVV, the P2–P2’ cleavage site amino acids of TGF-α (LAVV) were replaced with those of BTC (FLYQ) (Fig. 6A). Fig. 6B confirms that these epitope-
Identification of the metalloproteases responsible for shedding EGF family growth factors has received considerable attention recently, particularly due to the importance of these enzymes in regulating EGFR signaling, including via GPCR transactivation. We and others (27, 31) have previously identified TACE as a major TGF-α convertase on the basis of genetic, cell biologic, and biochemical evidence. Here we extend previous findings to support identification of TACE as an AR and HB-EGF convertase. In contrast, our findings do not support a role for TACE in the shedding of BTC or in the constitutive release of EPR. However, TACE could have a role in regulated, PMA-inducible EPR shedding. While this paper was being reviewed, Sahin et al. (59) described evidence from studies of ADAM9, -12, -15, and TACE knockout cells that supported a role for TACE as a key mediator of both constitutive and PMA-regulated shedding of TGF-α, AR, HB-EGF, and EPR but not BTC or EGF. Thus, our results are in general agreement with their findings but differ with respect to a positive role for TACE in constitutive EPR shedding. Conceivably, this contradiction could be due to differences in cell populations assayed or used by Sahin et al. (59) of an AP-EPR chimeric reporter.

The finding that TACE cleaved the C-terminal juxtamembrane stalk of pro-ARecto in vitro (Fig. 3) extends our previous report (31) that cotransfection of TACE promoted AR shedding from EC-2 cells and is consistent with results from several laboratories, including a recent report (59) demonstrating reduced AR shedding from primary TACE<sup>−/−</sup> fibroblasts. For example, antisense oligonucleotides to TACE but not ADAM9 or -10 blocked AR shedding and EGFR transactivation triggered by smoke-induced oxygen free radicals (36). Inhibition of TACE by RNA interference or expression of a dominant/negative enzyme also suppressed GPCR-stimulated AR release and EGFR signaling in squamous carcinoma cells (24). Recently, we found that mammary gland rudiments from TACE-deficient fetuses display impaired ductal outgrowth when transplanted into nude mice. A similar defect was observed upon transplantation of EGFR null mammary rudiments (60), and ductal development was stunted in pubescent female mice lacking AR.
Thus, the present findings add to a growing body of evidence implicating TACE as a major mediator of AR-induced biological responses.

On the other hand, the C-terminal site cleaved by TACE in vitro (Fig. 4) did not match the C terminus of the soluble human AR protein (183E2K184) purified from TPA-treated MCF-7 cell media by Shoyab et al. (61), which was only three amino acids downstream from the terminal Cys of the EGF-like motif. However, it has been suggested by others that this is not the bona fide C terminus of naturally secreted AR protein, because this form is much less potent with respect to EGFR activation and mitogenicity compared with recombinant AR proteins containing C-terminal extensions (62, 63). In particular, a recombinant AR containing three additional amino acids, which corresponds precisely to the form predicted from the in vitro TACE cleavage site, possessed 40-fold higher EGFR binding and mitogenic activities compared with the truncated 183E2K184 protein, and it corresponded closely to the respective activities of MCF-7 conditioned media (62). A soluble AR species, inconsistent in size with a C terminus of 183E2K184, was released from Madin-Darby canine kidney cells, suggesting that AR is cleaved more distally to the EGF-like domain (64). Moreover, the 183E2K184 position places cleavage closer to the EGF-like motif and further from the cell membrane than is observed with other EGF family ligands (Fig. 5). Thus, we propose that the site cleaved by TACE in vitro (mouse 180K2T181; equivalent to human 187K2T188) is physiologically relevant.

HB-EGF shedding has been the focus of considerable attention, due in part to its role in GPCR-induced EGFR transactivation as well as interest in the potential juxtacrine roles of pro-HB-EGF. However, the identity of the HB-EGF convertase has been controversial. Our previous findings indicate that TACE is an important physiologic regulator of HB-EGF shedding. HB-EGF knockout and TACE-deficient mice displayed indistinguishable cardiac valve and lung development defects (14, 35), and cotransfected TACE promoted shedding of HB-EGF from TACE-deficient cells (31). Moreover, HB-EGF shedding from primary fibroblasts was impaired in TACE-deficient cells (59). Here we additionally show that TACE cleaved pro-HB-EGFecto in vitro at a site in the juxtamembrane domain (Figs. 3 and 4) corresponding to the C terminus of the HB-EGF protein purified from TPA-stimulated Vero-H cells (54).

