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ADAMs 10 and 17 Represent Differentially Regulated Components of a General Shedding Machinery for Membrane Proteins Such as Transforming Growth Factor α, L-Selectin, and Tumor Necrosis Factor α

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Protein ectodomain shedding is a critical regulator of many membrane proteins, including epidermal growth factor receptor-ligands and tumor necrosis factor (TNF)-α, providing a strong incentive to define the responsible sheddases. Previous studies identified ADAM17 as principal sheddase for transforming growth factor (TGF)-α and heparin-binding epidermal growth factor, but Ca^{++} influx activated an additional sheddase for these epidermal growth factor receptor ligands in *Adam17*−/− cells. Here, we show that Ca^{++} influx and stimulation of the P2X7R signaling pathway activate ADAM10 as sheddase of many ADAM17 substrates in *Adam17*−/− fibroblasts and primary B cells. Importantly, although ADAM10 can shed all substrates of ADAM17 tested here in *Adam17*−/− cells, acute treatment of wild-type cells with a highly selective ADAM17 inhibitor (SP26) showed that ADAM17 is nevertheless the principal sheddase when both ADAMs 10 and 17 are present. However, chronic treatment of wild-type cells with SP26 promoted processing of ADAM17 substrates by ADAM10, thus generating conditions such as in *Adam17*−/− cells. These results have general implications for understanding the substrate selectivity of two major cellular sheddases, ADAMs 10 and 17.

INTRODUCTION

All ligands of the epidermal growth factor receptor (EGFR) are made as membrane-anchored precursors, which must be released from their membrane tether to activate the EGFR in a paracrine manner (for reviews, see Harris *et al*., 2003; Blobel, 2005). The EGFR has critical roles in development and in diseases such as cancer (Yarden and Sliwkowski, 2001; Gschwind *et al*., 2004), so it is important to understand how ligand availability is regulated and which enzymes are responsible for releasing EGFR ligands under various conditions. Moreover, many other membrane proteins are proteolytically released or shed from cells, including other growth factors, cytokines, and receptors (Hooper *et al*., 1997). Because the sheddases for other membrane proteins commonly have similar properties as EGFR ligand sheddases (e.g., Zheng *et al*., 2004; Weskamp *et al*., 2006; Kawaguchi *et al*., 2007), insight about the identity and properties of sheddases gained from studying shedding of EGFR ligands usually has general relevance, because they can provide a framework for understanding which enzymes are responsible for shedding of other membrane proteins, and how these enzymes are regulated.

Two membrane-anchored metalloproteinases, ADAMs 10 and 17, have emerged as key molecules in most of the shedding events characterized to date (for recent examples, see Weskamp *et al*., 2006; Chen *et al*., 2007; Kawaguchi *et al*., 2007; Li *et al*., 2007), including in the release of EGFR ligands (Peschon *et al*., 1998; Jackson *et al*., 2003; Hinkle *et al*., 2004; Sahin *et al*., 2004; Horiuchi *et al*., 2007b; Sahin and Blobel, 2007). Studies on the contribution of these two enzymes to constitutive and phorbol ester-stimulated EGFR ligand shedding have revealed a distinct substrate selectivity of ADAM10 and ADAM17 and also defined characteristic properties of these enzymes (Sahin *et al*., 2004; Horiuchi *et al*., 2007b). "Loss of function" studies with cells lacking different ADAMs demonstrated that ADAM17 is the principal constitutive and phorbol ester-stimulated sheddase for transforming growth factor (TGF)-α, amphiregulin (AR), epiregulin (EPR), epigen, and heparin-binding epidermal

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growth factor (HB-EGF) (Peschon *et al*., 1998; Jackson *et al*., 2003; Sahin *et al*., 2004; Sahin and Blobel, 2007). The physiological relevance of this enzyme for activation of these EGFR ligands during development was corroborated through studies of mice lacking ADAM17, which resemble mice lacking TGF-α, HB-EGF, AR, or EGFR (Peschon *et al*., 1998; Jackson *et al*., 2003; Sternlicht *et al*., 2005). ADAM10, in contrast, is responsible for the constitutive shedding of EGF and betacellulin (BTC), and its activity toward these substrates is not strongly enhanced by stimulation with phorbol 12-myristate 13-acetate (PMA) but instead can be stimulated by calcium influx (Sahin *et al*., 2004; Horiuchi *et al*., 2007b). Mice lacking ADAM10 die very early during embryogenesis, most likely due to defects in Notch signaling (Hartmann *et al*., 2002), so the contribution of ADAM10 to EGF and BTC signaling in development and adult animals remains to be determined. Nevertheless, inhibitors that are highly selective for ADAM10 over ADAM17 also block shedding of EGF in cell-based assays (Zhou *et al*., 2006), further supporting the notion that ADAM10 is critical for this process. Evaluating the role of these ADAMs in EGFR ligand shedding has thus provided insight into their regulation and defined characteristic properties of these major sheddases.

In light of previous loss of function studies, which demonstrated that ADAM17 is required for the constitutive and PMA stimulated shedding of TGF-α, AR, EPR and HB-EGF (Peschon *et al*., 1998; Jackson *et al*., 2003; Sahin *et al*., 2004), it was unexpected to find an activity in *Adam17−/−* cells, which was able to efficiently process these four EGFR-ligands upon stimulation with the calcium ionophore ionomycin (IM) (Horiuchi *et al*., 2007b). The critical role of shedding for activating EGFR-ligands prompted us to define the IM-stimulated sheddase for these proteins in *Adam17−/−* cells. Using several different activators and inhibitors of ectodomain shedding, we identified ADAM10 as the enzyme that can shed many substrates of ADAM17 in *Adam17−/−* cells. This observation raised the critical question about the physiological relevance of these two ADAMs in shedding various substrates in cells expressing both ADAM10 and 17. We therefore addressed whether both enzymes have largely equivalent or redundant functions, or whether one is the principal sheddase for substrates that can, in principle, be cleaved by both enzymes. Finally, we were interested in learning about the implications of our results for the development of ADAM17-selective pharmacological inhibitors, and whether ADAM10 can process substrates of ADAM17 in the presence of such inhibitors.

MATERIALS AND METHODS

Cell Lines and Reagents

Adam17−/− and *Adam10−/−* mouse embryonic fibroblasts (mEFs) were from embryonic day (E)13.5 and E9.5 embryos, respectively (Reddy *et al*., 2000; Hartmann *et al*., 2002). *Adam10/17−/−* mEFs were from Reiss and Saftig (unpublished data), and A431 and Chinese hamster ovary (CHO) cells were from American Type Culture Collection (Manassas, VA). CHO cells were grown in DMEM/F-12; all other cell lines were grown in DMEM, with antibiotics and 5% fetal calf serum (FCS), or with 10% FCS plus high glucose for A431 cells. All reagents were from Sigma-Aldrich unless otherwise indicated. Ionomycin was from Calbiochem (San Diego, CA). The hydroxamate inhibitor GI254023X (GI; 10-fold selective for ADAM10 over ADAM17; Hundhausen *et al*., 2003; Weskamp *et al*., 2006) was from David Becherer (GlaxoSmithKline, Research Triangle Park, NC), and marimastat was from Ouathek Ouerfelli (Sloan-Kettering Institute, New York, NY). The ADAM17-selective inhibitor SP26 (Mazzola *et al*., 2008) was from Schering Plough (Kenilworth, NJ). Anti-Phospho-extracellular signal-regulated kinase (ERK) were from Cell Signaling Technology (Beverly, MA), anti-ERK2 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-human CD23 (30X) was described previously (Weskamp *et al*., 2006). Tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2 were from Dr. G. Murphy (University of Cambridge, Cambridge, United Kingdom), Calbiochem, or R&D Systems (Minneapolis, MN). TIMP-3 was from Dr. R. Black (Amgen, Seattle, WA). Antibodies for flow cytometry were from BD Biosciences Pharmingen, (San Diego, CA).

