Substrate Selectivity of Epidermal Growth Factor-Receptor Ligand Sheddases and their Regulation by Phorbol Esters and Calcium Influx[□]

Keisuke Horiuchi,^{*,†} Sylvain Le Gall,^{*} Marc Schulte,[‡] Takafumi Yamaguchi,[†] Karina Reiss,[‡] Gillian Murphy,[§] Yoshiaki Toyama,[†] Dieter Hartmann,^{||} Paul Saftig,[‡] and Carl P. Blobel^{*¶}

^{*}Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY 10021; [†]Biochemical Institute, Christian-Albrechts University, D-24098 Kiel, Germany; [‡]Department of Orthopedic Surgery, Keio University, School of Medicine, Tokyo, 160-8582 Japan; [§]Cambridge Institute for Medical Research, Cambridge CB2 2XY, United Kingdom; ^{||}Department for Human Genetics, K.U. Leuven and Flanders Interuniversity Institute for Biotechnology (VIB-4), 3000 Leuven, Belgium; and [¶]Departments of Medicine and of Physiology and Biophysics, Weill Medical College of Cornell University, New York, NY 10021

Submitted January 6, 2006; Revised September 25, 2006; Accepted October 19, 2006
Monitoring Editor: Ben Margolis

Signaling via the epidermal growth factor receptor (EGFR), which has critical roles in development and diseases such as cancer, is regulated by proteolytic shedding of its membrane-tethered ligands. Sheddases for EGFR-ligands are therefore key signaling switches in the EGFR pathway. Here, we determined which ADAMs (a disintegrin and metalloprotease) can shed various EGFR-ligands, and we analyzed the regulation of EGFR-ligand shedding by two commonly used stimuli, phorbol esters and calcium influx. Phorbol esters predominantly activate ADAM17, thereby triggering a burst of shedding of EGFR-ligands from a late secretory pathway compartment. Calcium influx stimulates ADAM10, requiring its cytoplasmic domain. However, calcium influx-stimulated shedding of transforming growth factor α and amphiregulin does not require ADAM17, even though ADAM17 is essential for phorbol ester-stimulated shedding of these EGFR-ligands. This study provides new insight into the machinery responsible for EGFR-ligand release and thus EGFR signaling and demonstrates that dysregulated EGFR-ligand shedding may be caused by increased expression of constitutively active sheddases or activation of different sheddases by distinct stimuli.

INTRODUCTION

Members of the ADAM family of membrane-anchored peptidases are key regulators of epidermal growth factor receptor (EGFR) signaling by converting membrane-anchored precursors of EGFR-ligands into soluble growth factors (for review, see Blobel, 2005). These, in turn, have essential roles in development and diseases such as cancer (Yarden and Sliwkowski, 2001; Gschwind *et al.*, 2004). The best example of the essential physiological role of ectodomain shedding in the EGFR pathway are mice lacking ADAM17, a critical sheddase for the EGFR-ligands transforming growth factor (TGF) α , heparin binding epidermal growth factor (EGF)-like growth factor (HB-EGF), and amphiregulin (AR) (Peschon *et al.*, 1998; Jackson *et al.*, 2003; Sternlicht *et al.*, 2005). *Adam17*^{−/−} mice have developmental defects resembling those in animals lacking TGF α , HB-EGF, AR, or the EGFR (Peschon *et al.*, 1998; Jackson *et al.*, 2003; Sternlicht *et al.*, 2005). Thus, although the membrane-anchored forms of TGF α ,

HB-EGF, AR and the EGFR are present in *Adam17*^{−/−} mice, these growth factors are inactive in tissues in which their function is required for development. Moreover, EGFR-dependent proliferation and migration of cancer cells and TGF α -dependent EGFR-signaling are blocked by a metalloprotease inhibitor that prevents ectodomain shedding of EGFR-ligands (Dong *et al.*, 1999; Borrell-Pages *et al.*, 2003). Finally, a “knockin” deletion of the HB-EGF cleavage site leads to similar defects in heart valve development as those in *Hb-egf*^{−/−} mice and *Adam17*^{−/−} mice (Jackson *et al.*, 2003; Yamazaki *et al.*, 2003).

Central questions raised by the essential role of proteolysis as regulator of the EGFR-signaling pathway are which enzymes are involved in this process and how they are regulated. Identification of EGFR-ligand sheddases that are constitutively active or respond to different stimuli and understanding the basis of their regulation are therefore important for unraveling the mechanism underlying upstream regulation of EGFR-signaling by ligand release. Previous “loss of function” studies with cells lacking various ADAMs have uncovered a critical role for ADAM17 in shedding TGF α , HB-EGF, AR, epiregulin (EPR), and for ADAM10 in shedding EGF and betacellulin (BTC) (Peschon *et al.*, 1998; Merlos-Suarez *et al.*, 2001; Sunnarborg *et al.*, 2002; Sahin *et al.*, 2004; Sanderson *et al.*, 2005). The physiological relevance of these studies is borne out by the essential physiological role of ADAM17 in releasing TGF α , HB-EGF, and AR during

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-01-0014>) on November 1, 2006.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: Carl P. Blobel (blobelc@hss.edu).

mouse development (see above). In addition, studies with ADAM-deficient cells, small interfering RNA, dominant-negative ADAMs, and overexpressed ADAMs have provided evidence for a role of other ADAMs in EGFR-ligand shedding (Izumi *et al.*, 1998; Yan *et al.*, 2002; Gschwind *et al.*, 2003; Kurisaki *et al.*, 2003; Schafer *et al.*, 2004; Horiuchi *et al.*, 2005).

Although loss of function experiments can uncover which enzyme is indispensable for releasing a given substrate in a given cell type, "gain of function" experiments can provide important information about which enzyme(s) are able to contribute to the release of EGFR-ligands when they are dysregulated or overexpressed. Indeed, several ADAMs are dysregulated in diseases such as cancer (Kveiborg *et al.*, 2005; Mazzocca *et al.*, 2005; Peduto *et al.*, 2005, 2006) and could therefore conceivably contribute to tumorigenesis, especially if they are able to shed EGFR-ligands or other membrane proteins with roles in cancer. In addition to overexpression, another potential cause of dysregulated EGFR-ligand shedding includes inappropriate activation of sheddases. Previous studies have shown that phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), can activate ADAM17-dependent shedding of a variety of substrates (for review, see Black *et al.*, 2003; Blobel, 2005). Yet, little is known about the underlying mechanism. Even though the cytoplasmic domain of ADAM17 can be phosphorylated (Diaz-Rodriguez *et al.*, 2002; Fan *et al.*, 2003), and this can enhance the maturation of ADAM17 (Soond *et al.*, 2005), the cytoplasmic domain of ADAM17 is not required for PMA-stimulated shedding of tumor necrosis factor (TNF)- α (Reddy *et al.*, 2000; Doedens *et al.*, 2003). ADAM10, in contrast, is only weakly stimulated by PMA, if at all (Sahin *et al.*, 2004; Sanderson *et al.*, 2005). Instead, ADAM10-dependent shedding of CD44, BTC, and N-cadherin is stimulated by calcium ionophores (Nagano *et al.*, 2004; Reiss *et al.*, 2005; Sanderson *et al.*, 2005). The main goal of this study was to identify which enzymes can be part of the core machinery that regulates EGFR-signaling via release of its ligands and to determine which EGFR-ligand sheddases respond to phorbol esters and calcium ionophores, and how these responses might be regulated. New insight into the role of ADAMs in EGFR-ligand shedding have implications for understanding the upstream regulation of EGFR signaling, a critical pathway for development and a validated target for treatment of cancer, and also for other pathways affected by ADAM-dependent ectodomain shedding, including neurogenesis, heart development, Alzheimer's disease, and angiogenesis.