Other metalloproteases, including ADAM9, -10, and -12, have also been proposed as candidate HB-EGF convertases.
Dominant negative ADAM9 (MDC9/meltrin-γ) inhibited stimulus-induced shedding of HB-EGF in Vero-H cells (38), although ADAM9-null mice did not display an overt phenotype, and cells derived from them shed HB-EGF normally (65). Additionally, a dominant negative ADAM9 did not interfere with HB-EGF cleavage-dependent GPCR transactivation of EGFR (19), and as described here, ADAM9 did not cleave pro-HB-EGFecto in vitro (data not shown). Dominant negative ADAM10 and/or antisense oligonucleotides blocked GPCR-induced HB-EGF shedding and EGFR transactivation, respectively, in COS7 (23) and mucosal epithelial cells (39). However, whereas ADAM10 cleaved the juxtamembrane stalk of pro-HB-EGFecto in our studies (Fig. 3), the cleavage site was located several residues downstream of the C terminus of mature HB-EGF, closer to the transmembrane domain than is observed with other EGF family precursors. Because ADAM10 knockout mice died at embryonic day 9.5 exhibiting multiple defects, including severely delayed heart development (66), a direct comparison to the later and more restricted valvogenesisis defects displayed by HB-EGF knockout is not possible. ADAM12 has been linked to the role of HB-EGF in experimentally induced cardiac hypertrophy. A dominant negative ADAM12 blocked GPCR-induced HB-EGF shedding from cardiomyocytes, and a novel metalloprotease inhibitor of HB-EGF shedding bound ADAM12 and also attenuated both HB-EGF shedding and hypertrophic changes in a mouse model (21). Like HB-EGF null mice, a significant proportion of mice lacking ADAM12 die prior to weaning, and fibroblasts from ADAM12-null mice did not shed HB-EGF in response to TPA (67). However, cardiac defects were not observed in this model. ADAM19 knockout mice displayed enlarged cardiac valves, replicating at least part of the HB-EGF-deficient phenotype (68). On the other hand, cell-based studies using primary fibroblasts from ADAM19, -10, and -9/12/15 knockout mice indicated that HB-EGF shedding is not impaired in these cells. Moreover, a batastat-insensitive activity was also implicated in shedding (59). Finally, MMP-3 (69) and MMP7/matrilysin (70) have also been put forth as candidate HB-EGF convertases. Conceivably, multiple proteases could contribute to HB-EGF shedding in a cell type- and stimulus-dependent manner.

In contrast to its actions on the HB-EGF and AR precursors (31), TACE did not enhance the constitutive ectodomain shedding of either BTC or EPR from cotransfected EC-2 cells, and it did not efficiently cleave the juxtamembrane stalks of either ectodomain in vitro. Constitutive shedding of BTC and EPR were metalloprotease-dependent, as TAPI-2 blocked production of the soluble media forms (Fig. 2A). However, TACE may have a role in PMA-stimulated EPR shedding (Fig. 2B). Most interesting, CHO cells that normally express TACE (25) did not efficiently release BTC and instead accumulated membrane-associated forms (Fig. 7B). Our results also confirm the report from a recent study that secretion of a transfectected BTC/alkaline phosphatase fusion protein was not stimulated by phorbol esters (50), an observation that distinguishes BTC processing from that of other EGF family members, including EPR (42). These observations raise the possibility that pro-BTC is acted upon by another metalloprotease or instead functions predominately through juxtacrine actions involving membrane-associated forms. Unlike TACE-deficient mice, BTC knockout mice did not display an overt phenotype (14). Identifying the metalloprotease(s) responsible for C-terminal cleavage of BTC, EPR, and EGF will be key to understanding the biology of these growth factors. Recently, Sahin et al. (59) identified ADAM10 as a BTC sheddase, although we were unable to confirm ADAM10 cleavage of BTCecto in vitro (data not shown).

