

Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands

Umut Sahin,^{1,2} Gisela Weskamp,¹ Kristine Kelly,^{1,3} Hong-Ming Zhou,¹ Shigeki Higashiyama,⁴ Jacques Peschon,⁵ Dieter Hartmann,⁶ Paul Saftig,⁷ and Carl P. Blobel¹

¹Cell Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

²Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, RI 02912

³Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10021

⁴Department of Medical Biochemistry, Ehime University School of Medicine, Ehime 791-0301, Japan

⁵Amgen Inc., Seattle, WA 98101

⁶Department for Human Genetics, K.U. Leuven and Flanders Interuniversity Institute for Biotechnology (VIB-4), 3000 Leuven, Belgium

⁷Biochemical Institute, Christian-Albrechts University, D-24098 Kiel, Germany

All ligands of the epidermal growth factor receptor (EGFR), which has important roles in development and disease, are released from the membrane by proteases. In several instances, ectodomain release is critical for activation of EGFR ligands, highlighting the importance of identifying EGFR ligand sheddases. Here, we uncovered the sheddases for six EGFR ligands using mouse embryonic cells lacking candidate-releasing enzymes (a disintegrin and metalloprotease [ADAM] 9, 10, 12, 15, 17, and 19). ADAM10 emerged as the main sheddase of EGF and betacellulin, and ADAM17 as the major convertase of epireg-

ulin, transforming growth factor α , amphiregulin, and heparin-binding EGF-like growth factor in these cells. Analysis of *adam9/12/15/17^{-/-}* knockout mice corroborated the essential role of *adam17^{-/-}* in activating the EGFR in vivo. This comprehensive evaluation of EGFR ligand shedding in a defined experimental system demonstrates that ADAMs have critical roles in releasing all EGFR ligands tested here. Identification of EGFR ligand sheddases is a crucial step toward understanding the mechanism underlying ectodomain release, and has implications for designing novel inhibitors of EGFR-dependent tumors.

Introduction

The epidermal growth factor receptor (EGFR) signaling pathway has critical functions in development and in diseases such as cancer (Yarden and Sliwkowski, 2001). Ligands of the EGFR comprise a family of structurally and functionally related integral membrane proteins that can be proteolytically processed and released from cells (Harris et al., 2003). EGFR ligands include EGF (Cohen, 1965), heparin-binding EGF-like growth factor (HB-EGF; Higashiyama et al., 1991), TGF α (Derynck et al., 1984), betacellulin (Shing et al., 1993), amphiregulin (Shoyab et al., 1989), epiregulin (Toyoda et al., 1995), and epigen (Strachan et al., 2001). Although membrane-bound EGFR ligands can engage in juxtacrine signaling (Brachmann et al., 1989; Wong et al.,

1989; Higashiyama et al., 1991), a metalloprotease activity is critical for activation of EGFR signaling under a variety of circumstances. For example, EGFR-dependent proliferation and migration of a mammary epithelial cell line can be inhibited by the metalloprotease inhibitor batimastat (BB94), and this inhibition is rescued by addition of soluble EGF (Dong et al., 1999). Furthermore, activation of TGF α and potentially other EGFR ligands during mouse development depends on the presence of functional ADAM17 (Peschon et al., 1998). Moreover, the metalloprotease-dependent release of HB-EGF as well as amphiregulin from cells has been described as a key step in the transactivation of the EGFR by different G protein-coupled receptors (GPCRs; Prenzel et al., 1999; Gschwind et al., 2003; Lemjabbar et al., 2003). Production

Address correspondence to Carl P. Blobel, Cell Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, Box 368, 1275 York Avenue, New York, NY 10021. Tel.: (212) 639-2915. Fax: (212) 717-3047. email: c-blobel@ski.mskcc.org

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Abbreviations used in this paper: ADAM, a disintegrin and metalloprotease; AP, alkaline phosphatase; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; MEF, mouse embryonic fibroblast; TACE, TNF α -converting enzyme.

Table I. Genotype of offspring from matings of *adam9*^{+/-}*15*^{+/-} parents

Genotype of offspring	Expected	Observed
	%	%
9 ^{+/+} 15 ^{+/+}	6.25	5.5 (10)
9 ^{+/+} 15 ^{+/-}	12.5	15.0 (27)
9 ^{+/+} 15 ^{-/-}	6.25	5.0 (9)
9 ^{+/-} 15 ^{+/+}	12.5	12.8 (23)
9 ^{+/-} 15 ^{+/-}	25.0	23.9 (43)
9 ^{+/-} 15 ^{-/-}	12.5	12.8 (23)
9 ^{-/-} 15 ^{+/+}	6.25	5.5 (10)
9 ^{-/-} 15 ^{+/-}	12.5	15.5 (27)
9 ^{-/-} 15 ^{-/-}	6.25	4.4 (8)

Numbers in parentheses, # (total of 180).

of soluble HB-EGF by keratinocytes is up-regulated in response to wounding, and a metalloprotease inhibitor that blocks release of EGFR ligands from these cells abolishes their migration in vitro, and wound healing in vivo (Tokumaru et al., 2000). Shedding of HB-EGF also has an important role in heart development (Jackson et al., 2003; Yamazaki et al., 2003) and in a mouse model of myocardial hypertrophy, which can be prevented through a metalloprotease inhibitor (Asakura et al., 2002). A recent report demonstrates that even juxtacrine activation of the EGFR by TGF α on an adjacent cell can require a metalloprotease activity (Borrell-Page et al., 2003). Finally, all three EGFR ligands in *Drosophila* (Spitz, Gurken, and Keren) are activated via cleavage of their transmembrane anchors (Lee et al., 2001; Urban et al., 2001; Ghiglione et al., 2002; Tsruya et al., 2002; Shilo, 2003). However, in *Drosophila*, different proteolytic enzymes, the Rhomboid-type proteases, have been implicated in this process (Urban et al., 2002). Thus, proteolytic processing of EGFR ligands is emerging as a critical step in their functional regulation under several different circumstances.

Metalloproteases of the ADAM (a disintegrin and metalloprotease) family are thought to be responsible for shedding of certain EGFR ligands. ADAMs are membrane-anchored glycoproteins with diverse functions, including critical roles in fertilization, neurogenesis, angiogenesis, and in shedding of membrane-bound proteins from cells (Black and White, 1998; Schlöndorff and Blobel, 1999; Primakoff and Myles, 2000; Seals and Courtneidge, 2003). Mice lacking ADAM17 die perinatally and resemble mice lacking TGF α (Mann et al., 1993), HB-EGF (Iwamoto et al., 2003; Jackson et al., 2003), and the EGFR (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Peschon et al., 1998). Consistent with these observations, ADAM17-deficient cells have been shown to be defective in shedding of TGF α , HB-EGF, and amphiregulin (Peschon et al., 1998; Merlos-Suarez et al., 2001; Sunnarborg et al., 2002). However, in addition to ADAM17, three other ADAMs have been linked to HB-EGF shedding. Overexpression of ADAM9 increases HB-EGF shedding in VeroH cells, whereas a mutant form of ADAM9 that is presumably unfolded and retained in the ER decreases HB-EGF shedding (Izumi et al., 1998); yet no defect in HB-EGF shedding was observed in cells lacking ADAM9 (Weskamp et al., 2002). Furthermore, ADAM12

Table II. Genotype of offspring from matings of *adam9*^{-/-}*15*^{-/-}*12*^{+/-} parents

Genotype of offspring	Percent
9 ^{-/-} 15 ^{-/-} 12 ^{+/+}	30.1 (50)
9 ^{-/-} 15 ^{-/-} 12 ^{+/-}	44.0 (73)
9 ^{-/-} 15 ^{-/-} 12 ^{-/-}	25.9 (43)

Numbers in parentheses, # (total of 166).

reportedly has a role in HB-EGF shedding in the heart (Asakura et al., 2002) and in the down-regulation of cell-associated HB-EGF after stimulation with the phorbol ester PMA (Kurisaki et al., 2003). ADAM10 is the fourth ADAM to be implicated in HB-EGF shedding as part of the crosstalk between GPCRs and the EGFR (Lemjabbar and Basbaum, 2002; Yan et al., 2002). The remaining EGFR ligands, EGF, betacellulin, epiregulin, and epigen, are also known to be shed from cells, yet little information is available about the responsible enzyme(s) (Dempsey et al., 1997; Harris et al., 2003).