MATERIALS AND METHODS

Cell Lines and Reagents

The *Adam17*^{-/-} fibroblast cell line (E2 cells) and the *Adam10*^{-/-} fibroblast cell line were derived from E13.5 and E9.5 embryos, respectively (Reddy *et al.*, 2000; Hartmann *et al.*, 2002). Immortalized fibroblasts derived from *Adam 9*, *12*, *15*^{-/-} (*T*^{-/-}), *Adam19*^{-/-}, and wild-type E13.5 embryos were generated by transfecting primary mouse embryonic fibroblast (mEF) cells from the appropriate mouse lines (Sahin *et al.*, 2004) with a vector carrying Large-T antigen. All cell lines were grown in DMEM, supplemented with 5% fetal calf serum and antibiotics. The antiserum directed against ADAM17 was described previously (Schlöndorff *et al.*, 2000). Anti-ADAM10 cytoplasmic domain antibodies were from Triple Point Biologics (Forest Grove, OR); anti-human placental alkaline phosphatase antibodies, 8B6 (immunofluorescence), and 8A9 (Western blot) were from Sigma-Aldrich (St. Louis, MO) and Dako North America (Carpinteria, CA), respectively; anti-hemagglutinin (HA) antibodies (3F10) were from Roche Diagnostics (Mannheim, Germany); Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG were from Molecular Probes (Leiden, The Netherlands); anti-Myc antibodies (9E10) were from Upstate Biotechnology (Lake Placid NY); Cy3-conjugated AffiPure donkey anti-rat IgG was from Jackson ImmunoResearch Laboratories

(West Grove, PA); and fluorescein isothiocyanate-labeled wheat germ agglutinin from *Tritium vulgaris* for use as a Golgi marker was from Sigma-Aldrich. Ionomycin (IM), trifluoperazine dimaleate, and brefeldin-A (BFA) were obtained from Calbiochem (San Diego, CA). Batimastat (BB94) was kindly provided by Dr. D. Becherer (GlaxoSmithKline, Research Triangle Park, NC). All other reagents were obtained from Sigma-Aldrich unless otherwise indicated. Tissue inhibitor of metalloproteinases (TIMPs) 1 and 2 were kindly provided by Dr. Gillian Murphy (University of Cambridge, Cambridge, United Kingdom), and TIMP3 was kindly provided by Dr. Roy Black (Amgen, Seattle, WA).

Expression Vectors

The expression vectors for wild-type ADAMs and AP-tagged EGFR ligands have been described previously (Sahin *et al.*, 2004; Zheng *et al.*, 2004; Horiuchi *et al.*, 2005). The HA-tagged TGF α expression vector was kindly provided by Dr. D. Lee (University of Georgia, Athens, GA) (Briley *et al.*, 1997). Chimera of AP-tagged TGF α and EGF, and TGF α and BTC were generated by polymerase chain reaction (PCR) and cloned into pAPtag5 (GenHunter, Nashville, TN) (see Figure 6 for the illustration of each AP-tagged chimeric ligand). Cytoplasmic domain-deleted mutants, ADAM17b and ADAM10b, were generated by PCR and cloned into pCDNA3-6MT (a vector containing 6 copies of the myc-tag recognized by the 9E10 monoclonal antibody [mAb], MEQKLI-SEEDLINE, as well as two short unique sequences, MESLGDLT and RPLEPLEL) and pCDNA4/V5-His (Invitrogen, Carlsbad, CA), respectively. The chimera between ADAM10 and ADAM17 were generated by fusion PCRs by using murine ADAM10 and ADAM17 cDNAs as a template.

Cell Culture, Transfection, and Shedding Assay

Immortalized fibroblast cell lines and COS-7 cells were transfected with the indicated plasmids as described previously (Sahin *et al.*, 2004; Zheng *et al.*, 2004). Fresh Opti-MEM (Invitrogen) medium with or without the indicated reagents was added the next day after transfection and incubated for the designated time period. AP activity in the supernatant and cell lysate was measured by colorimetry (Sahin *et al.*, 2004; Zheng *et al.*, 2004). When a given AP-tagged ligand and protease were cotransfected simultaneously, at least two identical wells were prepared, and the ratio between AP activity in the supernatant (from either the cells treated with a given reagent or nontreated cells) and the cell lysate (from the cells incubated in fresh Opti-MEM without any additional reagents) was calculated for normalization. The ratio reflects the efficiency of the shedding activity of a given protease against a given AP-tagged ligand. All experiments were repeated at least three times with similar results.

Metabolic Labeling

COS-7 cells were plated on six-well plates and transfected with either ADAM17^{HA} or ADAM17b^{Myc} in a pcDNA3 expression vector. Twenty-four hours after transfection, cells were starved for 30 min in cysteine- and methionine-free growth medium containing 100 μ Ci/ml Redivue Promix ³⁵S-Cys/Met (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Cells were incubated for 3 h, washed twice with phosphate-buffered saline (PBS), and incubated in Opti-MEM with or without PMA for 2 h, and then washed twice with PBS and lysed in 0.5 ml of lysis buffer (1% Triton X-100, PBS, protease inhibitor cocktail, and 1,10-phenanthroline). Epitope-tagged ADAM17 was immunoprecipitated with either anti-HA antibody (for ADAM17^{HA}) or anti-Myc antibody (for ADAM17b^{Myc}) as described previously (Schlöndorff *et al.*, 2000).

Enzymatic Deglycosylation

N-linked carbohydrate residues were removed using endoglycosidase H (EndoH) or peptide N-glycosidase F (PNGase F) following the manufacturer's instructions (New England Biolabs, Ipswich, MA). The deglycosylated samples were then separated by SDS-PAGE and analyzed by Western blot (Schlöndorff *et al.*, 2000).

Cell Surface Labeling

Transfected COS-7 cells were washed twice in ice-cold PBS, and the cell surface molecules were biotinylated with 1 mg/ml nonmembrane-permeable biotinylation reagent EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL) following the manufacturer's instructions. The cells were then washed with 100 mM glycine/PBS and lysed in lysis buffer. Samples were immunoprecipitated either with anti-HA antibody (for ADAM10^{HA} and ADAM17^{HA}), anti-ADAM17 cytoplasmic domain antibody (for endogenous ADAM17) (Schlöndorff *et al.*, 2000), or anti-ADAM10 cytoplasmic domain antibody (for endogenous ADAM10). Immunoprecipitated material was separated by SDS-PAGE and analyzed by Western blot.

Immunofluorescence Microscopy

Immortalized mEF cells were plated on an eight-well chamber slide (Nalge Nunc International, Rochester, NY) and transfected with EGFR-ligand expression vectors and/or ADAM expression vectors as described above. After 24 h

Figure 1. Evidence for a contribution of multiple ADAMs to EGFR-ligand shedding. AP-tagged EGFR-ligands (A, TGF α ; B, EPR; C, HB-EGF; D, AR; E, EGF; and F, BTC) were cotransfected either with empty vector (–) or with an expression vector for ADAM8 (A8), ADAM9 (A9), ADAM10 (A10), ADAM12 (A12), ADAM15 (A15), ADAM17 (A17), or ADAM19 (A19) into *Adam17*^{–/–} cells (A–D) or *Adam10*^{–/–} cells (E and F). Shedding activity is presented as a ratio of the AP activity in the supernatant and the cell lysate as described in *Materials and Methods*. Black column, constitutive shedding; white column, PMA-stimulated shedding (25 ng/ml). The shedding activity of ADAM19 toward TGF α , EPR, HB-EGF, and AR was described previously (Horiuchi *et al.*, 2005).

of transfection, cells were treated with PMA or IM as indicated and then washed with PBS, fixed with 4% paraformaldehyde/PBS for 5 min, and permeabilized with 0.1% Triton X-100/PBS. Fixed cells were blocked with 0.1% bovine serum albumin (BSA)/PBS, incubated with primary antibody for 30 min, washed three times in 0.1% BSA/PBS, and incubated with secondary antibody for 30 min. Cells mounted in Aquatex (EM Scientific, Gibbstown, NJ) were viewed and photographed with Nikon Eclipse E600 (objective lens, 60 $\times$ /0.80), Qimaging Retiga Exi charge-coupled device camera, and Qcapture acquisition software. Digital images were processed and merged using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Statistical Analysis

A *t* test for two samples assuming equal variances was used to calculate the *p* values. *p* values smaller than 0.05 were considered statistically significant.

RESULTS

Gain of Function EGFR-Ligand Shedding Experiments with ADAM 8, -9, -10, -12, -15, -17, and -19

Previous loss of function experiments in mouse embryonic fibroblasts identified ADAM 10 and -17 as the main EGFR-ligand sheddases. Because ADAMs are known to be dysregulated in diseases such as cancer, we performed gain of function experiments with ADAM 8, -9, -10, -12, -15, -17, or -19 to determine which of these proteases can cleave which EGFR-ligands when overexpressed. To facilitate detecting EGFR-ligand sheddase activity for these ADAMs, all experiments were performed in cells lacking the major endogenous sheddase for each ligand (ADAM17 for TGF α , HB-EGF, AR, and EPR; ADAM10 for EGF and BTC; Sahin *et al.*, 2004). Constitutive and PMA-stimulated shedding of TGF α and EPR from *Adam17*^{–/–} cells could be rescued by ADAM17, but not ADAM 8, -9, -10, -12, or -15 (Figure

1A). Shedding of HB-EGF and AR was increased by overexpressing ADAM17 and ADAM8, but not the other ADAMs tested here (Figure 1, C and D). ADAM17-dependent shedding of HB-EGF and AR was consistently enhanced by PMA, albeit not to the same degree as in wild-type control mEF cells or COS-7 cells, whereas ADAM8-dependent release of these ligands was not. Processing of EGF in *Adam10*^{–/–} cells was increased by ADAM 8, -9, -10, -12, -17, and -19, but not by ADAM15 (Figure 1E, expression of mature ADAM15 was confirmed by Western blot analysis; data not shown). EGF shedding was not significantly stimulated by PMA in any of the rescue experiments, including those with ADAM17. Finally, ADAM 8, -10, -12, -17, and -19 could also shed BTC, whereas overexpressed ADAM 9 or -15 did not, although the expression of mature forms of both ADAMs was confirmed by Western blot analysis (data not shown). In all cases where increased shedding was observed, it did not require stimulation. Thus, these experiments demonstrate which ADAMs can cleave which EGFR-ligands constitutively, which may be particularly relevant for diseases where ADAM expression is up-regulated (please note, however, that these experiments do not allow a quantitative comparison of how efficiently different ADAMs shed various EGFR-ligands).