Evidence that TACE is a major C-terminal convertase for some EGF family members (e.g. TGF-α, AR, and HB-EGF) but not others (BTC and EPR) raises the question as to what structural features are recognized by sheddases. Because the sequence and length of juxtamembrane stalks of EGF family precursors (Fig. 5) are not conserved, sheddase action must be specified by other structural features. For example, substitution of stalk sequences from normally shed proteins for those of integral membrane proteins not subject to shedding conferred constitutive shedding in the latter (71, 72). Moreover, the length of the stalk may be an important determinant, because mutagenesis of the TNF precursor (73) or the growth hormone receptor (74) revealed a minimum stalk length for efficient processing, presumably reflecting accessibility of the cleavage site to the relevant metalloprotease. The distance from the cleavage site to the membrane may be an important determinant of cleavage. Studies of Kit ligand processing in which the stalk sequence was reiterated revealed that cleavage occurred only at the most membrane proximal cleavage site (75), consistent with a mechanism specified by distance from the membrane. In contrast, studies of angiotensin-converting enzyme/CD4 (76) or L-selectin/B7.2 (72) chimeric proteins underscored the importance of the distal ectodomain as a primary determinant of efficient shedding. Moreover, although they share the same stalk, the testis angiotensin-converting enzyme isoform was shed more efficiently (77), presumably due to the presence of a distal structural motif that was recognized by the processing enzyme (78).

Undoubtedly, the sequence of the cleavage site must impact shedding efficiency as well. A study of macrophage colony-stimulating factor shedding indicated that both the scissile bond sequence and the distance from the membrane determined cleavage (79). The fact that TACE cleaves TNF > TGF-α > AR/HB-EGF suggests it prefers small apolar amino acids in the P1 and P1’ positions (31). Our present results are in keeping with these indications, because lengthening the pro-BTC stalk either directly or by substituting the pro-TGF-α stalk stimulated BTC shedding, whereas the converse manipulations to pro-TGF-α reduced TGF-α shedding. The effect of lengthening the pro-BTC stalk was enhanced by simultaneously substituting the preferred P2–P2’ recognition site of pro-TGF-α. Thus, the combination of stalk length and scissile bond sequence determines the efficiency of EGF family shedding.

In addition to cleaving the juxtamembrane stalks of pro-TGF-α, pro-AR, and pro-HB-EGF, TACE also cleaved these precursors at one or more N-terminal sites. In the case of pro-TGF-α, we previously established that TACE correctly cleaved the physiologic N-terminal processing site in vitro (31). Here, we showed that TACE cleaved mouse pro-ARecto in vitro at a site (99Q Δ V100) corresponding to one of two N termini identified for human AR purified from cell culture (53). Human HB-EGF purified from cell culture also displayed heterogeneous N termini corresponding to (31R ω G32, 62R ω D63, 72L ω R73, and 73R ω V74) (55–58). TACE cleaved mouse HB-EGF in vitro two residues downstream of (31R ω G32) to produce a protein that retained the heparin binding domain. Whether this corresponds to a bona fide N terminus of the murine protein is presently unclear. TACE also cleaved the N-terminal regions of pro-BTC and pro-EPR (Figs. 2 and 3), albeit less efficiently, thus precluding identification of the cleavage sites. Most interesting, TACE invariably cleaved the N-terminal regions of EGF family precursors more rapidly and with greater efficiency than it cleaved the stalk regions. This observation agrees with an earlier cell-based study that revealed more rapid processing of the pro-TGF-α N-terminal site compared with the stalk cleavage site (80).

The possibility that TACE mediates both N- and C-terminal
TACE-dependent Shedding of EGFR Family

clavage events in the EGFR family raises the interesting question as to how this membrane-anchored protease cleaves distant sites separated by the EGFR-like motif and, in some cases, additional sequences. Identifying the mechanism and recognition elements required for TACE-dependent N-terminal cleavage events may provide more insight into the requirements and relative inefficiency of C-terminal cleavage.

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