A crucial step toward understanding the mechanism underlying proteolytic cleavage of EGFR ligands (and its potential role in their activation) is to identify the responsible enzyme(s). In previous papers, different cell types and different approaches were used to analyze shedding of some EGFR ligands (see previous paragraphs), including antisense oligonucleotides and overexpression of both wild-type and putative dominant-negative ADAM constructs. Here, we chose a genetically defined system that is less prone to potential artifacts to evaluate the role of ADAMs in EGFR ligand shedding. To address potential compensatory or redundant functions between ADAMs 9, 12, 15, and 17, we generated *adam9/12/15*^{-/-} and *adam9/12/15/17*^{-/-} mice. Furthermore, we used cells isolated from wild-type, *adam9/12/15*^{-/-}, *adam10*^{-/-}, *adam17*^{-/-}, *adam19*^{-/-}, or *adam9/12/15/17*^{-/-} mice to evaluate how loss of one or more widely expressed ADAMs affects the shedding of different EGFR ligands. This paper represents the first systematic characterization of EGFR ligand processing using mouse cells that lack one or more candidate sheddase of the ADAM family of metalloproteases.

Results

Generation of *adam9/12/15*^{-/-} triple knockout mice to assess potential compensatory or redundant roles of these candidate EGFR ligand sheddases in mouse development

ADAMs 9 and 12 have previously been implicated as sheddases for HB-EGF (Izumi et al., 1998; Asakura et al., 2002; Kurisaki et al., 2003). To evaluate potential redundant or compensatory roles of ADAMs 9 and 12, as well as the related ADAM 15 in development and in the shedding of HB-EGF and other EGFR ligands, we generated double knockout mice (*adam9/15*^{-/-}) and triple knockout mice (*adam9/12/15*^{-/-}) as described in the Materials and methods. Table I shows that *adam9/15*^{-/-} double knockout mice were born with the expected Mendelian ratio from matings of doubly heterozygous parents. *adam9/15*^{-/-} mice were viable and fertile, and did not display any evident spontaneous pathological phenotypes (see Materials and methods for details).

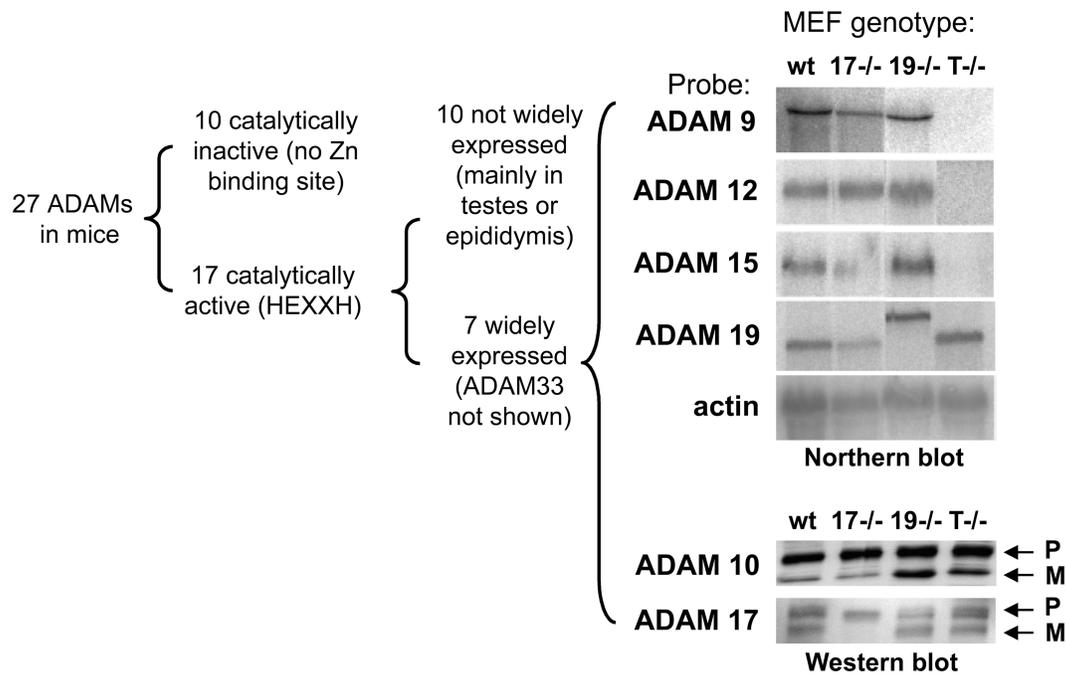


Figure 1. Expression of widely expressed and catalytically active ADAMs in MEFs. ADAMs are grouped by expression pattern and presence or absence of a catalytic site (HEXXH) in the metalloprotease domain. 27 ADAMs have been identified in mice, of which 10 lack an HEXXH sequence, and are presumably not catalytically active. Out of 17 ADAMs with an HEXXH sequence, 10 are mainly expressed in the testes or epididymis, or are not widely expressed (J.M. White, University of Virginia, Charlottesville, VA; http://www.people.virginia.edu/~7EJw7g/Table_of_the_ADAMs.html). Six of the seven widely expressed HEXXH-containing ADAMs were included in this paper. The top right panel shows a Northern blot analysis of the expression of ADAMs 9, 12, 15, and 19 in primary MEFs. The ADAM19 mRNA in *adam19*^{-/-} cells is larger than in the other cells analyzed because the ADAM19 gene is disrupted by insertion of a secretory gene trap (Zhou et al., 2004). The bottom right panel is a Western blot depicting expression of ADAMs 10 and 17 in the primary embryonic fibroblasts used here. Both pro- and mature ADAM10 are expressed in all primary mEFs analyzed here, and pro- and mature ADAM17 are expressed in wild-type, *adam19*^{-/-}, and *adam9/12/15*^{-/-} cells. Note that the exon containing the Zn²⁺-binding catalytic site of ADAM17 is deleted in *adam17*^{-/-} cells (ADAM17^{ΔZnΔZn}). This will most likely impair proper protein folding, resulting in retention of mutant ADAM17 in the ER by chaperones and subsequent degradation (Suzuki et al., 1998). wt, wild type; 17^{-/-}, *adam17*^{-/-}; 19^{-/-}, *adam19*^{-/-}; T^{-/-}, *adam9/12/15*^{-/-} triple knockout; P, pro-form; M, mature.

Triple knockout mice lacking ADAMs 9, 12, and 15 were generated by mating *adam9/15*^{-/-} parents carrying one mutant ADAM12 allele (*adam9/15*^{-/-}*12*^{+/-}). The genotype of offspring from these matings was Mendelian with respect to the mutant ADAM12 allele (Table II), and *adam9/12/15*^{-/-} triple knockout mice were viable and fertile and did not display any evident pathological phenotypes (see below, and Materials and methods for details).