PMA-dependent Stimulation of EGFR-Ligand Shedding

Among the widely expressed and catalytically active ADAMs tested here, ADAM17 emerged as the only PMA-responsive sheddase, at least with respect to release of TGF α , HB-EGF, AR, and EPR. We therefore focused on ADAM17 and two of

Figure 2. Effect of PMA on the maturation of ADAM17 in COS-7 cells. (A) COS-7 cells transfected with HA-tagged ADAM17 (A17^{HA}, left) or not transfected (right) were incubated with 500 ng/ml PMA for 10 and 30 min followed by labeling of molecules on the cell surface with a membrane-impermeable biotin. Samples immunoprecipitated with anti-HA antibodies (left, Anti-HA) or anti-ADAM17 antibodies (right, Anti-A17) were detected with Avidin-horseradish peroxidase (HRP) (top). The membrane shown in the top left panel was then stripped and reprobed with anti-ADAM17-cytotail antibodies (bottom left). Bottom right, Western blot analysis of ADAM17 in COS-7 cells. Open arrow, pro-ADAM17; black arrowhead, mature ADAM17. Please note that the small amounts of biotinylated pro-ADAM17 in both top panels are sensitive to EndoH, suggesting that they were labeled through leakage of the biotin reagent (data not shown). Experiments performed at lower PMA (25 ng/ml) concentrations yielded essentially identical results (data not shown). (B) COS-7 cells were transfected with HA-tagged ADAM17 (A17^{HA}, lanes 1 and 2) or a Myc-tagged ADAM17 lacking its cytoplasmic domain (A17b^{Myc}, lanes 4 and 5, see Figure 4A for a diagram of these constructs) or empty vector [(-), lanes 3 and 6]. Cells were pulse labeled with S³⁵ for 3 h and then chased in fresh media with or without PMA (500 ng/ml) for 2 h. The lysates were immunoprecipitated with anti-HA or anti-Myc tag antibodies as indicated, separated by SDS-PAGE, and used for autoradiographic exposure of Kodak X-AR film. Please note that A17b^{Myc} contains six myc tags and therefore comigrates with A17^{HA} (see Materials and Methods for details). Open arrow, pro-ADAM17; black arrow, mature ADAM17.

its substrates, TGF α and AR, for an evaluation of how this phorbol ester regulates ectodomain shedding. Two recent studies reported that PMA enhances maturation and prodomain processing of ADAM17 within 10 min of PMA stimulation (Nagano *et al.*, 2004; Soond *et al.*, 2005). However, PMA stimulation did not detectably increase the levels of HA-tagged ADAM17 (Figure 2A, top left) or endogenous ADAM17 (Figure 2A, top right, pro-ADAM17, open arrowhead; mature ADAM17, black arrowhead) that could be labeled with a membrane-impermeable biotinylation reagent under the conditions used here, even though it strongly stimulated ADAM17-dependent ectodomain shedding in COS-7 cells (see below; Figure 3D). Moreover, PMA stimulation did not increase the maturation of HA-tagged ADAM17 or endogenous ADAM17 (Figure 2A, bottom). When experiments identical to those shown in Figure 2A were performed in simian virus 40 (SV40) transformed wild-type mouse embryonic fibroblasts, we also did not detect an increase in the maturation of endogenous or transfected HA-tagged ADAM17 after stimulation with PMA (see Supplemental Figure 1). In a pulse-chase experiment in COS-7 cells, the processing of wild-type HA-tagged ADAM17 as well as a Myc-tagged mutant ADAM17 lacking its cytoplasmic domain (A17b) was also not increased after PMA stimulation for 2 h (Figure 2B).

Because PMA did not have a detectable effect on ADAM17 maturation in COS-7 cells in this study, we asked whether it might instead act on EGFR-ligands. An immunofluorescence analysis showed that TGF α and ADAM17 colocalize in unstimulated cells in a perinuclear compartment (Figure 3A, a–c). The perinuclear staining of TGF α , overlaps with that of wheat germ agglutinin, a marker for the *trans*-Golgi network (TGN; data not shown). In addition, diffuse staining of TGF α in vesicles throughout the cell, presumably corresponding to endoplasmic reticulum (ER) staining, can be detected in longer exposures (data not shown). PMA stimulation did not affect the localization of ADAM17 or TGF α (Figure 3A, d–f). Interestingly, among the other EGFR-ligands whose shedding by ADAM17 can be stimulated by PMA, only EPR had a very similar localization as TGF α (Figure 3B, d–f). In contrast, HB-EGF and AR had a more prominent cell surface localization, although substantial amounts of these growth factors also colocalized with TGF α (Figure 3B, g–i). EGF and BTC, whose shedding is not stimulated by PMA, were mainly found on the cell surface, although there was some colocalization with TGF α

in intracellular compartments (Figure 3B, m–r). Because all ligands were detected via their alkaline phosphatase tag, we confirmed that two differently tagged forms of TGF α (AP and HA-tagged) colocalized intracellularly (Figure 3B, a–c), which corroborates that the AP-tag does not change the localization of this EGFR-ligand. Because immunofluorescence analysis showed that substrates of ADAM17 (TGF α , HB-EGF, EPR, and AR) accumulate either in a perinuclear compartment or on the cell surface, the predominant subcellular localization of EGFR-ligands is presumably not a main determinant of the response of ADAM17 to PMA stimulation.

Another possible mechanism underlying PMA stimulation is increased transport of EGFR-ligands through the secretory pathway. The AP-tag contains *N*-linked carbohydrate residues; therefore, acquisition of resistance to EndoH indicates that AP-tagged EGFR-ligands have passed through the *medial*-Golgi apparatus. In AP-tagged TGF α , its major proform at 75 kDa (Figure 3C, a, black arrow) was completely EndoH sensitive, whereas only the small amount of a slower migrating form of pro-TGF α at ~79 kDa was resistant to EndoH (Figure 3C, a, open arrow). Both forms could be deglycosylated by PNGase F (Figure 3C, a, asterisk), which removes all *N*-linked carbohydrate residues. Similar experiments with AR showed substantial concentrations of EndoH-resistant pro-AR (Figure 3C, b, open arrow) as well as small amounts of EndoH-sensitive pro-AR (Figure 3C, b, asterisk). Thus, more pro-AR than pro-TGF α accumulates after passage through the *medial*-Golgi apparatus, which is consistent with the immunofluorescence studies described above. PMA stimulation only removed the small amount of EndoH-resistant 79-kDa pro-TGF α (Figure 3D, a, lanes 1 and 2, white arrow), whereas the 75-kDa EndoH-sensitive form of TGF α seemed unaffected (Figure 3D, a, lane 3, white arrow). Processing of the 79-kDa pro-TGF α could be prevented by the hydroxamate metalloprotease inhibitor BB94 in PMA-stimulated cells (Figure 3D, a, lane 4). In contrast to TGF α , a majority of pro-AR was removed after PMA stimulation, and this could also be blocked by BB94 (Figure 3D, b). Evidently, PMA only or mainly triggers shedding of EndoH-resistant EGFR-ligands that have already passed the medial Golgi apparatus, and has little or no effect on the intracellular maturation of these ligands.