Expression Vectors

The expression vectors for ADAMs 10 and 17, for alkaline phosphatase (AP)-tagged proteins, and for human CD23 have been described previously (Sahin *et al*., 2004; Weskamp *et al*., 2006; Horiuchi *et al*., 2007b). The human P2X7R cDNA (ATCC no. 10658792) was subcloned into pcDNA3.1-Zeo+ (Invitrogen, Carlsbad, CA). The pEF expression plasmid for dominant-negative (DN) ADAM10 lacking its metalloprotease domain consists of the leader sequence and part of the prodomain linked to the disintegrin, transmembrane, and cytoplasmic domains, as described previously (Pan and Rubin, 1997). An inactive ADAM9E>A was used as control for ADAM10-DN experiments. For expression of short hairpin RNA (shRNA), the mouse ADAM10 sequence (5′-GACAGUUCAACCUACGAAU-3′) followed by a nine nucleotide noncomplementary spacer (TCTCTTGAA) and the reverse complement sequence were inserted into the pSUPER vector (provided by Dr. T. Brummelkamp, Whitehead Institute, Cambridge, MA) after digestion with BglII and HindIII.

Cell Culture, Transfection, and Ectodomain Shedding Assays

Fibroblasts were transiently transfected with Lipofectamine 2000 and CHO cells with Lipofectamine and the indicated plasmids as described previously (Sahin *et al*., 2004; Zheng *et al*., 2004). Shedding assays were performed the day after transfection, except for shRNA and ADAM10-DN experiments, which were done 3 d after transfection. Chronic inactivation with 3 μM SP26 was for 2 d, with one change of medium, followed by a typical shedding experiment. For individual shedding experiments, cells were washed with DMEM, which was replaced after 1 h by fresh DMEM with or without activators or inhibitors of shedding, and then cells were incubated for 30 min to 4 h as indicated previously (Sahin *et al*., 2004). AP activity in the supernatant and cell lysates was measured by colorimetry (Sahin *et al*., 2004; Zheng *et al*., 2004). The ratio between the AP activity in the supernatant and the total AP activity in the cell lysate plus supernatant was calculated from two identically prepared wells, and averaged. The ratio reflects the activity of a given sheddase toward a given AP-tagged protein. CD23 shedding assays were performed in Opti-MEM, and the same wells were used to collect supernatants after 1 h of constitutive shedding and 1 h of stimulated shedding, as described previously (Weskamp *et al*., 2006).

Generation of $CD19^{Cre/+}Adam17^{flox/flox}$, Mmp7−/−Adam17−/− Mice and mEF Cells

Cd19(Cre/Cre) mice (C.Cg-$Cd19^{tm1(cre)Cgn}$ Igh^{b}/J, The Jackson Laboratory, Bar Harbor, ME) were crossed with $Adam17^{flox/flox}$ mice (Horiuchi *et al*., 2007a) to generate $Cd19^{Cre/+}Adam17^{flox/flox}$ mice. These were crossed with $Adam17^{flox/flox}$ mice to produce littermates that were $Adam17^{flox/flox}$ and either wild-type or $Cd19^{Cre/+}$ at the *Cd19* locus.

Mmp7−/− mice (B6.129-$Mmp7^{tm1Lmm}$/J; Jackson labs, Bar Harbor, ME) were crossed with *Adam17+/−* mice (Horiuchi *et al*., 2007a) to produce *Mmp7−/− Adam17+/−* animals, which were viable and fertile. These were bred to obtain *Mmp7−/−Adam17−/−* double knock-out mice, which were born with the expected Mendelian ratio (*Mmp7−/−Adam17−/−*: 23.3%; *Mmp7−/− Adam17+/−*: 50.0%; *Mmp7−/−Adam17+/+*: 26.7%; n = 30). E13.5 mEFs were prepared as described previously (Sahin *et al*., 2004). All mouse experiments were approved by the Institutional Animal Use and Care Committee of the Hospital for Special Surgery, New York, NY.

Shedding of CD23 and L-Selectin from Adam17+/+ or Adam17−/− B Cells

$CD19^{Cre/+}Adam17^{flox/flox}$ mice (see above) were used to generate *Adam17−/−* B cells as follows. Spleen cell suspensions prepared from 4- to 5-mo-old mice were subjected to Ficoll-Hypaque density gradient centrifugation to remove erythrocytes and dead cells. Splenocytes (1 million cells/ml) were incubated for 40 min in RPMI 1640 medium with the indicated reagents and then washed in phosphate-buffered saline (PBS) and stained for 30 min on ice first with a monoclonal antibody to mouse Fcγ receptor to avoid nonspecific antibody binding, and then with phenotype-specific fluorescent antibodies (anti-CD90.1.2 Allophycocyanine for T cells and anti-B220 fluorescein isothiocyanate for B cells), and with phycoerythrin (PE)-conjugated anti-L-Selectin or anti-CD23. Cytometry was performed with a FACSCalibur (BD Biosciences, San Jose, CA) and data were analyzed with CellQuest software (BD Biosciences). $B220^{+}$ $CD90^{-}$ cells were considered to represent B cells, and $B220^{-}$ $CD90^{+}$ cells were considered to represent T cells. The relative amount of L-Selectin on the surface of the entire B or T cells population was calculated by multiplying the percentage of L-$Selectin^{+}$ cells (L-$Selectin^{low}$ to L-$Selectin^{high}$) in B or T cells, with its corresponding mean of fluorescence in the L-$Selectin^{+}$ subpopulation. CD23 cell surface levels were calculated similarly,