Ectodomain shedding of EGFR ligands in mouse embryonic fibroblasts

To further explore the role of ADAMs in EGFR ligand shedding, we turned to cell-based assays using cells isolated from triple knockout *adam9/12/15*^{-/-} mice, as well as from animals lacking ADAM10, ADAM17, or ADAM19, and wild-type controls. This allowed us to evaluate the contribution of all but one of the widely expressed and catalytically active ADAMs (ADAMs 9, 10, 12, 15, 17, and 19; see Fig. 1; ADAM33 was not included in this work) to the shedding of the EGFR ligands TGF α , amphiregulin, epiregulin, HB-EGF, betacellulin, and EGF. The general approach was to transfect cells with alkaline phosphatase (AP)-tagged forms of EGFR ligands, and then to quantitate shedding by measuring AP activity released into the culture supernatant, or by an in-gel detection of the released AP domain (Weskamp et

al., 2002; Zheng et al., 2002; see Materials and methods for details). Because immortalization of primary cells can significantly affect the expression pattern of ADAMs and other genes (unpublished data), shedding experiments were performed with primary E13.5 mouse embryonic fibroblasts (MEFs) whenever this was possible. The only exceptions were *adam10*^{-/-} and *adam10*^{+/-} cell lines, which were immortalized because *adam10*^{-/-} embryos die early in embryogenesis (E9.5) (Hartmann et al., 2002). Northern or Western blot analyses confirmed that primary mEFs from wild-type mice indeed express all ADAMs analyzed here (ADAMs 9, 10, 12, 15, 17, and 19; see Fig. 1). Furthermore, Northern blots of RNA isolated from primary *adam*^{-/-} cells confirmed the absence of wild-type RNA for the corresponding targeted ADAM(s). Finally, Western blot analysis confirmed that ADAM10 is expressed in all *adam*^{-/-} primary mEFs, and that no mature ADAM17 is produced in *adam17*^{-/-} cells.

As differences in expression levels between tissue culture wells or separate experiments might affect the interpretation of these experiments, each data point was derived from two consecutive measurements of AP activity shed from a single transfected well (see Fig. 2 A). Stimulation of EGFR ligand shedding in a given well was determined by collecting medium after 1 h from unstimulated cells, and then after 1 h from the same cells stimulated with the phorbol ester PMA, a

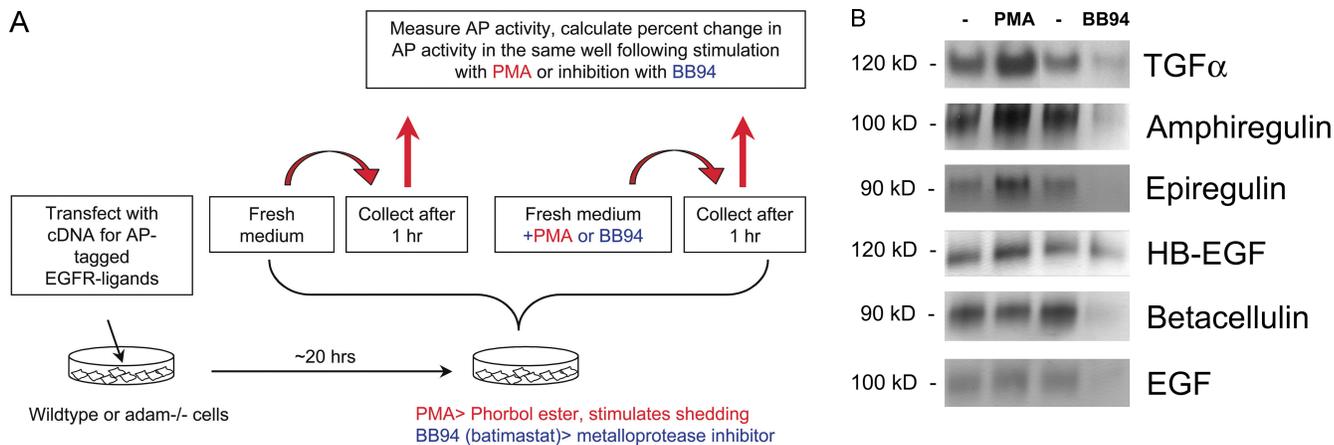


Figure 2. Shedding of EGFR ligands in wild-type primary MEFs. (A) Diagram of a typical shedding experiment (see text for details). (B) Detection of shed AP-tagged EGFR ligands after renaturation in SDS gels (see Materials and methods for details). The left lane shows the AP-tagged forms of TGF α , amphiregulin, epiregulin, HB-EGF, betacellulin, and EGF released in 1 h into the supernatant of a single well each of transfected mEF under resting conditions. The next lane shows the EGFR ligands released in 1 h from the same well after addition of PMA, a phorbol ester that stimulates ectodomain shedding. The third lane shows EGFR ligands released from a separate well in 1 h under resting conditions, and the fourth lane shows the released EGFR ligands in that same well after addition of the hydroxamate-based metalloprotease inhibitor batimastat (BB94).

commonly used activator of ectodomain shedding (Massague and Pandiella, 1993; Hooper et al., 1997; see Fig. 3 A). This was used to calculate the increase in shedding in a given well during PMA stimulation. The batimastat-sensitive constitutive shedding was determined similarly, by measuring the decrease in AP activity in media collected after 1 h in the presence of batimastat and comparing it to the AP activity released from the same well collected 1 h before treatment (see Fig. 4 A). This single-well assay minimizes possible effects caused by different transfection levels. Nevertheless, the absolute values for constitutive shedding from different wild-type or primary *adam*^{-/-} cells expressing a given EGFR ligand were also determined to provide a reference point for the comparison of total unstimulated shedding levels.

Shedding of EGFR ligands in wild-type cells

Shedding of EGFR ligands was first evaluated in wild-type MEFs. As shown in Fig. 2 B, unstimulated mEFs shed basal amounts of TGF α , amphiregulin, epiregulin, HB-EGF, betacellulin, and EGF. In the case of TGF α , amphiregulin, epiregulin, and HB-EGF, shedding was stimulated relatively strongly by PMA, whereas shedding of betacellulin and EGF was only weakly enhanced by PMA (see also Fig. 3 A). Treatment with the metalloprotease inhibitor batimastat strongly reduced both PMA-stimulated (unpublished data) and constitutive shedding of all EGF family members except HB-EGF. Although stimulated HB-EGF shedding was effectively inhibited by batimastat (unpublished data), constitutive release was only weakly affected (see also Fig. 4 A), suggesting that the predominant constitutive HB-EGF sheddase in primary mEFs is not a batimastat-sensitive metalloprotease, and is distinct from the sheddase(s) of other EGFR ligands.

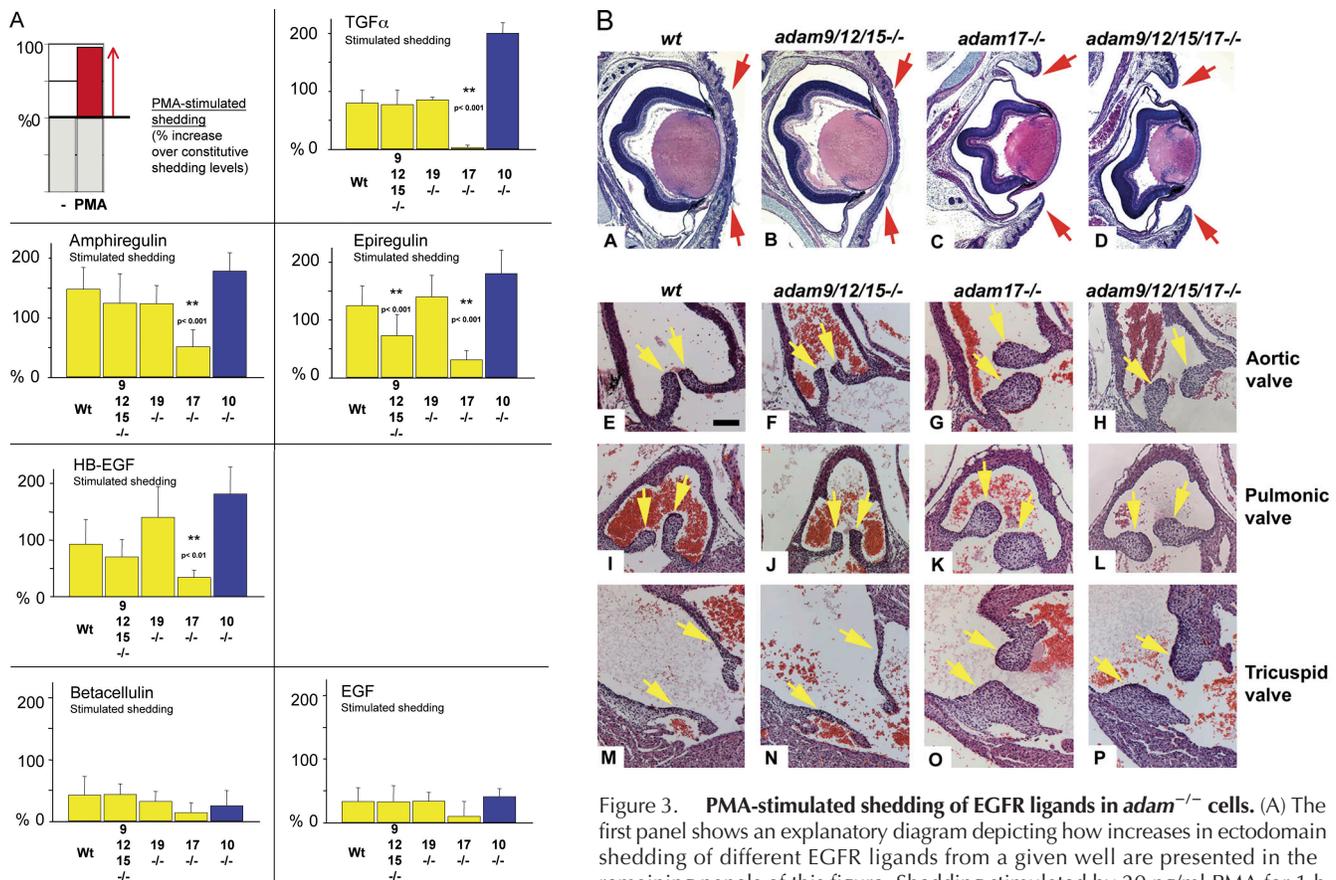
PMA-stimulated shedding of EGFR ligands in *adam*^{-/-} cells