To more directly address the role of PMA in increasing the intracellular maturation and transport of ADAM17 or its substrates to the *trans*-Golgi network, we tested whether

Figure 3. Subcellular localization of EGFR-ligands and effect of PMA stimulation on shedding of TGF α and AR (A) AP-tagged TGF α and ADAM17-cotransfected in wild-type mEF cells were incubated in the absence (a–c) or presence of PMA (25 ng/ml, d–f) for 10 min and double stained with anti-AP mAb (detected with a fluorescein-labeled secondary antibody) or an anti-ADAM17 rabbit polyclonal antibody (detected with a rhodamine labeled secondary antibody). Experiments were performed at higher 500 ng/ml PMA concentration with similar results (data not shown). Bar, 20 μ m. (B) The AP-tagged EGFR-ligands (TGF α , a–c; EPR, d–f; HB-EGF, g–i; AR, j–l; EGF, m–o; and BTC, p–r) were cotransfected with HA-tagged TGF α in wild-type mEF cells and double-stained with anti-AP monoclonal antibodies (visualized with a fluorescein labeled secondary antibody) or anti-HA monoclonal antibodies (detected with rhodamine labeled secondary antibodies, see *Materials and Methods* for details. Bar, 20 μ m. (C) Cell lysates from COS-7 cells transfected with TGF α (a) or AR (b) were treated with EndoH (E–H) or PNGase F (P–N); immunoblots were probed with anti-AP antibodies. Open arrow, proform; black arrow mature form; asterisk, endoglycosidase-digested proteins. (D) TGF α - (a) or AR (b)-transfected COS-7 cells were incubated with 500 ng/ml PMA and/or 1 μ M BB94 for 4 h. The supernatant was collected and analyzed by colorimetry as described in *Materials and Methods*, and the lysates were subjected to immunoblot analysis with an anti-AP antibody. Open arrow, proform; black arrow mature form. (E) TGF α (a) or AR (b) transfected COS-7 cells were incubated with 25 ng/ml PMA and/or 5 μ g/ml BFA for 4 h. The supernatant was collected every hour and replaced with fresh media containing the same concentration of the indicated reagents. BFA-treated cells were preincubated with BFA for 7 min. Red asterisk, $p < 0.05$ between PMA and PMA + BFA-treated cells; blue asterisk, $p < 0.05$ between nontreated and BFA-treated cells. (F) COS-7 cells transfected with soluble AP were incubated with or without (5 μ g/ml) BFA for 30 min.

BFA, a fungal toxin that blocks the secretory pathway by collapsing the Golgi apparatus into the ER (Lippincott-Schwartz *et al.*, 1989), blocks PMA stimulated shedding of these EGFR-ligands. Remarkably, BFA treatment for 1 h had little effect on the PMA-stimulated shedding of AR (8% decrease) or TGF α (28% decrease), and it did not detectably affect their constitutive release (Figure 3E, a and b). Under

identical conditions, BFA strongly reduced the release of soluble alkaline phosphatase (AP) (84%; Figure 3F). PMA-stimulated shedding of TGF α and AR decreased substantially in the second hour in the absence of BFA, and it returned to constitutive levels after 3 h (Figure 3E). BFA had a more pronounced effect on stimulated shedding after 3–4 h, suggesting that it prevents replenishment of pro-TGF α

Figure 4. Rescue of constitutive and PMA-stimulated shedding of TGF α in *Adam17*^{-/-} cells by chimera between ADAM10 and 17 (A) Diagram of chimeras between ADAM10 and ADAM17 (K, KUZ or kuzbanian, ADAM10; T, TACE, TNF α -converting enzyme, ADAM17). Domains of ADAM10 and ADAM17 are represented by solid bars and open bars, respectively. An HA-epitope was added to ADAM10, ADAM17, and all the chimeric constructs to facilitate their detection and comparison of transfection levels. A 6xMyc tag and V5-His tag were added to the Δ -cyto mutants ADAM17b and ADAM10b, respectively. (B) TGF α was cotransfected with a wild-type or mutant ADAMs in *Adam17*^{-/-} cells as indicated. The cells were incubated with or without PMA (25 ng/ml) for 1 h, and TGF α shedding is presented as a ratio of the AP activity in the supernatant and the cell lysate as described in *Materials and Methods*.

and pro-AR once the EndoH-resistant pool of these molecules is consumed. Secretion of soluble AP was not stimulated by PMA (data not shown), providing additional evidence against a general effect of PMA on the constitutive secretory pathway under the conditions used here. Thus, short-term PMA stimulation affects only or mainly ADAM17 as well as substrates that have already passed the medial-Golgi apparatus, without a requirement for increased intracellular maturation. Finally, when we analyzed the concentration of endogenous ADAM17 by Western blot in wild-type SV40-transformed mEF cells treated with 25 ng/ml PMA, in the presence or absence of BFA, we found similar amounts of pro-ADAM17 and mature ADAM17 in cells treated with PMA or PMA/BFA for up to 2 h as in untreated cells (data not shown). Thus, even though a higher concentration of PMA (100 ng/ml) stimulated down-regulation of mature ADAM17 (Doedens and Black, 2000), the lower concentration of PMA used in this experiment (25 ng/ml) did not.

Chimera between ADAM10 and -17 Address the Role of Different ADAM Domains in PMA-stimulated Shedding of TGF α

Because ADAM17 responds strongly to 1-h PMA stimulation, whereas ADAM10 does not, we used chimera between mouse ADAM17 and ADAM10 to determine which domains of ADAM17 are essential for PMA-stimulated shedding of TGF α via rescue experiments in *Adam17*^{-/-} cells (see Figure 4 A for a diagram of the chimera, and a key to the nomenclature, T, TNF- α converting enzyme/ADAM17; K, kuzbanian/ADAM10). Constitutive and PMA-stimulated TGF α shedding was restored by wild-type ADAM17 as well as mutants in which the transmembrane domain and cytoplasmic domains of ADAM17 had been replaced by the corresponding domains of ADAM10 (TTKK), and to a lesser extent (~50%) by ADAM17 lacking its cytoplasmic domain (A17b, TTT; Figure 4B). None of the other chimera in which only the metalloprotease or disintegrin domains had been swapped (KTTT, TKKK, and TKTT), were able to rescue PMA-stimulated TGF α shedding. The protein expression levels resulting from transfections of COS-7 cells with 1 μ g

each of plasmids coding for wild-type ADAM10 or ADAM17 or either of the four mutants was determined by Western blot analysis. As shown in Supplemental Figure 2A, wild-type ADAM17 and TKTT had the highest expression, ADAM10, TKKK, and TTKK were expressed at intermediate levels, whereas the expression of KTTT was low but still clearly detectable (please see Figure 2B for a side-by-side comparison of the expression of wild-type ADAM17 and ADAM17b). In addition, immunofluorescence analysis showed a comparable staining pattern of the four mutants (see Supplemental Figure 2B). Thus, PMA stimulation requires an intact ectodomain of ADAM17 but not its cytoplasmic or transmembrane domains.

Chimera between TGF α and EGF or BTC Define the Requirements for Stimulated Shedding by ADAM17

We next focused on chimera between TGF α and EGF or BTC to determine which domains of TGF α are required for stimulated shedding by ADAM17 (a diagram of the chimera and a key to the nomenclature is shown in Figure 5 A). Each chimera was cotransfected into *Adam17*^{-/-} cells with empty vector or with ADAM17. Overexpressed ADAM17 only slightly enhanced processing of EGF (Figure 5B; also see Figure 1) but could release relatively high amounts of TGF α from *Adam17*^{-/-} cells. Moreover, ADAM17-dependent shedding of TGF α was strongly stimulated by PMA (Figure 5B; also see Figure 1). Shedding of the chimera with the EGF ectodomain and TGF α transmembrane and cytoplasmic domain (ETT) was similar to that of TGF α , although there was a slight increase in constitutive shedding in the absence of ADAM17. Shedding of EET was inefficient and was only slightly increased upon rescue with wild-type ADAM17, whereas very little ADAM17-dependent shedding of TEE was observed. However, placing the ectodomain and juxtamembrane domain of TGF α on the cytoplasmic domain of EGF (TTE) restored ADAM17-dependent constitutive and PMA-induced shedding, although not to the same level as seen with wild-type TGF α . Moreover, ADAM17 induced slightly more shedding of TET compared with TEE. Finally, constitutive and stimulated ADAM17-

Figure 5. Chimera between TGFα and EGF or BTC define the requirements for selective PMA-dependent and constitutive shedding of TGFα by ADAM17. (A) Diagram of chimeras between TGFα and EGF or BTC. Domain components of EGF (or BTC) and TGFα are presented by open bars and solid bars, respectively. (B) TGFα-EGF chimera were transfected into *Adam17*^{-/-} cells together with empty vector (–) or ADAM17 (A17). The cells were incubated with or without 25 ng/ml PMA for 1 h. Similar experiments were performed with TGFα-BTC chimeras (C). Shedding activity is presented as a ratio of the AP activity between the supernatant and the cell lysates as described in *Materials and Methods*. The expression of each chimera was confirmed by immunofluorescence analysis (see Supplemental Figure 1).

dependent shedding of ETE was similar to that of TTE, demonstrating the cleavage site of TGFα is necessary and sufficient for constitutive and PMA-dependent processing by ADAM17. Shedding experiments with chimera between BTC and TGFα yielded largely comparable results (Figure 5C), with the exception that ADAM17 had little or no effect on shedding of two chimera in which the ectodomain of BTC was positioned next to the cleavage site of TGFα (BTT, BTB), suggesting that the EGF-like domain in BTC blocks access to the TGFα cleavage site. The expression of all chimeras was confirmed by immunofluorescence analysis (see Supplemental Figure 3).