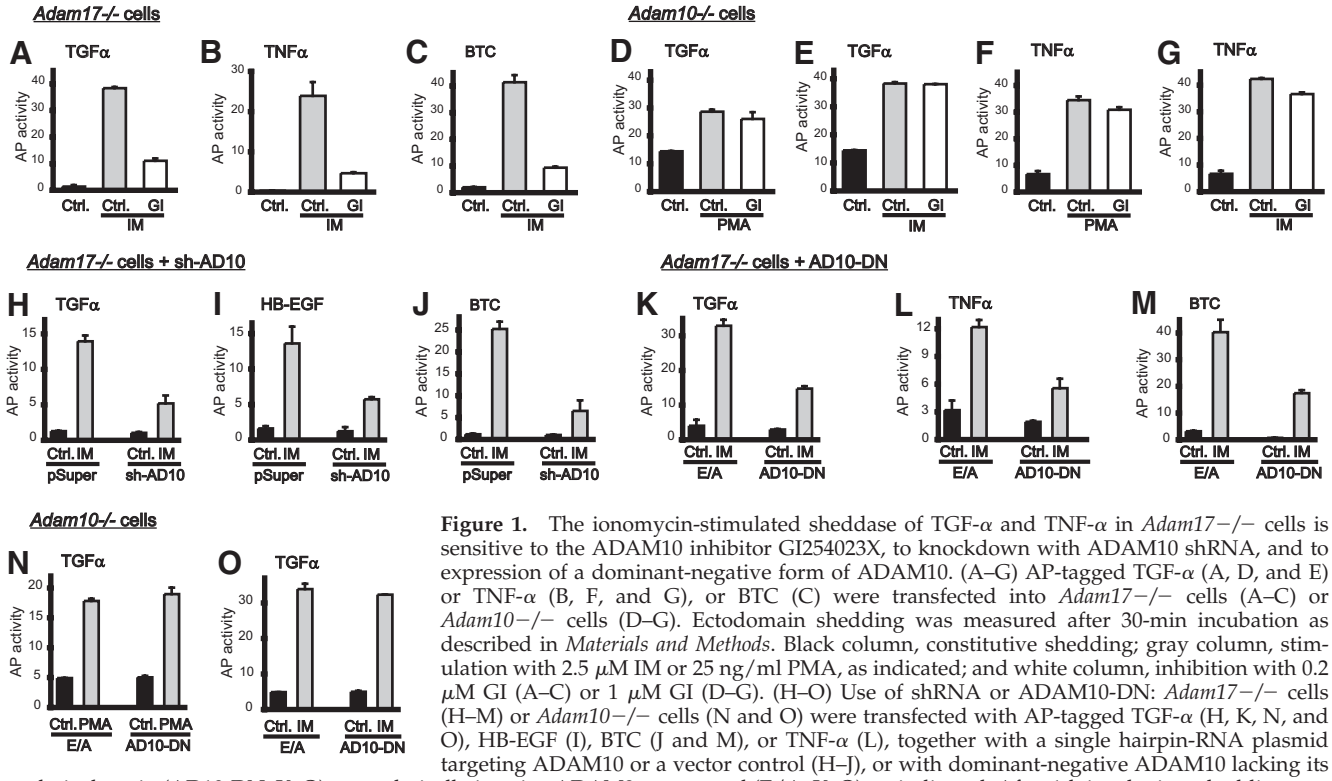

Figure 1. The ionomycin-stimulated sheddase of TGF-α and TNF-α in *Adam17−/−* cells is sensitive to the ADAM10 inhibitor GI254023X, to knockdown with ADAM10 shRNA, and to expression of a dominant-negative form of ADAM10. (A–G) AP-tagged TGF-α (A, D, and E) or TNF-α (B, F, and G), or BTC (C) were transfected into *Adam17−/−* cells (A–C) or *Adam10−/−* cells (D–G). Ectodomain shedding was measured after 30-min incubation as described in *Materials and Methods*. Black column, constitutive shedding; gray column, stimulation with 2.5 μM IM or 25 ng/ml PMA, as indicated; and white column, inhibition with 0.2 μM GI (A–C) or 1 μM GI (D–G). (H–O) Use of shRNA or ADAM10-DN: *Adam17−/−* cells (H–M) or *Adam10−/−* cells (N and O) were transfected with AP-tagged TGF-α (H, K, N, and O), HB-EGF (I), BTC (J and M), or TNF-α (L), together with a single hairpin-RNA plasmid targeting ADAM10 or a vector control (H–J), or with dominant-negative ADAM10 lacking its catalytic domain (AD10-DN; K–O) or catalytically inactive ADAM9 as a control (E/A; K–O), as indicated. After 1-h incubation, shedding was measured as described in *Materials and Methods*. Black columns, constitutive shedding; and gray columns, stimulation with 2.5 μM IM or 25 ng/ml PMA, as indicated.

but because its expression level varied greatly from one mouse to another, data were further normalized to the untreated control to allow comparisons of individual experiments.

RESULTS

Sensitivity of the Calcium Influx-stimulated Sheddase for TGF-α in Adam17−/− Cells to Metalloproteinase Inhibitors

To characterize the ionomycin (IM)-stimulated sheddase for TGF-α in *Adam17−/−* cells, we compared its response to hydroxamate-type metalloproteinase inhibitors to that of ADAMs 10 and 17. As a selective assay for ADAM10, we used constitutive and IM-stimulated shedding of BTC, because this is abolished in *Adam10−/−* cells and can be rescued by transfection with ADAM10 (Sahin *et al*., 2004; Horiuchi *et al*., 2007b). Moreover, constitutive and PMA-stimulated shedding of TGF-α served as selective readout for ADAM17, because both activities are strongly reduced in *Adam17−/−* cells and can be rescued by reintroduction of ADAM17 (Sahin *et al*., 2004; Horiuchi *et al*., 2007b).

In *Adam17−/−* cells, IM-stimulated shedding of TGF-α was sensitive to the ADAM10-selective inhibitor GI at a concentration that blocks ADAM10 but not ADAM17 (0.2 μM; Figure 1A) (Weskamp *et al*., 2006). Moreover, IM-stimulated shedding of TNF-α (Figure 1B) and of the ADAM10-substrate BTC (Figure 1C) in *Adam17−/−* cells was sensitive to 0.2 μM GI. In parallel experiments with *Adam10−/−* cells, PMA and IM-stimulated shedding of TGF-α (Figure 1, D and E) and TNF-α (Figure 1, F and G) were not inhibited by 1 μM GI, consistent with ADAM17 functioning as major sheddase for these substrates. Finally, we found that several other substrates, whose constitutive and PMA-dependent processing depends on ADAM17 (amphiregulin, epiregulin, and HB-EGF; Sahin *et al*., 2004), neuregulin 1β1 and 1β2 (Horiuchi *et al*., 2005), intercellular adhesion molecule (ICAM) (Tsakadze *et al*., 2006), and L-Selectin (Li *et al*., 2006) were shed from *Adam17−/−* cells after IM stimulation, and the increase in shedding of these substrates could also be blocked by 0.2 μM GI (Supplemental Figure 1).

Calcium-stimulated TGF-α Shedding in Adam17−/− Cells Is Blocked by ADAM10-shRNA or Dominant-Negative ADAM10

To further assess whether the IM-stimulated sheddase of TGF-α in *Adam17−/−* cells could be ADAM10, we coexpressed single-hairpin RNA against mouse-ADAM10 (AD10-shRNA) with TGF-α, HB-EGF, or BTC in *Adam17−/−* cells. This resulted in a robust decrease (40–60%) in the IM-stimulated shedding of these substrates compared with a vector control (Figure 1, H–J). In addition, we found that a dominant-negative ADAM10 (AD10-DN) lacking its metalloproteinase domain inhibited IM-stimulated shedding of TGF-α, TNF-α, and BTC in *Adam17−/−* mEF cells compared with mock-transfected control cells (data not shown) or compared with control cells coexpressing an inactive ADAM (ADAM9E/A mutant, E/A; Figure 1, K–M). When ADAM10-DN was expressed in *Adam10−/−* cells as a control for nonspecific effects, it did not affect the constitutive, PMA-stimulated or IM-stimulated shedding of TGF-α (Figure 1, N and O), which depend on ADAM17 (see below and Figures 2, A–C, G, and H, and 3 B, D, and F).

Previous studies have shown that ADAM17 is the principal constitutive sheddase of TGF-α, HB-EGF, AR, and EPR in primary mouse embryonic fibroblasts (Sahin *et al*., 2004).