The potential role of different ADAMs as EGFR ligand sheddases was then addressed in *adam*^{-/-} mEFs. When we evalu-

ated PMA-stimulated EGFR ligand shedding in triple knockout *adam9/12/15*^{-/-} cells, no significant decrease in the release of HB-EGF, TGF α , amphiregulin, EGF, or betacellulin compared with wild-type cells was observed (Fig. 3 A). However, there was a statistically significant reduction in stimulated shedding of epiregulin in *adam9/12/15*^{-/-} cells (41.8%), suggesting that one or more of these ADAMs contributes to stimulated epiregulin processing. Similarly, we found no evidence for a major role of ADAM19 in stimulated shedding of the EGFR ligands tested here (Fig. 3 A). The reason for the slight increase in HB-EGF shedding in *adam19*^{-/-} cells compared with wild-type controls remains to be determined.

ADAM17 has been implicated in the shedding of TGF α and HB-EGF in immortalized embryonic fibroblasts and primary keratinocytes (Peschon et al., 1998; Merlos-Suarez et al., 2001; Sunnarborg et al., 2002), and of amphiregulin in primary keratinocytes (Sunnarborg et al., 2002). Consistent with these results, we observed a significant reduction of PMA-induced shedding of TGF α (89%), amphiregulin (65.8%), and HB-EGF (58.1%) in *adam17*^{-/-} compared with wild-type mEFs (Fig. 3 A). Furthermore, shedding of epiregulin was also strongly decreased in *adam17*^{-/-} cells (75.7%), providing the first evidence for a critical role of ADAM17 in stimulated shedding of this EGFR ligand. For TGF α , amphiregulin, and epiregulin, PMA-dependent ectodomain shedding in *adam17*^{-/-} cells was increased when wild-type ADAM17 was cotransfected, confirming that the defect in PMA-dependent shedding is indeed due to loss of ADAM17 (unpublished data).

ADAM10 has been implicated in shedding of HB-EGF as part of a pathway for crosstalk between a GPCR and the EGFR (Lemjabbar and Basbaum, 2002; Yan et al., 2002). As shown in Fig. 3, PMA-stimulated shedding of HB-EGF, TGF α , amphiregulin, and epiregulin is not decreased in *adam10*^{-/-} cells, suggesting that ADAM10 is not required for the PMA-stimulated shedding of these EGFR ligands. The enhanced stimulated shedding of these EGFR ligands



in the same well for 1 h. The next panels only show the PMA-dependent increase in shedding over constitutive levels for each EGFR ligand and each *adam*^{-/-} cell type. Data from primary mEFs (yellow bars) are compiled from separate experiments using cells from three or more litters for each *adam*^{-/-} mouse line. Only *adam*¹⁰^{-/-} and control *adam*¹⁰^{+/-} cells were immortalized (blue bars). Overall, at least four separate wells were evaluated per EGFR ligand. The results indicate that ADAM17 is the major stimulated sheddase for TGF α , amphiregulin, epiregulin, and HB-EGF. ADAMs 9, 12, or 15 (or a combination of two or more of these ADAMs) also contribute to stimulated epiregulin shedding. On the other hand, the shedding of betacellulin and EGF is only weakly stimulated by PMA. Because the increase in stimulated shedding is small, no statistically significant differences in stimulated shedding of betacellulin or EGF was seen in *adam*^{-/-} cells compared with wild-type controls. (B) Histological analysis of sectioned hematoxylin and eosin-stained eyes and eyelids (A–D), aortic valves (E–H), pulmonic valves (I–L), and tricuspid valves (M–P) of newborn wild-type (A, E, I, and M), *adam*^{9/12/15}^{-/-} (B, F, J, and N), *adam*¹⁷^{-/-} (C, G, K, and O), and *adam*^{9/12/15/17}^{-/-} (D, H, L, and P) mice. Eyelids of wild-type and *adam*^{9/12/15}^{-/-} mice are closed at birth (A and B), whereas those of *adam*¹⁷^{-/-} and *adam*^{9/12/15/17}^{-/-} mice are open (C and D). The aortic, pulmonic, and tricuspid valves of *adam*^{9/12/15}^{-/-} mice (F, J, and N) resemble those of wild-type mice (E, I, and M, respectively), whereas these valves are thickened and misshapen in *adam*¹⁷^{-/-} (G, K, and O) and *adam*^{9/12/15/17}^{-/-} quadruple knockout mice (H, L, and P). The valve defects in *adam*^{9/12/15/17}^{-/-} quadruple knockout mice, which also include thickened and misshapen mitral valves (not depicted), are comparable to those in *adam*¹⁷^{-/-} mice. Eyelids in A–D marked by red arrows, heart valves in E–P marked by yellow arrows. Bar (E–P), 100 μ m.

in *adam*¹⁰^{-/-} (Fig. 3 A) and *adam*¹⁰^{+/-} cells (unpublished data) compared with the primary mEFs is presumably a consequence of immortalization.

Generation of *adam*^{9/12/15/17}^{-/-} quadruple knockout mice, and evaluation of PMA-stimulated EGFR ligand shedding in *adam*^{9/12/15/17}^{-/-} cells

Although ADAM17 is essential for the majority of stimulated shedding of HB-EGF in mEF cells, a residual amount of PMA-stimulated HB-EGF shedding is seen in the absence of ADAM17. Could this residual shedding depend on ADAMs 9 or 12, both of which have been implicated in HB-EGF shedding, or on the related ADAM15? To address this issue, we generated quadruple knockout mice lacking ADAMs 9, 12, 15, and 17 (see Materials and methods for

details). Similar to *adam*¹⁷^{-/-} mice, *adam*^{9/12/15/17}^{-/-} quadruple knockout mice that were born had open eyes and died in the first day after birth. The percentage of *adam*^{9/12/15/17}^{-/-} quadruple knockout embryos at E18.5 generated by mating *adam*⁹^{-/-} *adam*¹²^{-/-} *adam*¹⁵^{-/-} *adam*¹⁷^{-/-} parents was somewhat lower than the percentage of *adam*¹⁷^{-/-} embryos at E17.5–18.5 produced by mating *adam*¹⁷^{+/-} mice (Table III; Peschon et al., 1998).