Calmodulin Inhibitor- and Calcium Ionophore-stimulated Shedding of EGF and BTC by ADAM10

Because ADAM17 lacking its cytoplasmic domain could still be stimulated by PMA (also see Reddy *et al.*, 2000), arguing that a direct interaction with cytoplasmic molecules is not necessary for this effect, we tested whether ADAM 10 or -17 require their cytoplasmic domain to respond to a different stimulus, the calcium ionophore IM, and the calmodulin inhibitor trifluoroperazine (TFP). Both have previously been shown to activate ADAM10 (Nagano *et al.*, 2004; Sanderson *et al.*, 2005). We found that shedding of EGF and BTC in *Adam10*^{-/-} cells rescued with wild-type ADAM10 was enhanced by TFP (Figure 6A). Little effect of TFP on EGF or BTC shedding was seen in *Adam10*^{-/-} cells transfected with empty vector or the inactive ADAM10 E > A mutant,

corroborating that TFP-stimulated shedding of EGF and BTC depends mainly on ADAM10 (Figure 6A).

A previous study suggested that IM stimulates shedding of CD44 by enhancing the intracellular maturation of ADAM10 (Nagano *et al.*, 2004). However, stimulation with IM for 10 or 30 min did not affect the maturation of HA-tagged ADAM10 or endogenous ADAM10 that could be cell surface biotinylated or detected by Western blot (Figure 6B). Furthermore, in an immunofluorescence analysis, the subcellular localization of ADAM10 and BTC was indistinguishable in IM-treated mEF cells compared with untreated controls (Figure 6C, a–f), and both ADAM10 and ADAM17 colocalize in these cells (Figure 6E, g–i).

The ability of ADAM10 to rescue IM-stimulated shedding of EGF and BTC in *Adam10*^{-/-} cells provided an opportunity to test how well chimera between ADAM10 and -17 (see Figure 4A for a key to the constructs) are able to rescue shedding of the EGFR-ligands EGF or BTC in *Adam10*^{-/-} cells. Only wild-type ADAM10 strongly enhanced shedding of EGF and BTC after addition of IM (Figure 6D). Among the chimera tested here, only the deletion mutant A10b, which lacks the cytoplasmic domain of ADAM10, could partially rescue the IM induced shedding of EGF (~40%; Figure 6D) and BTC (~25% Figure 6E; see Supplemental Figure 2, A and B, for a comparison of the relative amounts of wild-type ADAM10 and -17 and of the chimera expressed in COS-7 cells).

Figure 6. Calcium ionophore and calmodulin inhibition stimulates ADAM10-dependent shedding of EGF and BTC. (A) EGF and BTC were transfected into *Adam10*^{-/-} cells together with either empty vector (-), wild-type ADAM10 (A10), or the catalytically inactive mutant ADAM10E > A (A10EA). The cells were incubated with or without TFP (25 μ M) for 30 min. (B) COS-7 cells transfected with HA-tagged ADAM10 (A10HA, left) or untransfected controls (right) were incubated with IM (2.5 μ M) for 10–30 min followed by labeling with a nonmembrane-permeable biotinylation reagent. The lysates were immunoprecipitated with either anti-HA antibodies (left, anti-HA) or anti-ADAM10 antibodies (anti-A10), and biotinylated proteins were detected with Avidin-HRP (top). Subsequently, the membrane on the top left was stripped and reprobed with anti-ADAM10 antibodies (bottom left). Bottom right, Western blot analysis of COS-7 cells probed with anti-ADAM10 antibodies. Open arrow, pro-ADAM10; black arrow, mature ADAM10; asterisk, nonspecific band. (C) AP-tagged BTC and HA-tagged ADAM10 cotransfected into wild-type mEF cells were incubated with (d–f) or without (a–c) IM (2.5 μ M) for 10 min and then stained with anti-AP and anti-HA antibody. The secondary antibody against the AP-tag was labeled with fluorescein and against the HA-tag with rhodamine. (g–i) ADAM17 and HA-ADAM10 were cotransfected and stained with anti-ADAM17 antibody and anti-HA antibody, respectively. The secondary antibody against ADAM17 was fluorescein labeled and against the HA-tag was rhodamine labeled. Bar, 20 μ m. (D) EGF was cotransfected with wild-type or chimeric ADAM expression constructs in *Adam10*^{-/-} cells (see Figure 4A for a diagram of the ADAM chimeras). The cells were incubated with or without IM (5 μ M) for 30 min. Similar experiments were performed with BTC (E). (F) Western blot analysis of ADAM10 (lanes 1 and 2) or the ADAM10 IQ > AA mutant (lanes 3 and 4) expressed in COS-7 cells. Lanes 1 and 3 show Western blots of the cell lysates expressing wild-type or mutant protein, and lanes 2 and 4 show Western blots of material that bound to calmodulin (CaM) agarose beads. Open arrowhead: pro-ADAM10, black arrowhead: mature ADAM10. The IQ > AA mutation leads to a strong reduction of the ratio between mature ADAM10 and pro-ADAM10. (G) Shedding of EGF (left) or BTC (right) in *Adam10*^{-/-} cells transfected with wild-type ADAM10 (A10), the ADAM10 IQ > AA mutant (IQ), or a pcdNA3 vector as control (-), in the presence or absence of IM (5 μ M). Despite the low level of mature ADAM10 IQ > AA compared with wild-type ADAM10 (F), ADAM10 IQ > AA rescues constitutive and ionomycin-induced shedding of EGF and BTC as well as wild-type ADAM10.

A **B** **C** **D**

Figure 7. TFP-stimulated shedding of TGF α , HB-EGF, AR, and EPR in the absence of ADAM17. (A) TGF α , HB-EGF, AR, and EPR are EGFR ligands that require ADAM17 for constitutive and PMA-stimulated shedding. These four EGFR-ligands were transfected into *Adam17*^{-/-} cells, and the transfected cells were incubated either with 25 ng/ml PMA, 25 μ M TFP, or 25 μ M TFP and BB94 (1 μ M), or in medium with no additions (-) for 30 min. (B) TGF α and HB-EGF were transfected into ADAM-deficient cell lines (*Adam9,12,15*^{-/-} triple knockout cells, *9/12/15*^{-/-}; *Adam8*^{-/-}, *A8*^{-/-}; *Adam19*^{-/-}, *A19*^{-/-}; *Adam10*^{-/-}, *A10*^{-/-}). The cells were incubated with or without 25 μ M TFP for 30 min. (C) The constitutive and PMA-stimulated shedding of TGF α in wild-type SV40-transformed mEF cells is inhibited by ~70% in the presence of 30 nM TIMP3 compared with 1 μ M BB94 but not by 1 nM TIMP3 or by 1 nM or 30 nM TIMP1 and -2, which is consistent with the role of ADAM17 in PMA-dependent TGF α shedding. This experiment is representative of three separate experiments in which one tissue culture well was used per TIMP concentration due to limited availability of TIMP2. (D) The sheddase for TGF α , which is stimulated by calcium influx (2.5 μ M ionomycin) in *Adam17*^{-/-} cells, is strongly inhibited by 1 and 10 nM TIMP1, -2, and -3 and thus has a clearly distinct TIMP inhibitor profile in cell-based assays compared with ADAM17. This experiment is representative of three separate experiments with at least two wells each per TIMP concentration. AP activity in the supernatant was determined by colorimetric analysis as described in *Materials and Methods*.