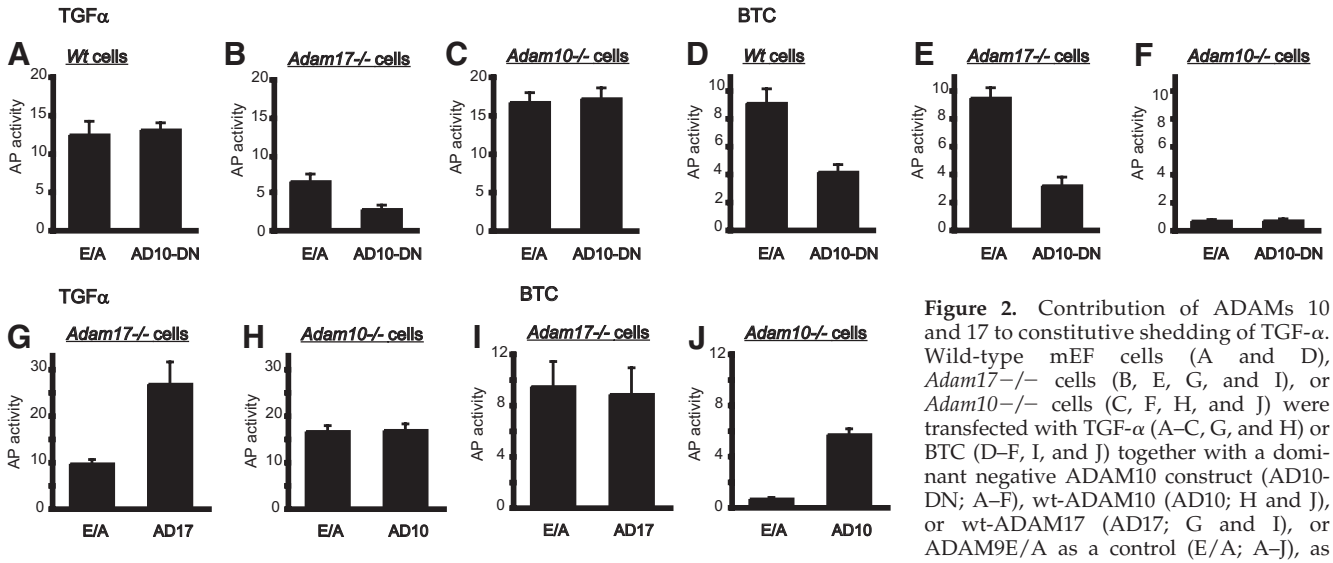

Figure 2. Contribution of ADAMs 10 and 17 to constitutive shedding of TGF-α. Wild-type mEF cells (A and D), *Adam17−/−* cells (B, E, G, and I), or *Adam10−/−* cells (C, F, H, and J) were transfected with TGF-α (A–C, G, and H) or BTC (D–F, I, and J) together with a dominant negative ADAM10 construct (AD10-DN; A–F), wt-ADAM10 (AD10; H and J), or wt-ADAM17 (AD17; G and I), or ADAM9E/A as a control (E/A; A–J), as indicated. The effect of cotransfected ADAMs on constitutive EGFR-ligand-shedding was determined after 4 h of incubation as described in *Materials and Methods*.

To determine whether ADAM10 also contributes to constitutive shedding of these molecules, we tested how ADAM10-DN affected TGF-α shedding in unstimulated cells. ADAM10-DN inhibited the constitutive shedding of TGF-α in *Adam17−/−* cells (Figure 2B), but not in wild-type (*wt*) controls (Figure 2A) or in *Adam10−/−* cells (Figure 2C), which both express ADAM17. Conversely, constitutive BTC shedding was decreased when ADAM10-DN was coexpressed in *wt* cells (Figure 2D) and *Adam17−/−* mEF cells (Figure 2E), thus in cells expressing ADAM10, whereas ADAM10-DN did not affect the residual BTC shedding seen in *Adam10−/−* cells (Figure 2F). In rescue experiments, expression of ADAM17 in *Adam17−/−* cells increased constitutive TGF-α shedding (Figure 2G), whereas expression of ADAM10 in *Adam10−/−* cells did not (Figure 2H), consistent with a dominant role of ADAM17 in TGF-α constitutive shedding. In similar rescue experiments with BTC, constitutive shedding could be increased by transfecting *Adam10−/−* cells with ADAM10 (Figure 2J) but not by expressing ADAM17 in *Adam17−/−* cells (Figure 2I).

To further explore the requirement for ADAMs 10 and 17 for constitutive shedding, we used *Adam10/17−/−* double-deficient cells (Reiss and Saftig, unpublished data). Introducing ADAM10 into *Adam10/17−/−* cells restored constitutive BTC shedding, whereas ADAM17 did not (Figure 3A). In contrast, expression of either ADAM10 or 17 increased constitutive shedding of TGF-α and L-Selectin, with higher levels of both shed proteins observed in the presence of ADAM17 (Figure 3, B and C). With respect to stimulated shedding, PMA-dependent release of TGF-α (Figure 3D) and L-Selectin (data not shown) in *Adam10/17−/−* cells was only rescued by ADAM17 but not ADAM10. Shedding of BTC was not sensitive to PMA stimulation, regardless of whether ADAM10 or ADAM17 were coexpressed (Figure 3E). Finally, IM stimulation of *Adam10/17−/−* cells rescued with ADAM10 strongly enhanced shedding of TGF-α (Figure 3F), L-Selectin (data not shown) and of BTC (Figure 3G), demonstrating that ADAM10 can shed TGF-α in the absence of ADAM17. In contrast, IM stimulation of *Adam10/17−/−* cells expressing ADAM17 strongly increased shedding of TGF-α (Figure 3F) and L-Selectin (data not shown) but not of BTC (Figure 3G), corroborating that calcium influx also activates ADAM17. In *Adam10/17−/−* cells, ADAM10 rescued the IM-stimulated shedding of other proteins whose principal PMA-stimulated sheddase is ADAM17 (TNF-α, ICAM, and L-Selectin; Figure 3, H–J; HB-EGF, data not shown). Together, these experiments confirm that IM-stimulated shedding of TGF-α and several other proteins is mediated by ADAM10 when ADAM17 is absent.

Stimulation of ADAM10-dependent Shedding by a Physiologically Relevant Signaling Pathway

The stimuli described above, IM and PMA, are useful to define the characteristic properties ("fingerprint") of protease activities in cell-based assays (Overall and Blobel, 2007), but they are not physiological stimuli. We therefore tested whether a ligand-activated receptor-signaling pathway can also stimulate ADAM10 to serve as an alternative protease for ADAM17 substrates. Previous studies showed that shedding of CD23, a substrate for ADAM10 (Weskamp *et al*., 2006), is stimulated by activation of the P2X7 receptor in B cells (Gu *et al*., 1998). CHO cells express endogenous P2X7R (Michel *et al*., 1998), whereas mEF cells do not respond to activators of this receptor (Figures 4, D–F, and 5, A–E). Shedding of CD23 from CHO cells was stimulated by IM or dibenzoyl ATP (BzATP) (Michel *et al*., 1998), an agonist of P2X7R but not by the ADAM17 activator PMA. Moreover, the BzATP-stimulated CD23 shedding was sensitive to 0.75 μM GI, consistent with an ADAM10-mediated processing (Figure 4A). Similarly, BTC shedding from CHO cells was also stimulated by BzATP and was sensitive to 0.75 μM GI and to marimastat (Figure 4B) and to coexpression of ADAM10-DN (Figure 4C). When P2X7R was coexpressed with BTC in *Adam17−/−* cells, addition of BzATP enhanced BTC shedding, and this was sensitive to 0.75 μM GI (Figure 4D). When these experiments were repeated in *Adam10−/−* cells (Figure 4E) and *Adam10/17−/−* cells (Figure 4F), no stimulation of BTC shedding by BzATP was observed, strongly suggesting that ADAM10 is responsible for this P2X7R-stimulated shedding.