To determine whether the loss of ADAMs 9, 12, and 15 exacerbates known defects in EGFR signaling in *adam*¹⁷^{-/-} mice, we performed a histopathological examination of wild-type, *adam*^{9/12/15}^{-/-}, *adam*¹⁷^{-/-}, and *adam*^{9/12/15/17}^{-/-} E18.5 embryos. As shown in Fig. 3 B (panels A–D), the open-eye phenotype in *adam*¹⁷^{-/-} mice that results from lack of TGF α activation (Peschon et al., 1998) is similar in *adam*^{9/}

Table III. Offspring of matings of *adam9/12/15^{-/-}17^{+/-}* parents

Genotype of E18.5 embryos	Percent
<i>9^{-/-}15^{-/-}12^{-/-}17^{+/+}</i>	36.7 (36)
<i>9^{-/-}15^{-/-}12^{-/-}17^{+/-}</i>	49.0 (48)
<i>9^{-/-}15^{-/-}12^{-/-}17^{-/-}</i>	14.3 (14) [4]
Genotype of E17.5–18.5 embryos	
<i>17^{+/+}</i>	23.3 (23)
<i>17^{+/-}</i>	57.3 (59)
<i>17^{-/-}</i>	20.4 (21) [4]

Genotype of E18.5 embryos from matings of *adam9^{-/-}15^{-/-}12^{-/-}17^{+/-}* parents. Numbers in parentheses, # (total of 98). Genotype of E17.5–18.5 embryos from matings of *adam17^{+/-}* parents, taken from Peschon et al. (1998). Numbers in parentheses, # (total of 103). Nonviable embryos are indicated in brackets.

12/15/17^{-/-} quadruple knockout mice, whereas it is not seen in *adam9/12/15^{-/-}* triple knockout mice. Furthermore, Jackson et al. (2003) have described a defect in morphogenesis of the semilunar heart valves and the tricuspid and mitral valves in *adam17^{-/-}* mice (Fig. 3 B, panels E–P; mitral valve not depicted), which resembles the thickened and misshapen valves seen in *hb-egf^{-/-}* mice and in mice with a knock-in mutation that abolishes HB-EGF shedding (Iwamoto et al., 2003; Yamazaki et al., 2003). As shown in Fig. 3 B, the heart valves in *adam9/12/15^{-/-}* triple knockout mice are indistinguishable from those in wild-type mice, and again, the defects in heart valve morphogenesis in *adam17^{-/-}* mice are comparable to the defects in *adam9/12/15/17^{-/-}* quadruple knockout mice. A morphometric analysis of all heart valves of six *adam9/12/15/17^{-/-}* E18.5 embryos also did not show an increased size

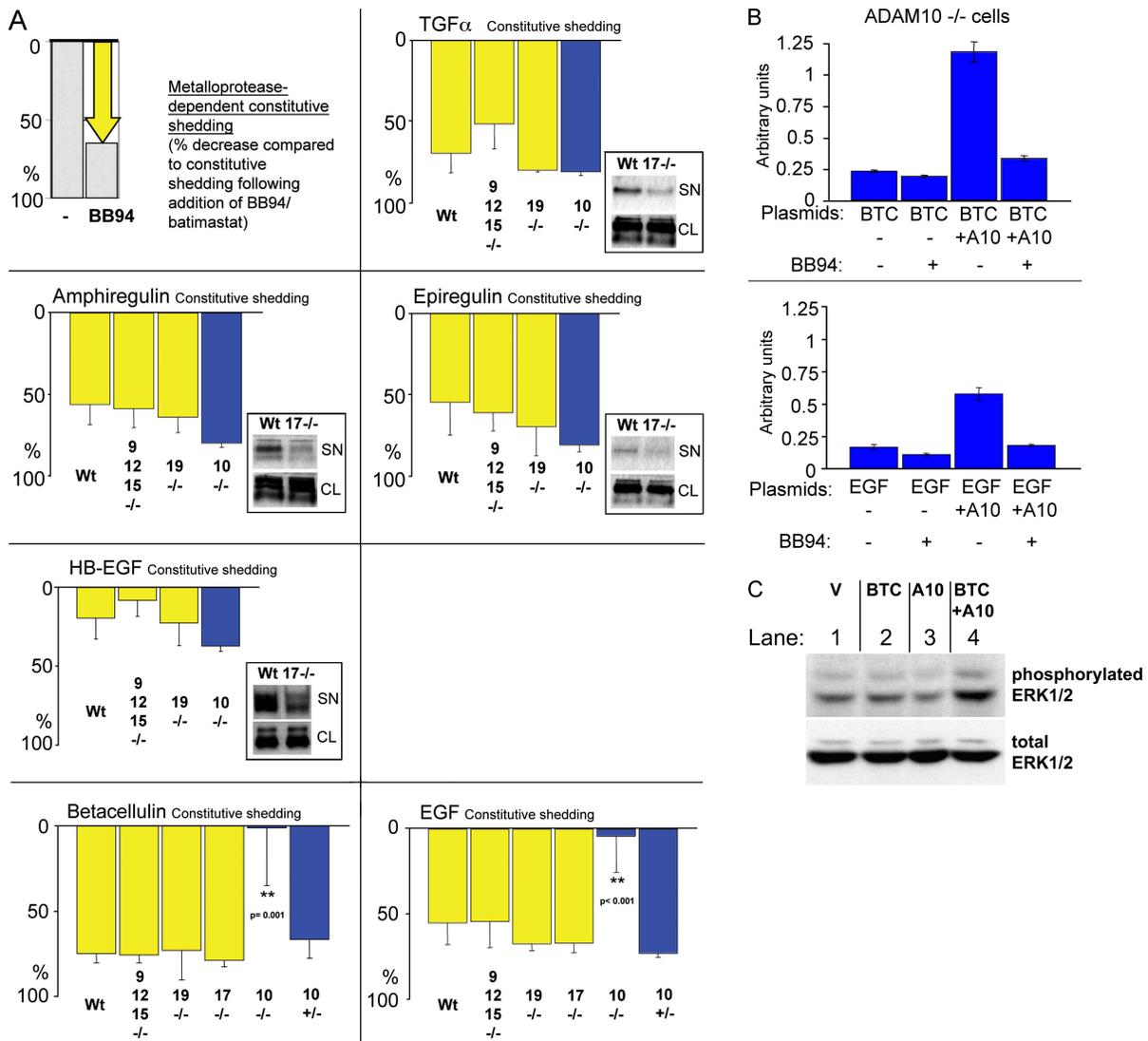


Figure 4. Batimastat-sensitive constitutive shedding of EGFR ligands in *adam^{-/-}* cells. (A) A diagram indicating how the batimastat-sensitive component of ectodomain shedding of different EGFR ligands from a given well of resting cells was determined. Shedding of EGFR ligands in 1 h from resting cells is used as a reference to determine the percentage of batimastat-sensitive constitutive shedding (percent decrease after addition of batimastat). The next panels show the batimastat-sensitive decrease in shedding of each EGFR ligand in each *adam^{-/-}* cell type. As in Fig. 3, separate experiments were performed with cells from three or more litters for each *adam^{-/-}* mouse line. At least six separate wells were analyzed for *adam10^{-/-}* and control *adam10^{+/-}* cells, which were immortalized (blue bars). Several wells were evaluated in each experiment for each lot of cells and for each EGFR ligand. The results show that ADAMs 9, 10, 12, 15, and 19 are not essential for the batimastat-

compared with six *adam17^{-/-}* E18.5 embryos (unpublished data). When we performed shedding experiments with *adam9/12/15/17^{-/-}* quadruple knockout mEFs, the residual amount of PMA-stimulated HB-EGF shedding was comparable to what is observed in *adam17^{-/-}* cells (percent increase in HB-EGF shedding after PMA stimulation in *adam9/12/15/17^{-/-}* mEF: $33.3 \pm 26.5\%$, $n = 16$; four embryos, 2–6 wells analyzed per embryo); compared with $33.7 \pm 12.9\%$ in *adam17^{-/-}* mEF; Fig. 3 A). Together, these results argue against a significant contribution of ADAMs 9, 12, or 15 to the shedding of HB-EGF in these cells.