The cytoplasmic domain of ADAM10 from several species (human, bovine, murine, and rat) contains a sequence resembling the calcium-independent IQ consensus binding site for calmodulin (IQXXRXXXXR; for review, see (Bahler and Rhoads, 2002), sequence in murine ADAM10: IQQP-PRQRPRE). HA-tagged ADAM10 and a mutant in which the

IQ sequence was mutated to AA (IQ > AA) were transfected into COS-7 cells, and the expressed proteins were visualized by Western blot analysis with anti-HA antibodies (Figure 6F, lanes 1 and 3). Similar levels of the proform of wild-type ADAM10 and the IQ > AA mutant were detected; however, only very little mature ADAM10 IQ > AA was present

compared with wild-type ADAM10. This suggests that the IQ sequence has a role in maturation of ADAM10, or in stabilizing the mature protein. Cell lysates expressing wild-type ADAM10 or the IQ > AA mutant were incubated with calmodulin/agarose beads, and the bound material was subjected to Western blot analysis with antibodies against the HA-tag. This showed that only the proform of wild-type ADAM10 as well as of the ADAM10 IQ > AA mutant bound to immobilized calmodulin (Figure 6F, lanes 2 and 4), demonstrating that the IQ sequence is not essential for the interaction between calmodulin and pro-ADAM10. Further studies will be necessary to understand the involvement of the IQ sequence in the maturation or turnover of ADAM10 (please note that the effects of the IQ > AA mutation might be unrelated to any putative interaction of ADAM10 with calmodulin). Nevertheless, the availability of a mutant with impaired maturation or decreased stability of the mature protein provided an additional opportunity to test whether the decreased levels of mature ADAM10 IQ > AA compared with wild-type ADAM10 affect its response to calcium influx. *Adam10*^{-/-} cells were cotransfected with EGF or BTC and either wild-type ADAM10, the IQ > AA mutant, or an empty pcDNA3 vector as control. The decreased levels of mature ADAM10 IQ > AA did not affect the ability of this mutant to rescue IM stimulated shedding of EGF or BTC compared with wild-type ADAM10 (Figure 6G), suggesting that other sequences in the cytoplasmic domain are responsible for the response of ADAM10 to calcium influx.

Calmodulin Inhibitor Stimulates Shedding of TGF α , HB-EGF, AR, and EPR in the Absence of ADAM17

As shown above and in Figure 7A, PMA-stimulated shedding of TGF α , HB-EGF, AR, and EPR is abolished in *Adam17*^{-/-} cells. Remarkably, shedding of these four EGFR-ligands could be strongly stimulated by TFP in *Adam17*^{-/-} cells by an activity that was sensitive to BB94 (Figure 7A). When we measured TFP stimulated shedding of TGF α and HB-EGF in cells lacking one or more of the widely expressed and catalytically active ADAMs that are candidate sheddases (ADAM8, -9, -10, -12, -15, and -19), a significant increase in shedding of TGF α and HB-EGF in response to TFP was seen in all cases (Figure 7B), arguing against a major role of these ADAMs in TFP-stimulated shedding of TGF α and HB-EGF. Thus, different stimuli (calmodulin inhibition versus PMA) can activate distinct sheddases for these four EGFR-ligands in mouse embryonic cells.

To learn more about the identity of the calcium-activated sheddase(s) for TGF α , we compared the inhibitor profile of TIMP1, -2, and -3 toward PMA-stimulated shedding of TGF α in wild-type mEF cells to the profile of the sheddase activated by calcium influx in *Adam17*^{-/-} cells. Constitutive and PMA-stimulated shedding of TGF α was not blocked by up to 30 nM TIMP1 and -2 or by 1 nM TIMP3, but it was inhibited between 50 and 70% by 30 nM TIMP3 compared with the inhibition with 1 μ M BB94 (Figure 7C). The calcium influx induced shedding of TGF α in *Adam17*^{-/-} cells after addition of 2.5 μ M IM was strongly inhibited by TIMP1, -2, and -3 at concentrations as low as 1 nM, as well as by 1 μ M BB94, suggesting the involvement of an MMP that is sensitive to all three TIMPs (Figure 7D).

DISCUSSION

Ectodomain shedding has a crucial role in regulating EGFR signaling by releasing EGFR-ligands from their membrane tether. Previous studies have implicated different members of the ADAM family of cell surface peptidases in ectodo-

main shedding EGFR-ligands, and loss of function studies defined a key role for ADAM10 and -17. The gain of function experiments performed here reveal which other ADAMs are able to contribute to shedding of EGFR-ligands when overexpressed or dysregulated. Moreover, this study explores how two commonly used activators of ectodomain shedding, phorbol esters or calcium ionophores and calcium/calmodulin inhibitors, affect the function of the major EGFR-ligand sheddases, ADAM10 and -17.

Gain of Function Studies

The "gain of function" studies demonstrate that several catalytically active ADAMs (ADAM8, -9, -10, -12, -17, and -19) should, in principle, have the ability to contribute to EGFR-signaling via releasing EGF. Moreover, they define which enzymes are able to cleave which EGFR-ligands when overexpressed or dysregulated (summarized in Table 1). Several ADAMs are known to be up-regulated in human or mouse tumors (see, for example, Kveiborg *et al.*, 2005; Mazzocca *et al.*, 2005; Peduto *et al.*, 2005, 2006; and references therein), where they could conceivably contribute to processing of EGFR-ligands and EGFR activation (other substrates or functions of ADAMs could also contribute to tumor growth). It should be emphasized that the increased shedding by overexpressed ADAMs was seen in the absence of further stimulation. This suggests that increased expression levels of these ADAMs will presumably be an important determinant of their relative contribution to EGFR-ligand shedding.

The ability of several ADAMs to cleave EGF and BTC when overexpressed raises the question of why ADAM10 emerged as the major sheddase for EGF and BTC in loss of function studies in mEF cells, in which most ADAMs tested here are expressed (Sahin *et al.*, 2004). Perhaps endogenous ADAM10 is required for shedding of EGF and BTC in mEF cells because it is expressed at higher levels, or it has higher relative activity or both compared with the other ADAMs. The contribution of other ADAMs to EGF and BTC shedding might thus be more pronounced, for example, in cells or tissues where their expression relative to ADAM10 is higher than in mEF cells. It will be interesting to further explore how the relative contribution of different candidate sheddases to EGFR-ligand release in different types of cells and tissues is determined.

PMA-stimulated EGFR-Ligand Shedding

Even though several ADAMs possess constitutive sheddase activity, the regulation of ectodomain shedding by phorbol esters has been considered a hallmark feature of this process (Massague and Pandiella, 1993). In addition, ectodomain shedding can be regulated by other stimuli, including calcium ionophores, calmodulin inhibitors, cholesterol depletion, and stimulation of G protein-coupled receptors (GPCR) or the mitogen-activated protein (MAP) kinase pathway (see Blobel, 2005, and references therein). The results of this study support the notion that ADAM17 is the main enzyme that responds to short-term PMA stimulation. It is tempting to speculate that the catalytic activity of ADAM17 is up-regulated by PMA through cytoplasmic phosphorylation of serine/threonine residues. Although this notion is supported by several studies (Diaz-Rodriguez *et al.*, 2002; Fan *et al.*, 2003; Soond *et al.*, 2005), we found that the transmembrane domain and cytoplasmic domains of ADAM17 are not required for its response to PMA stimulation. It should be noted that our results are consistent with a previous study in which the cytoplasmic domain of ADAM17 was found to be dispensable for PMA-stimulated shedding of TNF- α (Reddy

Table 1. Summary of results of a previous loss of function study (Sahin *et al.*, 2004) as well as the current study with respect to which ADAMs participate in the release of individual EGFR-ligands under different conditions in mouse embryonic cells, as indicated

	Loss of function (constitutive or PMA)	Gain of function (overexpression)	Loss of function (calmodulin inhibitor)
TGF α	ADAM17 (PMA)	ADAM17	Not ADAM8, -9, -10, -12, -15, -17, -19
AR	ADAM17 (PMA)	ADAM8, -17	Not ADAM8, -9, -10, -12, -15, -17, -19
EPR	ADAM17 (PMA)	ADAM17	Not ADAM8, -9, -10, -12, -15, -17, -19
HB-EGF	ADAM17 (PMA)	ADAM8, -17	Not ADAM8, -9, -10, -12, -15, -17, -19
BTC	ADAM10 (constitutive)	ADAM8, -10, -12, -17, -19	ADAM10
EGF	ADAM10 (constitutive)	ADAM8, -9, -10, -12, -17, -19	ADAM10

et al., 2000). Moreover, under the conditions used in here, PMA did not affect maturation of ADAM17 or its transport to the cell surface at concentrations that strongly stimulated ADAM17-dependent TGF α shedding.