When the ADAM17-substrates ICAM or TNF-α were transfected together with P2X7R in mEF cells, BzATP stimulated

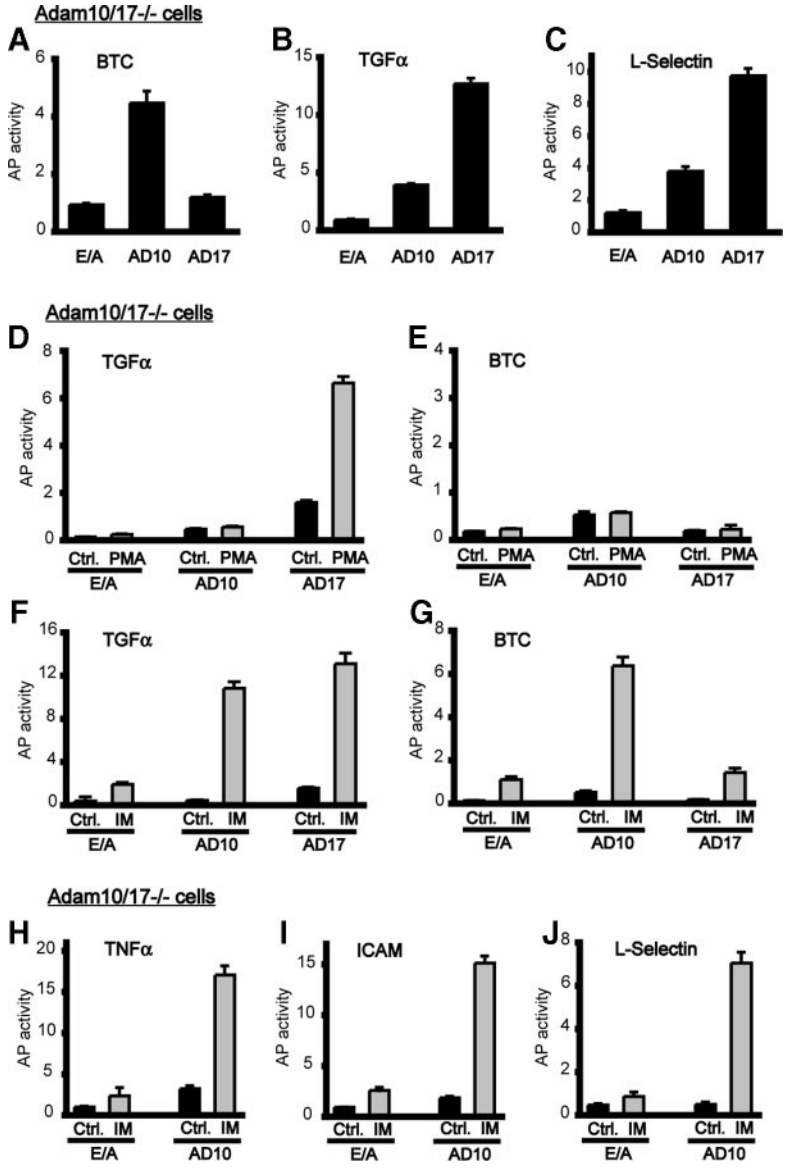

Figure 3. In cells lacking both ADAM10 and -17, transfection of ADAM10 rescues the Ionomycin-stimulated shedding of proteins, for which ADAM17 is the major constitutive and PMA-stimulated sheddase. *Adam10/17−/−* double-knockout cells were transiently transfected with AP-tagged BTC (A, E, and G), TGF-α (B, D, and F), L-Selectin (C and J), TNF-α (H), or ICAM (I), together with wild-type ADAM10 (AD10; A–J) or wild-type ADAM17 (AD17; A–G), or ADAM9E/A control (E/A; A–J). The ability of the transiently cotransfected ADAMs to rescue the defective constitutive shedding of BTC (A), TGF-α (B), and L-Selectin (C) was assessed after 4 h of incubation, and the ability of ADAMs to rescue shedding after stimulation with PMA (D and E) or IM (F–J) was assessed after 30 min of incubation, as described in *Materials and Methods*. Black columns, constitutive shedding; and gray columns, stimulation with 2.5 μM IM or 25 ng/ml PMA, as indicated.

their shedding from both *Adam17−/−* (Figure 5, A and D) and *Adam10−/−* cells (Figure 5, B and E). The BzATP-stimulated shedding was sensitive to 0.75 μM GI in *Adam17−/−* cells, which express endogenous ADAM10, but not in *Adam10−/−* cells, which express endogenous ADAM17. The BzATP-stimulation did not increase ICAM shedding in *Adam10/17−/−* double-knockout cells (Figure 5C). These results are similar to those obtained with IM stimulation (Figure 3) and are consistent with a P2X7R stimulated processing of the substrates by both ADAM10 and ADAM17, in *Adam17−/−* and *Adam10−/−* cells, respectively. However, in CHO cells, which express P2X7R and both proteases, the ADAM10 inhibitor GI did not detectably affect shedding of TGF-α, TNF-α, or ICAM when stimulated with BzATP (Figure 5, F–H), corroborating that a contribution of ADAM10 to the shedding of these substrates is most evident in the absence of ADAM17.

ATP and Calcium-stimulated Shedding of the ADAM17-Substrate L-Selectin from Primary Adam17−/− B Cells

To assess whether activators of ADAM10 can lead to the release of endogenous substrates of ADAM17 from primary cells ex vivo, we analyzed shedding of L-Selectin (Li *et al.*, 2006) from ADAM17-deficient B cells that were stimulated with ionomycin or by activation of the P2X7R. For this purpose, we generated mice that carried a floxed *Adam17* and expressed the B cell specific CD19-Cre to obtain animals that lack ADAM17 in B cells ($CD19^{Cre/+}Adam17^{flox/flox}$ mice; see *Materials and Methods* for details). FACS analysis was used to evaluate the expression of L-Selectin on the surface of T cells or B cells from $CD19^{+/+}Adam17^{flox/flox}$ controls (Figure 6, A and B) or from $CD19^{Cre/+}Adam17^{flox/flox}$ mice (Figure 6, A and D). The level of cell surface L-Selectin as determined by the total fluorescence dramatically increased in the B cells from $CD19^{Cre/+}Adam17^{flox/flox}$ mice compared with the controls $CD19^{+/+}Adam17^{flox/flox}$ (Figure 6, B and D), in agreement with the deletion of ADAM17 by the Cre-recombinase, and as reported for $Adam17^{\Delta}Zn/^{\Delta}Zn$-chimeric mice (Li *et al.*, 2006). After treatment with PMA, IM, or ATP, we observed an almost complete decrease in L-Selectin on the surface of T cells from $CD19^{+/+}Adam17^{flox/flox}$ mice and from $CD19^{Cre/+}Adam17^{flox/flox}$ mice (Figure 6, A and C). This decrease was not seen in B cells from $CD19^{Cre/+}Adam17^{flox/flox}$