Constitutive shedding of EGFR ligands in *adam^{-/-}* cells

Next, we evaluated the batimastat-sensitive component of constitutive shedding of EGFR ligands in the presence or absence of different ADAMs. No significant difference in the batimastat-sensitive constitutive shedding of all six ligands tested here was observed in *adam9/12/15^{-/-}* or *adam19^{-/-}* cells compared with wild-type controls (Fig. 4 A). Furthermore, constitutive shedding of TGF α , amphiregulin, epiregulin, and HB-EGF was also not affected in *adam10^{-/-}* cells.

Because the shedding assay used here relies on measurements of the percent increase (see above) or decrease in shedding after treatment compared with unstimulated shedding, it is important to ensure that the levels of unstimulated shedding are indeed similar in different *adam^{-/-}* and wild-type cells. In experiments where wild-type cells were evaluated simultaneously with *adam9/12/15^{-/-}* or *adam19^{-/-}* cells, comparable amounts of each EGFR ligand analyzed here were released from unstimulated cells (unpublished data). However, the overall levels of constitutive shedding for TGF α , amphiregulin, epiregulin, and HB-EGF (but not EGF and betacellulin) were reduced in *adam17^{-/-}* cells compared with wild-type controls (Fig. 4 A, insets; unpublished data). This demonstrates that ADAM17 has a key role in both stimulated and constitutive shedding of these EGFR ligands.

Interestingly, when we measured the batimastat-sensitive constitutive shedding of EGF and betacellulin, we found that it was abrogated in two independent *adam10^{-/-}* cell lines compared with control *adam10^{+/-}* cells and the primary mEF cells (Fig. 4 A; unpublished data). This resulted in a strong decrease in overall unstimulated constitutive shedding of betacellulin (87.5%) and EGF (49.7%) from *adam10^{-/-}*

sensitive constitutive shedding of TGF α , amphiregulin, epiregulin, and HB-EGF. The absolute levels of constitutive release of each EGFR ligand were comparable between wild-type, *adam9/12/15^{-/-}*, and *adam19^{-/-}* cells (not depicted). However, the levels of constitutive shedding of TGF α , amphiregulin, epiregulin, and HB-EGF were significantly reduced in *adam17^{-/-}* cells compared with wild-type controls (see inset gel figures; SN, supernatant; CL, cell lysate), indicating that ADAM17 is also the major constitutive sheddase for these ligands. Data on the batimastat-sensitive shedding in *adam17^{-/-}* cells are not included in the graph because addition of batimastat further decreases the small amount of constitutive shedding in *adam17^{-/-}* cells. Thus, another metalloprotease besides ADAM17 apparently makes a very minor contribution to shedding of these four substrates. The batimastat-sensitive shedding of betacellulin and EGF was very similar in *adam9/12/15^{-/-}*, *adam19^{-/-}*, *adam17^{-/-}*, and *adam10^{+/-}* mEFs compared with wild-type controls. Although the absolute levels of constitutive shedding from these cells were also very similar (not depicted), constitutive shedding of betacellulin from *adam10^{-/-}* cells was decreased by 87.5%, whereas shedding of EGF was decreased by 49.7% compared with *adam10^{+/-}* cells. In the absence of ADAM10, the remaining small amount of constitutive shedding was not inhibitable by batimastat. (B) Batimastat-sensitive shedding of betacellulin (BTC) and EGF from *adam10^{-/-}* cells can be rescued by cotransfection with wild-type ADAM10 (A10; each bar represents the results from six tissue culture wells). These results confirm that the defect in EGF and betacellulin shedding in *adam10^{-/-}* cells is indeed due to the absence of ADAM10. (C) Constitutive phosphorylation of ERK1/2 in *adam10^{-/-}* cells transfected with the pcDNA3 vector (V, lane 1) is not increased by transfection with BTC (lane 2) or ADAM10 (lane 3). However, ERK1/2 phosphorylation is increased when BTC and ADAM10 are cotransfected (lane 4), demonstrating that ADAM10 is critical for BTC-dependent EGFR signaling in these cells. The bottom panel shows the same blot reprobed with antibodies against total ERK1/2 to confirm equal loading in all lanes.

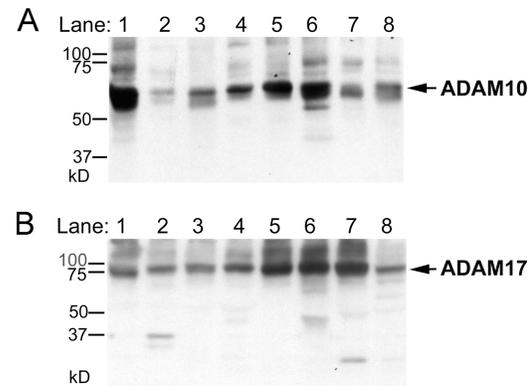


Figure 5. Evaluation of ADAM10 and ADAM17 protein levels in different mouse tissues. Western blots of mouse tissue extracts were probed with a polyclonal antiserum against ADAM10 (A) or ADAM17 (B). Equal amounts of Con A–enriched glycoproteins from the following tissues were loaded per lane: brain (lane 1), skeletal muscle (lane 2), kidney (lane 3), heart (lane 4), lung (lane 5), spleen (lane 6), testis (lane 7), and liver (lane 8). The arrow indicates the position of ADAM10 in A, and of ADAM17 in B.

cells compared with *adam10^{+/-}* cells (unpublished data). Constitutive shedding of betacellulin and EGF could be rescued with wild-type ADAM10, confirming that the defect in shedding in *adam10^{-/-}* cells is indeed due to the lack of ADAM10 (Fig. 4 B). Next, we evaluated the role of ADAM10 in betacellulin-dependent EGFR signaling in *adam10^{-/-}* cells. When either ADAM10 or betacellulin were introduced in *adam10^{-/-}* cells, there was no increase in phosphorylation of ERK1/2, a commonly used indicator for activation of the EGFR (Fig. 4 C). However, when wild-type ADAM10 was cotransfected with betacellulin in *adam10^{-/-}* cells, ERK1/2 phosphorylation was increased (Fig. 4 C). Thus, EGFR signaling via transfected betacellulin depends on the presence of functional ADAM10 in these cells. Together, these results are the first to identify the major sheddase for EGF and betacellulin in mouse embryonic cells, and thus also to uncover two novel substrates for ADAM10.

To address whether the results obtained in mEF cells could in principle also be relevant for other cells and tissues, Western blot analysis of the expression of ADAMs 10 and 17 in different mouse tissues was performed (Fig. 5). This

confirmed that both ADAMs are widely expressed, even though their expression levels vary. Thus, it is likely that both ADAMs 10 and 17 are expressed in the cells and tissues in which the ligands analyzed in this paper exert their function as activators of EGFR signaling.

Discussion

Protein ectodomain shedding of EGFR ligands can be critical for their functional activation. All EGFR ligands analyzed in this paper are synthesized as membrane-anchored precursors, and were initially identified as soluble biologically active growth factors (Cohen, 1962, 1965; de Larco and Todaro, 1978; Todaro et al., 1980; Shoyab et al., 1989; Higashiyama et al., 1991; Shing et al., 1993; Toyoda et al., 1995). A key step in elucidating the mechanism underlying the proteolytic release of EGFR ligands is the identification of the responsible sheddases. Although ADAM17 has been implicated in the shedding of TGF α , HB-EGF, and amphiregulin (Merlos-Suarez et al., 2001; Sunnarborg et al., 2002; Jackson et al., 2003), no information was previously available about the identity of the sheddases for epiregulin, EGF, and betacellulin. Furthermore, three other ADAMs (9, 10, and 12) had been implicated in HB-EGF shedding (Izumi et al., 1998; Asakura et al., 2002; Lemjabbar and Basbaum, 2002), raising questions about their individual contributions to HB-EGF release. To test the hypothesis that the sheddases for epiregulin, EGF, and betacellulin are also ADAMs, and to further evaluate the contribution of different ADAMs to HB-EGF shedding, we studied the release of these proteins from cells lacking one or more members of this family of metalloproteases. Moreover, even though ADAM17 has been linked to the shedding of TGF α and amphiregulin, we included these EGFR ligands in our paper both to investigate whether other ADAMs may participate in the shedding of these two proteins and as a positive control to validate the assay used here. Finally, we determined the effects of targeted deletions of up to four ADAMs that are candidate sheddases (ADAMs 9, 12, 15, and 17) on mouse development.