A separate hypothesis to explain the unusual mechanism underlying PMA-stimulated shedding was that PMA might promote the interaction between substrates and ADAM17. However, two ADAM17 substrates, TGF α and AR, had very different subcellular localizations to begin with, and their subcellular localization as well as that of ADAM17 did not undergo major changes after PMA stimulation. Instead, Western blot analysis showed that PMA stimulation triggers a burst of shedding, which rapidly consumes the EndoH-resistant pro-TGF α and pro-AR. This suggests that it mainly affects pro-TGF α and pro-AR that have traversed the medial-Golgi apparatus. Moreover, treatment of cells with BFA, which leads to collapse of the Golgi network into the ER (Lippincott-Schwartz *et al.*, 1989) had little effect on PMA-stimulated shedding of TGF α and AR. Because BFA also blocks the transport of ADAM17 to the TGN, where it is activated by processing of its prodomain (Schlndorff *et al.*, 2000), increased maturation of ADAM17 or increased transport of its substrates through the secretory pathway cannot be responsible for the PMA-dependent burst in shedding. Domain swapping experiments between ADAM10 and -17 indicated that the intact ectodomain of ADAM17 is crucial for PMA-stimulated shedding of TGF α , either because a membrane-anchored accessory protein(s) must interact with the ectodomain of ADAM17 to regulate the PMA response (Black *et al.*, 2003) or because an intact ectodomain is necessary for proper presentation of the catalytic domain. Moreover, domain swapping experiments between TGF α and EGF showed that the substrate cleavage site was necessary and sufficient for PMA-stimulated shedding (also see Arribas *et al.*, 1997; Zheng *et al.*, 2004). This strongly argues against substrate targeting by interactions between the EGF-like domain of TGF α and ADAM17. Further studies will be necessary to search for ADAM17-interacting proteins and to understand the fate of EndoH-resistant pro-TGF α and pro-AR as well as ADAM17 after PMA stimulation.

The observation that some EGFR-ligands displayed predominant cell surface localization in transfected cells (pro-EGF, pro-BTC, and pro-AR), whereas others had a mainly perinuclear localization (pro-TGF α , pro-HB-EGF, and pro-EPR) raises questions about how the subcellular localization of different EGFR-ligands is determined. The accumulation of pro-EGF and pro-BTC on the cell surface can most likely be explained, at least in part, by the relatively inefficient processing of these two EGFR-ligands compared with TGF α , even at similar expression levels. In addition, pro-TGF α is retained in the ER by PDZ-domain binding proteins (Fernandez-Larrea *et al.*, 1999), which would explain the high amounts of intracellular EndoH-sensitive pro-TGF α . TGF α also contains an endocytosis motif, which delivers

unprocessed pro-TGF α back to the TGN (Martinez-Arca *et al.*, 2005), where most ADAM17 resides (Schlndorff *et al.*, 2000). Interestingly, inhibiting proteasomal degradation almost doubled the constitutive shedding of TGF α in COS-7 cells and significantly increased the shedding of HB-EGF, but it had little effect on shedding of pro-AR, and none on pro-EGF and pro-BTC release (see Supplemental Figure 4). In light of the different localizations of overexpressed EGFR-ligands, it is tempting to speculate that the corresponding endogenous EGFR-ligands also have different properties in terms of their subcellular transport and final localization. The amount of cell surface accumulation of endogenous EGFR-ligands presumably depends on a combination of factors, including the expression level of the EGFR-ligand and its sheddase(s), the cleavage efficiency of the sheddase(s), transport of the EGFR-ligand out of the ER or endocytosis from the cell surface, and turnover by proteasomal degradation. These factors in turn could affect the relative contribution of an EGFR-ligand to juxtacrine versus paracrine EGFR signaling (for review, see Blobel, 2005).

Calcium Influx-dependent EGFR-Ligand Shedding

In addition to PMA, other commonly used stimuli for ectodomain shedding are calcium influx and inhibition of calcium calmodulin, both of which activated ADAM10-dependent shedding of EGF. However, under the conditions used here, IM did not affect the amount of mature ADAM10, its accumulation on the cell surface, or its perinuclear localization, even though it had previously been reported to induce maturation of ADAM10 and its translocation to the cell surface (Nagano *et al.*, 2004). Nevertheless, because IM clearly activated ADAM10-dependent shedding of EGF and BTC in this study, the mechanism underlying IM-dependent stimulation of ADAM10 is most likely independent of prodomain removal or relocalization. This notion is further supported by the finding that an ADAM10 carrying two cytoplasmic point mutations (IQ > AA) was able to fully rescue IM-dependent shedding in *Adam10*^{-/-} cells, even though only small amounts of the mature form of this mutant were expressed compared with its proform. However, deletion of the cytoplasmic domain of ADAM10 strongly decreased its response to IM stimulation (75% for BTC). Further studies will be necessary to define the role of the cytoplasmic domain of ADAM10 in its response to stimulation by calcium influx.

When we analyzed whether ADAM17 responds to IM or TFP, we found that calcium influx induces shedding of TGF α , HB-EGF, AR, and EPR in *Adam17*^{-/-} cells, even though the constitutive- and PMA-stimulated shedding of these EGFR-ligands depends mainly on ADAM17 (see above). The TFP-induced sheddase for these four growth factors was sensitive to BB94, suggesting that it is also a metalloprotease, but it was insensitive to inhibitors of the MAP kinase pathway (data not shown). Moreover, calcium

influx-stimulated shedding of TGF α in *Adam17*^{-/-} cells was inhibited by TIMP1, -2, and -3, strongly suggesting that it depends on a matrix metalloproteinase. The ADAMs included in this study are unlikely to be responsible for this activity, because TFP-stimulated shedding of TGF α and HB-EGF was not noticeably affected in cells lacking ADAM8, -9, -10, -12, -15, or -19. It should be emphasized that this yet to be identified sheddase is apparently unable to compensate for the loss of ADAM17 during development, because the phenotype of mice lacking ADAM17 closely resembles that of mice lacking TGF α , HB-EGF, and AR as well as the EGFR. Nevertheless, any enzyme that is capable of releasing EGFR-ligands should be able to contribute to EGFR activation when this enzyme is dysregulated. These results also raise questions about the relative contribution of this enzyme compared with ADAM17 and other ADAMs to cross-talk between GPCRs and the EGFR (Fischer *et al.*, 2003), at least in cases involving GPCR activated calcium-influx. It will be interesting to determine whether the TFP/IM-stimulatable TGF α sheddase is related to a TGF α sheddase that is activated by APMA in cells lacking ADAM17 (Merlos-Suarez *et al.*, 2001) and/or to an enzymatic activity that can process the C-terminal cleavage site peptide of TGF α in extracts of rat liver epithelial cell membranes that contain no detectable ADAM17 (Hinkle *et al.*, 2003).

Together, these findings establish several novel properties of EGFR-ligand sheddases. The gain of function studies demonstrate which ADAMs can constitutively process which EGFR-ligands when overexpressed (Table 1), which is likely to be highly relevant for pathologies where ADAMs are known to be dysregulated, such as inflammation or cancer. Moreover, we show that stimulation of ADAM17 by PMA leads to a burst of shedding from a post-Golgi compartment and does not require its cytoplasmic domain or increased maturation. These findings further constrain any models to explain the mechanism underlying the PMA stimulation of ADAM17. Conversely, the activation of ADAM10-dependent shedding of EGF and BTC by calcium influx is strongly reduced in a mutant lacking its cytoplasmic domain. Finally, the unexpected observation that a calmodulin inhibitor stimulates processing of TGF α , HB-EGF, AR, and EPR in cells lacking ADAM17 uncovers another sheddase for these substrates, whose constitutive and PMA-stimulated shedding in mEF cells depends on ADAM17 (Table 1). These results provide new insight into the regulation and substrate specificity of several ADAMs with key roles in development and disease. Moreover, in light of the critical role of ectodomain shedding in regulating the EGFR-signaling pathway, these findings uncover new opportunities for modulating the EGFR-signaling pathway.

ACKNOWLEDGMENTS

We thank Drs. R. Black and J. Peschon for providing *Adam17*^{-/-} mice and TIMP3; Dr. A. Sehara-Fujisawa for *Adam12*^{-/-} mice; Dr. S. Higashiyama for alkaline phosphatase-tagged EGFR-ligands; Dr. Gillian Murphy for providing TIMP1 and -2; Katya Bojilova and Arash Shirazi for technical assistance; and Dr. Gerd Blobel, Dr. Steven Swendeman, and members of C.P.B.'s laboratory for critically reading this manuscript. This work was supported by National Institutes of Health R01 GM-64750 (to C.P.B.), Deutsche Forschungsgemeinschaft SFB 415TPB9 (to P.S. and K.R.), and it was conducted in a facility constructed with support from Research Facilities Improvement Program, National Institutes of Health Grant C06-RR12538-01.