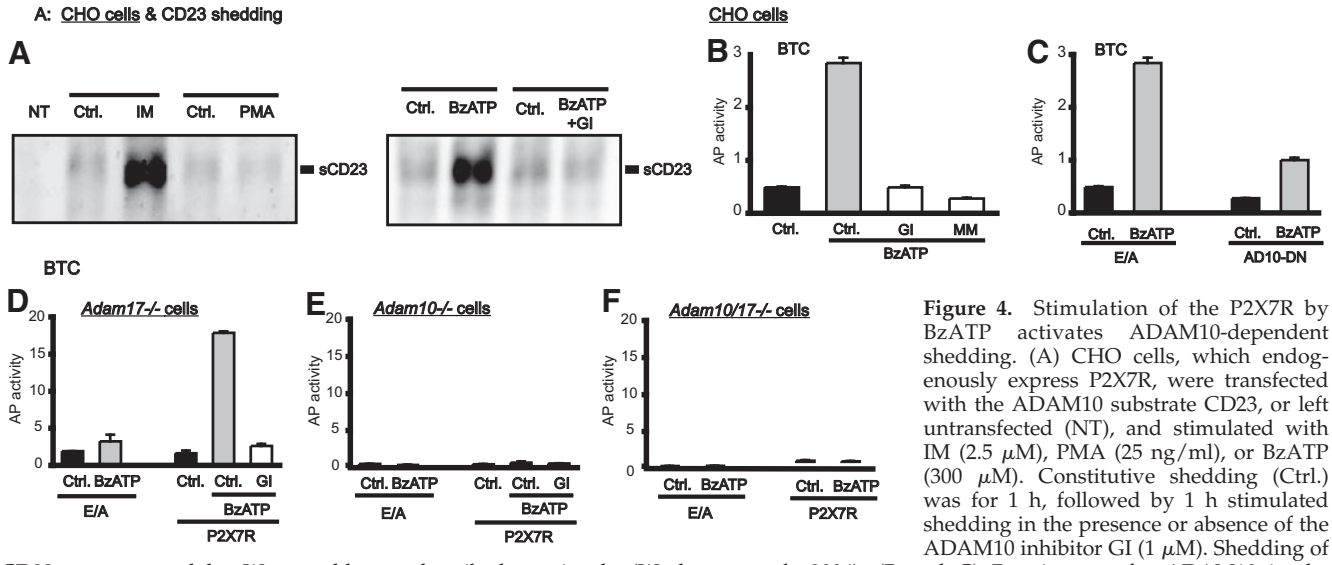

Figure 4. Stimulation of the P2X7R by BzATP activates ADAM10-dependent shedding. (A) CHO cells, which endogenously express P2X7R, were transfected with the ADAM10 substrate CD23, or left untransfected (NT), and stimulated with IM (2.5 μM), PMA (25 ng/ml), or BzATP (300 μM). Constitutive shedding (Ctrl.) was for 1 h, followed by 1 h stimulated shedding in the presence or absence of the ADAM10 inhibitor GI (1 μM). Shedding of CD23 was assessed by Western blot as described previously (Weskamp *et al*., 2006). (B and C) Requirement for ADAM10 in the P2X7R-mediated shedding of BTC in CHO. BTC was transiently transfected into CHO cells alone (B), or together with either ADAM9E/A (E/A) or dominant-negative ADAM10 (AD10-DN) (C). Shedding was assessed after 30 min in the presence or absence of the P2X7R agonist BzATP (B and C) with or without GI or marimastat (MM) (B). (D–F) The BzATP-stimulated shedding of BTC was investigated in *Adam−/−* mEF cells cotransfected with human P2X7R to corroborate that it depends on ADAM10. *Adam17−/−* cells (D), *Adam10−/−* cells (E), and *Adam10/17−/−* cells (F) were cotransfected with BTC and either ADAM9E/A (E/A) or P2X7R (D–F). Shedding was monitored for 30 min in the presence or absence of the P2X7R agonist BzATP, with or without GI, as described in *Materials and Methods* (D and E). Black columns, constitutive shedding; gray columns, stimulation with 300 μM BzATP; and white columns, inhibition with 1 μM GI or 3 μM MM.

mice that were stimulated with PMA, but a partial reduction in L-Selectin was achieved after treatment with IM or ATP (Figure 6D). Moreover, a partial shedding of CD23 from B cells isolated from $CD19^{+/+}Adam17^{flox/flox}$ (Figure 6E) and $CD19^{Cre/+}Adam17^{flox/flox}$ mice (Figure 6F) was detected upon treatment with IM or ATP, but not with PMA, so this was not affected by the presence of CD19-Cre. These results corroborate that ADAM10 can be activated by IM and ATP in B cells, just as in mEFs. Moreover, they suggest that only ADAM17 can completely deplete the cell surface L-Selectin pool, depending on the activating signal, which might be a prerequisite for an adequate immune response.

Selective Inhibitors for ADAM17 Demonstrate That the Ability of ADAM10 to Compensate for ADAM17 Develops over Time

The results presented above, which demonstrate that ADAM10 can efficiently process many ADAM17 substrates

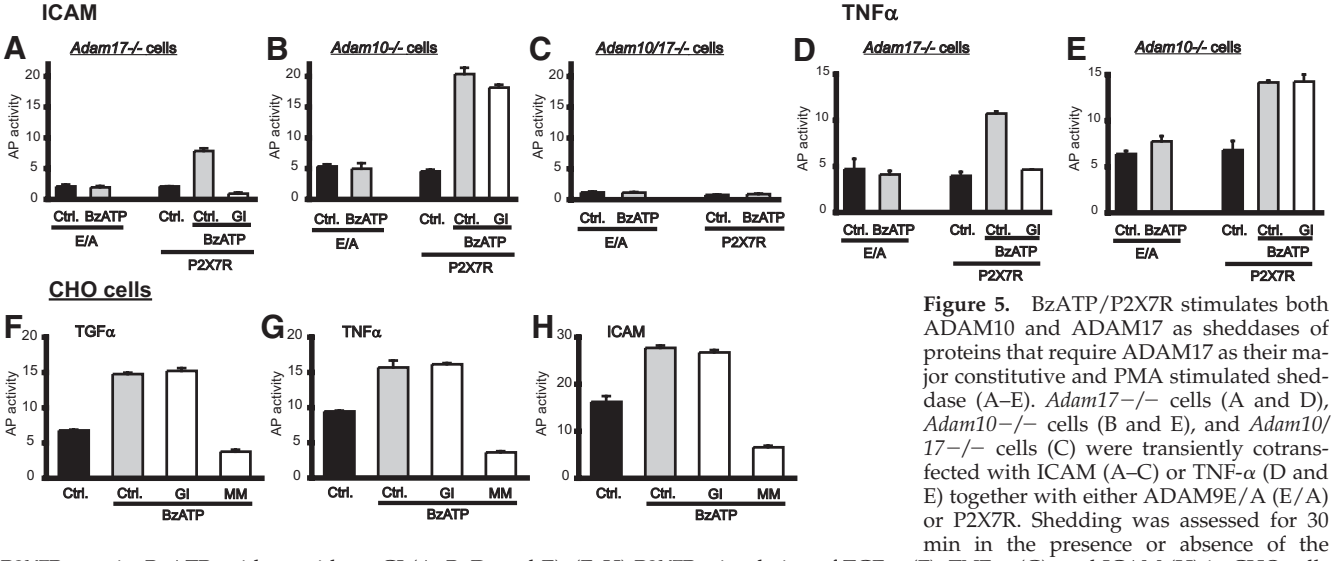

Figure 5. BzATP/P2X7R stimulates both ADAM10 and ADAM17 as sheddases of proteins that require ADAM17 as their major constitutive and PMA stimulated sheddase (A–E). *Adam17−/−* cells (A and D), *Adam10−/−* cells (B and E), and *Adam10/17−/−* cells (C) were transiently cotransfected with ICAM (A–C) or TNF-α (D and E) together with either ADAM9E/A (E/A) or P2X7R. Shedding was assessed for 30 min in the presence or absence of the P2X7R agonist BzATP, with or without GI (A, B, D, and E). (F–H) P2X7R stimulation of TGF-α (F), TNF-α (G), and ICAM (H) in CHO cells in the presence or absence of GI or marimastat (MM) (F–H). The AP activity was determined after 30 min of incubation as described in *Materials and Methods*. Black columns, constitutive shedding; gray columns, stimulation with 300 μM BzATP; and white columns, inhibition with 1 μM GI or 3 μM MM.