Ectodomain shedding experiments using these six major EGFR ligands in *adam*^{-/-} MEFs corroborated previous reports that ADAM17 has a major role in the shedding of TGF α , HB-EGF, and amphiregulin (Peschon et al., 1998; Merlos-Suarez et al., 2001; Sunnarborg et al., 2002). We found no evidence for a major contribution of other ADAMs besides ADAM17 to TGF α , HB-EGF, and amphiregulin shedding in these cells. Furthermore, epiregulin was identified as a novel ADAM17 substrate. Previous works have shown that *adam17*^{-/-} mice resemble *tgfa*^{-/-} mice (Peschon et al., 1998) in that they have open eyes at birth, as well as displaying similar vibrissae, hair, and skin defects (Mann et al., 1993). Furthermore, *adam17*^{-/-} mice also resemble *hb-egf*^{-/-} mice (Iwamoto et al., 2003) in that they have thickened aortic and pulmonic valves (Jackson et al., 2003). A similar phenotype is seen in mice with a mutation in the cleavage site of HB-EGF that abolishes its shedding (Yamazaki et al., 2003). Finally, the phenotype of *adam17*^{-/-} mice resembles that of *egfr*^{-/-} mice (Miettinen et al., 1995; Sibilina and Wagner, 1995; Threadgill et al., 1995; Peschon et al., 1998). Thus, genetic experiments have substan-

tiated that ADAM17 is also essential for the activation of EGFR ligands in vivo. It remains to be determined whether the lack of processing of amphiregulin or epiregulin (or both) also contributes to the phenotype of *adam17*^{-/-} mice.

ADAMs 9, 10, and 12 are also considered candidate HB-EGF sheddases (Izumi et al., 1998; Asakura et al., 2002; Kurisaki et al., 2003). However, although PMA-stimulated ectodomain shedding of HB-EGF was somewhat reduced in *adam9/12/15*^{-/-} cells, this reduction was not statistically significant. In addition, the residual PMA stimulation of HB-EGF shedding in *adam17*^{-/-} cells is most likely also not due to ADAMs 9, 12, or 15 because it remains unchanged in *adam9/12/15/17*^{-/-} quadruple knockout cells. In a previous paper, Kurisaki et al. (2003) reported a significant reduction in the down-regulation of cell-associated HB-EGF in phorbol ester-stimulated *adam12*^{-/-} cells compared with wild-type controls. This apparent discrepancy may be due to differences in cell preparation or experimental design. Nevertheless, the main conclusion from the side-by-side comparison of different *adam*^{-/-} cells isolated and cultured under identical conditions in this paper is that ADAM17 is the predominant PMA-stimulated HB-EGF sheddase in primary MEF cells.

The conclusion that ADAM17 has a critical role in shedding HB-EGF in vivo was further corroborated by an analysis of the role of these ADAMs during mouse development. As mentioned previously in this paper, *adam17*^{-/-} mice resemble *egfr*^{-/-}, *tgfa*^{-/-}, or *hb-egf*^{-/-} mice, whereas no similar defects were seen in *adam9/12/15*^{-/-} mice. Furthermore, the phenotype of *adam17*^{-/-} mice does not appear to be considerably exacerbated when ADAMs 9, 12, and 15 are also deleted. Together, these findings argue against major compensatory or redundant roles for ADAMs 9, 12, and the related ADAM 15 in the activation of TGF α , HB-EGF, or the EGFR during development. However, it cannot be ruled out that ADAMs 9, 12, or 15 contribute to shedding of EGFR ligands in cells or tissues where these enzymes and potential substrates are highly expressed. Further analyses will address which ADAMs are capable of cleaving EGFR ligands when overexpressed, and in which tissues candidate EGFR ligand sheddases besides ADAMs 10 and 17 are highly expressed together with EGFR ligands that they can cleave.

ADAM10 has also been implicated in HB-EGF shedding as part of a pathway that involves crosstalk between GPCRs and the EGFR (Lemjabbar and Basbaum, 2002; Yan et al., 2002; Lemjabbar et al., 2003). On the other hand, our results indicate that ADAM10 does not make a major contribution to PMA-stimulated or constitutive shedding of HB-EGF in the cells tested here. This is consistent with the notion that different stimuli may activate different ADAMs, such that HB-EGF shedding depends mainly on ADAM17 under the conditions used here, and mainly on ADAM10 when the appropriate GPCR is stimulated.

Little was previously known about the sheddases responsible for the release of EGF and betacellulin from cells. Here, we show that constitutive shedding of both EGF and betacellulin was strongly reduced in *adam10*^{-/-} cells compared with heterozygous controls, and could be rescued by reintroduction of wild-type ADAM10. Furthermore, stimulation of the EGFR by transfected betacellulin in *adam10*^{-/-} cells is only seen when these cells are rescued by cotransfection

with wild-type ADAM10. These results are the first to identify ADAM10 as the major sheddase for these two crucial EGFR ligands in mouse cells. Because ADAM10 is widely expressed, it is tempting to speculate that it may participate in the functional regulation of these two EGFR ligands in development and in diseases such as cancer.

In light of the genetic evidence for a key role of ADAM17 in activation of EGFR ligands in mice (Peschon et al., 1998; Sunnarborg et al., 2002; Jackson et al., 2003), it is surprising that no ADAM has been identified as an essential part of the EGFR pathway in *Drosophila* (Lee et al., 2001; Urban et al., 2001; Ghigliione et al., 2002; Tsuruya et al., 2002; Shilo, 2003). Instead, Rhomboids (integral membrane proteins with seven membrane-spanning domains) have been implicated in cleaving EGFR ligands (Urban et al., 2002), whereas reducing the expression of a putative ADAM17 orthologue in *Drosophila* via small interfering RNA did not block development of EGFR-dependent structures (Lee et al., 2001).

These results suggest that there are critical differences in the mechanism underlying proteolytic activation of EGFR ligands between flies and mice. However, the finding that all EGFR ligands tested here are processed by ADAM10 or ADAM17 in mEFs suggests a possible alternative explanation for these findings. *Drosophila* carry orthologues of ADAM10 (KUZ) and ADAM17 (AAF56986, the ADAM targeted by RNA interference in Lee et al. [2001]), as well as a third ADAM related to ADAM17 and KUZ with no evident orthologue in mammals (AAF56926). It is conceivable that two or three of these ADAMs fulfill redundant or compensatory roles in activation of EGFR ligands during development in *Drosophila*. This may only become apparent once two or three of these putative EGFR ligand sheddases are simultaneously inactivated. Conversely, the results in *Drosophila* suggest that it will be worthwhile to further investigate the potential role of Rhomboids and intramembrane proteolysis in EGFR ligand activation in mammals.