REFERENCES

Arribas, J., Lopez-Casillas, F., and Massague, J. (1997). Role of the juxtamembrane domains of the transforming growth factor- α precursor and the β -amy-

- loid precursor protein in regulated ectodomain shedding. *J. Biol. Chem.* 272, 17160–17165.
- Bahler, M., and Rhoads, A. (2002). Calmodulin signaling via the IQ motif. *FEBS Lett.* 513, 107–113.
- Black, R. A., *et al.* (2003). Substrate specificity and inducibility of TACE (tumour necrosis factor α -converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity. *Biochem. Soc. Symp.* 39–52.
- Blobel, C. P. (2005). ADAMs: key players in EGFR-signaling, development and disease. *Nat. Rev. Mol. Cell Biol.* 6, 32–43.
- Borrell-Pages, M., Rojo, F., Albanell, J., Baselga, J., and Arribas, J. (2003). TACE is required for the activation of the EGFR by TGF- α in tumors. *EMBO J.* 22, 1114–1124.
- Briley, G. P., Hissong, M. A., Chiu, M. L., and Lee, D. C. (1997). The carboxyl-terminal valine residues of proTGF α are required for its efficient maturation and intracellular routing. *Mol. Biol. Cell* 8, 1619–1631.
- Diaz-Rodriguez, E., Montero, J. C., Esparis-Ogando, A., Yuste, L., and Pandiella, A. (2002). Extracellular signal-regulated kinase phosphorylates tumor necrosis factor α -converting enzyme at threonine 735, a potential role in regulated shedding. *Mol. Biol. Cell* 13, 2031–2044.
- Doedens, J. R., and Black, R. A. (2000). Stimulation-induced down-regulation of tumor necrosis factor- α converting enzyme. *J. Biol. Chem.* 275, 14598–14607.
- Doedens, J. R., Mahimkar, R. M., and Black, R. A. (2003). TACE/ADAM-17 enzymatic activity is increased in response to cellular stimulation. *Biochem. Biophys. Res. Commun.* 308, 331–338.
- Dong, J., Opreko, L. K., Dempsey, P. J., Lauffenburger, D. A., Coffey, R. J., and Wiley, H. S. (1999). Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA* 96, 6235–6240.
- Fan, H., Turck, C. W., and Derynck, R. (2003). Characterization of growth factor-induced serine phosphorylation of tumor necrosis factor- α converting enzyme and of an alternatively translated polypeptide. *J. Biol. Chem.* 278, 18617–18627.
- Fernandez-Larrea, J., Merlos-Suarez, A., Urena, J. M., Baselga, J., and Arribas, J. (1999). A role for a PDZ protein in the early secretory pathway for the targeting of proTGF- α to the cell surface. *Mol. Cell* 3, 423–433.
- Fischer, O. M., Hart, S., Gschwind, A., and Ullrich, A. (2003). EGFR signal transactivation in cancer cells. *Biochem. Soc. Trans.* 31, 1203–1208.
- Gschwind, A., Fischer, O. M., and Ullrich, A. (2004). The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat. Rev. Cancer* 4, 361–370.
- Gschwind, A., Hart, S., Fischer, O. M., and Ullrich, A. (2003). TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J.* 22, 2411–2421.
- Hartmann, D., *et al.* (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for α -secretase activity in fibroblasts. *Hum. Mol. Genet.* 11, 2615–2624.
- Hinkle, C. L., Mohan, M. J., Lin, P., Yeung, N., Rasmussen, F., Milla, M. E., and Moss, M. L. (2003). Multiple metalloproteinases process protransforming growth factor- α (proTGF- α). *Biochemistry* 42, 2127–2136.
- Horiuchi, K., Zhou, H. M., Kelly, K., Manova, K., and Blobel, C. P. (2005). Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins beta1 and beta2. *Dev. Biol.* 283, 459–471.
- Izumi, Y., *et al.* (1998). A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* 17, 7260–7272.
- Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C., and Lee, D. C. (2003). Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* 22, 2704–2716.
- Kurisasi, T., *et al.* (2003). Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: involvement of meltrin alpha in adipogenesis and myogenesis. *Mol. Cell Biol.* 23, 55–61.
- Kveiborg, M., Frohlich, C., Albrechtsen, R., Tischler, V., Dietrich, N., Holck, P., Kronqvist, P., Rank, F., Mercurio, A. M., and Wewer, U. M. (2005). A role for ADAM12 in breast tumor progression and stromal cell apoptosis. *Cancer Res.* 65, 4754–4761.
- Lippincott-Schwartz, J., Yuan, L. C., Bonifacio, J. S., and Klausner, R. D. (1989). Rapid redistribution of Golgi proteins into the endoplasmic reticulum in cells treated with brefeldin A: evidence for membrane recycling from Golgi to ER. *Cell* 56, 801–813.

- Martinez-Arca, S., Bech-Serra, J. J., Hurtando-Kuttner, M., Borroto, A., and Arribas, J. (2005). Recycling of cell surface proTGF- α regulates EGFR activation. *J. Biol. Chem.* 280, 36970–36977.
- Massague, J., and Pandiella, A. (1993). Membrane-anchored growth factors. *Annu. Rev. Biochem.* 62, 515–541.
- Mazzocca, A., Coppari, R., De Franco, R., Cho, J. Y., Libermann, T. A., Pinzani, M., and Toker, A. (2005). A secreted form of ADAM9 promotes carcinoma invasion through tumor-stromal interactions. *Cancer Res.* 65, 4728–4738.
- Merlos-Suarez, A., Ruiz-Paz, S., Baselga, J., and Arribas, J. (2001). Metalloprotease-dependent protransforming growth factor- α ectodomain shedding in the absence of tumor necrosis factor- α -converting enzyme. *J. Biol. Chem.* 276, 48510–48517.
- Nagano, O., Murakami, D., Hartmann, D., De Strooper, B., Saftig, P., Iwatsubo, T., Nakajima, M., Shinohara, M., and Saya, H. (2004). Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. *J. Cell Biol.* 165, 893–902.
- Peduto, L., Reuter, V. E., Sehara-Fujisawa, A., Shaffer, D. R., Scher, H. I., and Blobel, C. P. (2006). ADAM12 is highly expressed in carcinoma-associated stroma and is required for mouse prostate tumor progression. *Oncogene* 25, 5462–5466.
- Peduto, L., Reuter, V. E., Shaffer, D. R., Scher, H. I., and Blobel, C. P. (2005). Critical function for ADAM9 in mouse prostate cancer. *Cancer Res.* 65, 9312–9319.
- Peschon, J. J., *et al.* (1998). An essential role for ectodomain shedding in mammalian development. *Science* 282, 1281–1284.
- Reddy, P., Slack, J. L., Davis, R., Cerretti, D. P., Kozlosky, C. J., Blanton, R. A., Shows, D., Peschon, J. J., and Black, R. A. (2000). Functional analysis of the domain structure of tumor necrosis factor- α converting enzyme. *J. Biol. Chem.* 275, 14608–14614.
- Reiss, K., Maretzky, T., Ludwig, A., Tousseyn, T., de Strooper, B., Hartmann, D., and Saftig, P. (2005). ADAM10 cleavage of N-cadherin and regulation of cell-cell adhesion and beta-catenin nuclear signalling. *EMBO J.* 24, 742–752.
- Sahin, U., Weskamp, G., Zhou, H. M., Higashiyama, S., Peschon, J. J., Hartmann, D., Saftig, P., and Blobel, C. P. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR-ligands. *J. Cell Biol.* 164, 769–779.
- Sanderson, M. P., Erickson, S. N., Gough, P. J., Garton, K. J., Wille, P. T., Raines, E. W., Dunbar, A. J., and Dempsey, P. J. (2005). ADAM10 mediates ectodomain shedding of the betacellulin precursor activated by p-aminophenylmercuric acetate and extracellular calcium influx. *J. Biol. Chem.* 280, 1826–1837.
- Schafer, B., Gschwind, A., and Ullrich, A. (2004). Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* 23, 991–999.
- Schlöndorff, J., Becherer, J. D., and Blobel, C. P. (2000). Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem. J.* 347, 131–138.
- Soond, S. M., Everson, B., Riches, D. W., and Murphy, G. (2005). ERK-mediated phosphorylation of Thr735 in TNF α -converting enzyme and its potential role in TACE protein trafficking. *J. Cell Sci.* 118, 2371–2380.
- Sternlicht, M. D., Sunnarborg, S. W., Kouros-Mehr, H., Yu, Y., Lee, D. C., and Werb, Z. (2005). Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. *Development* 132, 3923–3933.
- Sunnarborg, S. W., *et al.* (2002). Tumor necrosis factor- α converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J. Biol. Chem.* 277, 12838–12845.
- Yamazaki, S., *et al.* (2003). Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J. Cell Biol.* 163, 469–475.
- Yan, Y., Shirakabe, K., and Werb, Z. (2002). The metalloprotease kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. *J. Cell Biol.* 158, 221–226.
- Yarden, Y., and Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* 2, 127–137.
- Zheng, Y., Saftig, P., Hartmann, D., and Blobel, C. (2004). Evaluation of the contribution of different ADAMs to TNF α shedding and of the function of the TNF α ectodomain in ensuring selective stimulated shedding by the TNF α convertase (TACE/ADAM17). *J. Biol. Chem.* 279, 42898–42906.