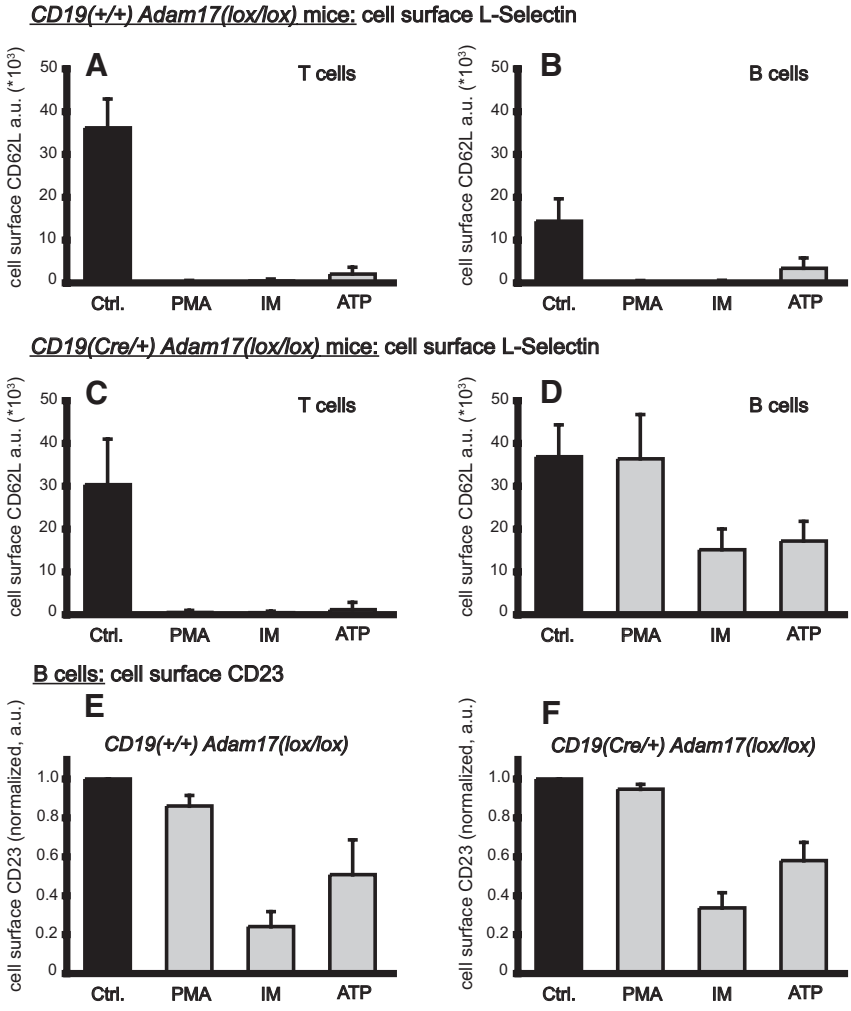

Figure 6. Shedding of the ADAM17 substrate L-Selectin from primary *Adam17*−/− B cells (A–D). Primary T cells (A and C) or B cells (B and D) were isolated from $CD19^{+/+}$ $Adam17^{flox/flox}$ mice (A and B) or $CD19^{Cre/+}$ $Adam17^{flox/flox}$ mice (C and D) as described in *Materials and Methods*, and the cell surface levels of the ADAM17 substrate L-Selectin were measured by FACS in untreated cells, and cells treated with PMA, IM, or ATP for 40 min. In B cells lacking ADAM17 (isolated from $CD19^{Cre/+}$ $Adam17^{flox/flox}$ mice; D), L-Selectin levels were not reduced by treatment with PMA, corroborating that ADAM17 was effectively inactivated, but they were decreased after stimulation with IM or ATP. (E and F) Shedding of the ADAM10 substrate CD23 served as a control for the presence of active ADAM10 in B cells from $CD19^{+/+}Adam17^{flox/flox}$ (E) or $CD19^{Cre/+}$ $Adam17^{flox/flox}$ mice (F). Black columns, control; and gray columns, cells stimulated with 25 ng/ml PMA, 0.5 μM IM, or 5 mM ATP as indicated. Data represent the average plus SEM of three separate experiments (n = 4 mice for each strain). a.u.: arbitrary units.

when ADAM17 is inactive, raised important questions about the relative contribution of ADAM10 to shedding of ADAM17 substrates in wild-type cells. To address this question, we analyzed shedding from wild-type cells treated with a highly selective inhibitor of ADAM17. When *wt* mEFs expressing TGF-α or L-Selectin were stimulated with PMA or IM, we found complete inhibition of the PMA- and IM-stimulated shedding by the highly ADAM17-selective inhibitor SP26 (Mazzola *et al*., 2008), whereas the ADAM10-selective GI had no detectable effect (Figure 7, A–D, see Supplemental Figure 2 for controls regarding the selectivity of these inhibitors toward ADAM10 and -17). Identical experiments performed with BTC confirmed that GI completely blocked IM-stimulated shedding of this ADAM10 substrate, whereas SP26 did not (Figure 7E). Similar profiles of inhibition were obtained for TGF-α when we repeated these experiments in CHO cells (Supplemental Figure 2). Together with our previous results (see above and Figures 2, A–C, G, and H; 5, F–H, and 6, B and D), these data demonstrate that ADAM10 does not significantly contribute to the shedding of substrates of ADAM17 when both ADAM10 and -17 are present, even though ADAM10 can efficiently shed ADAM17 substrates in *Adam17*−/− cells. Thus, ADAM17 is indeed the principal sheddase for ADAM17 substrates such as TGF-α, L-Selectin, or TNF-α, and ADAM10 only cleaves these substrates when ADAM17 is inactivated.

These findings raised questions about whether chronic treatment with selective inhibitors of ADAM17, which are being developed for treatment of cancer and rheumatoid arthritis, could generate conditions that mimic those in *Adam17*−/− mice. To directly address the possibility of compensatory changes in the cellular shedding mechanism, we performed essentially identical shedding experiments after a chronic incubation of *wt* mEF cells with SP26 for 2 d. Unlike the results shown in Figure 7, B and D, in which SP26 was used for acute inhibition, IM could stimulate shedding of TGF-α and L-Selectin despite the presence of SP26 after chronic treatment with this inhibitor, and the responsible sheddase was completely blocked by GI, consistent with a role for ADAM10 in this process (Figure 7, F and G; BTC shedding is shown as a control for ADAM10 activity under these conditions in Figure 7H, dimethyl sulfoxide as a control had no effect; data not shown). Evidently, ADAM10 does not make a detectable contribution to the shedding of ADAM17 substrates in wild-type cells treated with an ADAM17-selective inhibitor for 30 min to 2 h, but after prolonged treatment with this inhibitor, the contribution of ADAM10 to shedding of ADAM17 substrates is similar to what is seen in *Adam17*−/− cells. These experiments demonstrate that chronic inhibition of ADAM17 creates conditions under which ADAM10 can make a significant contribution to the IM-stimulated shedding of ADAM17 substrates in *wt* cells.