In summary, we report the first systematic analysis of the shedding of EGFR ligands in cells lacking one or more widely expressed and catalytically active ADAM. Our results uncover critical roles for both ADAM10 and ADAM17 in shedding of EGFR ligands in mEF cells. ADAM17 emerged as the major PMA-stimulated and constitutive sheddase of TGF α , amphiregulin, HB-EGF, and epiregulin, which is consistent with the essential role for ADAM17 in activation of the EGFR during development. Furthermore, ADAM10 was found to be the major batimastat-sensitive sheddase for betacellulin and EGF in mEF. Further experiments, including the generation of conditional *adam10*^{-/-} knockout mice, as well as knock-in mutations that abolish shedding of EGF and betacellulin, will be necessary to address the biological relevance of ADAM10 in shedding the endogenous forms of these EGFR ligands in vivo. The identification of different EGFR ligands as substrates for ADAM10 and ADAM17 sets the stage for the further analysis of how these ADAMs are regulated and how their substrate specificity is achieved. Because proteolysis of EGFR ligands may be critical for their functional activation, and signaling via the EGFR has been implicated in diseases such as cancer, ADAM10 and ADAM17 may be attractive targets for the design of drugs that modulate the action of these ligands.

Materials and methods

Generation of *adam9/15*^{-/-}, *adam9/12/15*^{-/-}, and *adam9/12/15/17*^{-/-} knockout mice

Mice lacking ADAMs 9, 12, or 15 have been described previously (Weskamp et al., 2002; Horiuchi et al., 2003; Kurisaki et al., 2003). To generate *adam9/15*^{-/-} double knockout mice, we mated *adam9*^{+/-} *15*^{+/-} doubly heterozygous parents. This produced offspring in the expected Mendelian ratio (Table I). *adam9/15*^{-/-} double knockout mice were then mated with *adam12*^{-/-} mice (provided by Dr. Fujisawa-Sehara, University of Kyoto, Kyoto, Japan) to produce *adam9*^{+/-} *12*^{+/-} *15*^{+/-} triple heterozygous parents. These were backcrossed with *adam9/15*^{-/-} mice to generate *adam9/15*^{-/-} *12*^{+/-} animals. When *adam9/15*^{-/-} *12*^{+/-} mice were crossed, the ratio of offspring was Mendelian with respect to the mutant ADAM12 allele (Table II). Genotyping was performed by Southern blot as described previously (Weskamp et al., 2002; Horiuchi et al., 2003; Kurisaki et al., 2003). All animals used in this work were of mixed genetic background (129Sv/C57Bl6).

The histopathological analysis of *adam9/15*^{-/-} mice was performed as described previously for *adam9*^{-/-} or *adam15*^{-/-} mice (Weskamp et al., 2002; Horiuchi et al., 2003). Histopathological analysis of *adam9/12/15*^{-/-} mice was performed by the Memorial Sloan-Kettering Cancer Center mouse phenotyping core. No abnormalities or pathological phenotypes were observed in *adam9/15*^{-/-} and *adam9/12/15*^{-/-} mice. Serial sections of tissues affected in *egfr*^{-/-}, *hb-egf*^{-/-}, and *tgfa*^{-/-} mice did not uncover any evident defects in *adam9/12/15*^{-/-} mice. Specifically, there were no defects in the development of the heart or its valves (Fig. 3 B, panels E–P), and also no defects in epithelia, intestine, lung, or in hair development. Finally, *adam9/12*^{-/-} double knockout and *adam9/12/15*^{-/-} triple knockout mice were indistinguishable from wild-type controls in their appearance and behavior during routine handling.

To generate *adam9/12/15/17*^{-/-} quadruple knockout mice, *adam9/12/15*^{-/-} triple knockout mice were mated with *adam17*^{+/-} animals. Offspring from this mating that were heterozygous for the targeted allele of all four ADAMs were identified by Southern blotting, and were backcrossed several times with *adam9/12/15*^{-/-} triple knockout mice to obtain *adam9*^{-/-} *12*^{-/-} *15*^{-/-} *17*^{+/-} mice. Crosses of *adam9*^{-/-} *12*^{-/-} *15*^{-/-} *17*^{+/-} mice produced litters with a similar distribution of the targeted ADAM17 allele at E18.5 to what has been reported from crosses of *adam17*^{+/-} mice (Table III; Peschon et al., 1998). Histopathological evaluation of newborn *adam9/12/15/17*^{-/-} quadruple knockout mice did not uncover any significant worsening of the developmental defects described for *adam17*^{-/-} mice. The cause for the slightly increased embryonic lethality of *adam9/12/15/17*^{-/-} mice compared with *adam17*^{-/-} mice (Table III) remains to be determined. Images of fixed and hematoxylin and eosin-stained heart sections mounted in Permout/Histoclear were acquired with Axiovision software via an Axiocam HRC camera mounted on an Axioplan2 microscope (software, camera, and microscope all from Carl Zeiss MicroImaging, Inc.). The objective was a Plan-Neofluar 10 \times /0.30 (44 03 30; Carl Zeiss MicroImaging, Inc.) lens. Images were processed with Adobe Photoshop[®] 7.0, and the surface area of heart valves in serial sections was measured using NIH Image 1.63 software.

Expression vectors for AP-tagged EGFR ligands

Plasmids encoding AP-tagged EGFR ligands were constructed by inserting partial cDNAs for human TGF α , amphiregulin, epiregulin, EGF, betacellulin, and HB-EGF into the 3' end of human placental AP cDNA on a pRc/CMV-based expression vector pAlPh. pAlPh contains an NH₂-terminally located HB-EGF signal sequence. In all cases, the junction between AP and the EGFR ligand was placed next to the membrane-proximal EGF repeat. Release of the AP module into the culture supernatant thus requires cleavage at the COOH-terminal cleavage site. It should be noted that the results obtained with AP-tagged EGFR ligands corroborate previous results that TGF α , HB-EGF, and amphiregulin are cleaved by ADAM17 (Peschon et al., 1998; Merlos-Suarez et al., 2001; Sunnarborg et al., 2002; Jackson et al., 2003). This validates the use of AP tags to measure ectodomain shedding for these substrates. In addition, the use of an AP tag has been independently validated using the TNF family members TNF α and TRANCE/OPGL (Zheng et al., 2002; Chesneau et al., 2003). This strongly suggests that an AP tag, which provides a sensitive and quantitative means of measuring ectodomain shedding, should not interfere with the shedding properties of the other EGFR ligands tested here.

Generation of primary MEFs

Primary MEFs were generated from wild-type or *adam*^{-/-} E13.5 embryos and were cultured as described previously (Weskamp et al., 2002). In ad-

dition to *adam9/12/15*^{-/-} triple knockout and *adam9/12/15/17*^{-/-} quadruple knockout mice, we also used *adam17*^{-/-} (Peschon et al., 1998) and *adam19*^{-/-} (Zhou et al., 2004) mice as well as wild-type controls of mixed genetic background (129Sv/C57Bl6) to generate the corresponding primary mEF cells. All genotyping was performed by Southern blot analysis. *adam10*^{-/-} fibroblast cell lines derived from E9.5 embryos have been described previously (Hartmann et al., 2002).

Northern blot analyses

Procedures for isolation of mRNA, gel electrophoresis, transfer to membranes, and generation of ³²P-labeled cDNA probes of the indicated ADAMs under high stringency were described previously (Weskamp and Blobel, 1994).

Transfections and shedding assays

cDNA constructs encoding AP-EGFR ligand fusion proteins were transfected with LipofectAMINE™ (Invitrogen). Fresh Opti-MEM (Invitrogen) medium was added the next day, incubated for 1 h, and then replaced with fresh medium containing either 20 ng/ml PMA or 1 μM batimastat (provided by D. Becherer, GlaxoSmithKline, Research Triangle Park, NC), which was also collected after 1 h. Evaluation of AP activity by SDS-PAGE or by colorimetric assays was performed as described previously (Zheng et al., 2002). No AP activity was present in conditioned media of nontransfected cells.

Western blot analysis

Western blot analysis of the expression of ADAM10 and ADAM17 in MEFs and in different mouse tissues was performed as described previously (Weskamp et al., 1996). The blots were probed with a polyclonal antiserum against ADAM10 (CHEMICON International) and against ADAM17 (Schlönörf et al., 2000).

Statistical analyses

t tests for two samples assuming equal variances were used to calculate the *P* values. *P* values <0.05 were considered statistically significant.

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