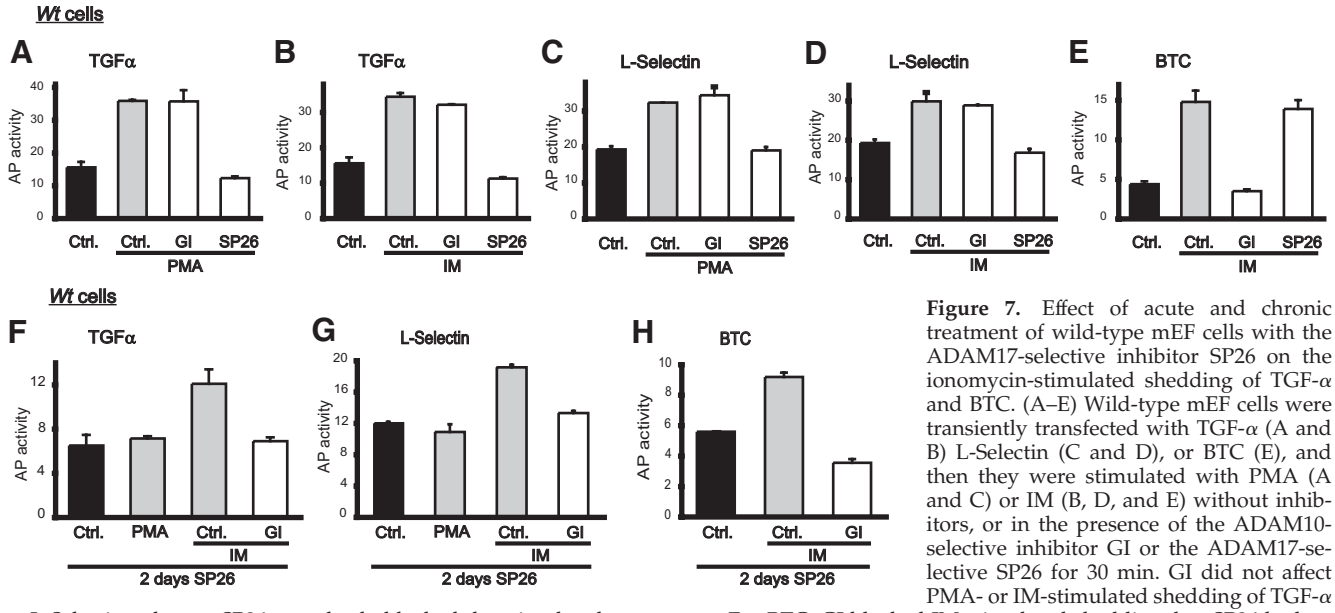

Figure 7. Effect of acute and chronic treatment of wild-type mEF cells with the ADAM17-selective inhibitor SP26 on the ionomycin-stimulated shedding of TGF-α and BTC. (A–E) Wild-type mEF cells were transiently transfected with TGF-α (A and B) L-Selectin (C and D), or BTC (E), and then they were stimulated with PMA (A and C) or IM (B, D, and E) without inhibitors, or in the presence of the ADAM10-selective inhibitor GI or the ADAM17-selective SP26 for 30 min. GI did not affect PMA- or IM-stimulated shedding of TGF-α or L-Selectin, whereas SP26 completely blocked the stimulated component. For BTC, GI blocked IM-stimulated shedding, but SP26 had no effect. (F–H) Pretreatment of wild-type mEF cells transfected with BTC (F), TGF-α (G), or L-Selectin (H) with SP26 (3 μM) for 2 d was followed by shedding experiments with 0.3 μM SP26. Prolonged treatment of wild-type mEFs transfected with TGF-α changed the response of these cells to IM, in that IM-stimulated ADAM10-dependent shedding was observed after chronic (2 d) but not acute inhibition (30 min; see above) of ADAM17. The ratio of AP activity was determined after 30 min of incubation as described in *Materials and Methods*. Black columns, constitutive shedding; gray columns, stimulation with 25 ng/ml PMA or 2.5 μM IM; and white columns, inhibition with 0.2 μM GI or 0.3 μM SP26.

DISCUSSION

Ectodomain shedding is critical for regulating the function of membrane proteins such as TNF-α and EGFR ligands. Because dysregulation of EGFR signaling occurs in diseases such as cancer, and TNF-α is a causative factor in rheumatoid arthritis, it is important to understand the underlying proteolytic machinery. Here, we used the ectodomain shedding of TNF-α, TGF-α, and other membrane proteins such as L-Selectin to identify ADAM10 as a sheddase that can, in principle, release these proteins almost as efficiently as their primary sheddase, ADAM17, but only in *Adam17−/−* cells stimulated with ionomycin. Nevertheless, despite the ability of ADAM10 to efficiently shed many substrates of ADAM17 in *Adam17−/−* cells, ADAM17 nevertheless clearly emerged as the functionally dominant enzyme, or "principal sheddase" for these substrates when both enzymes were present.

An important criterion for identifying ADAM10 as an efficient sheddase of ADAM17-substrates in *Adam17−/−* cells was its "fingerprint," defined as its characteristic response to activators and inhibitors of ectodomain shedding (Overall and Blobel, 2007). ADAM10 emerged as the relevant IM-stimulated sheddase for TGF-α and other membrane proteins in *Adam17−/−* cells by all criteria we could apply, including its response to an ADAM10-selective metalloprotease inhibitor (GI), ADAM10-shRNA, and dominant-negative ADAM10. In *Adam10/17−/−* double knockout cells, IM-stimulated shedding of TGF-α could be rescued by both ADAM10 and ADAM17, demonstrating that calcium influx activates both ADAMs. However, PMA-dependent shedding of TGF-α could only be rescued by ADAM17, corroborating that only ADAM17 responds to short-term stimulation with PMA. Interestingly, IM-stimulated ADAM10 is sensitive to low nanomolar concentrations of TIMPs 1, 2, and 3 in cell-based assays, whereas purified soluble ADAM10 is inhibited by TIMPs 1 and 3, but not TIMP2, in vitro (Amour *et al*., 2000) (Supplemental Figure 3). Evidently, ADAM10 has a different inhibitor profile in cell-based assays compared with biochemical assays. Because MMP7 is a known sheddase of TNF-α and other membrane proteins (Powell *et al*., 1999; Haro *et al*., 2000; Li *et al*., 2002; Lynch *et al*., 2005), we also ruled out its involvement in IM-stimulated shedding by using *Mmp7−/−/Adam17−/−* double-knockout cells (Supplemental Figure 4). Moreover, following up on a previous report of an aminophenylmercuric acetate (APMA)-activated TGF-α sheddase in CHO cells lacking functional ADAM17 (Merlos-Suarez *et al*., 2001), we showed that ADAM10-dependent TGF-α shedding can be activated by APMA in *Adam17−/−* cells (Supplemental Figure 5). Finally, we confirmed that TGF-α released by ADAM10 retains its biological activity (Supplemental Figure 6).

The stimuli described above, such as PMA, IM, and APMA, are pleiotropic and not physiological, so we also evaluated shedding activated by the P2X7 nucleotide receptor, which is involved in many physiological aspects of the immune response (Chen and Brosnan, 2006; Moore and MacKenzie, 2007). Experiments in *Adam−/−* mEFs as well as ADAM17-deficient primary B cells clearly established that both ADAMs 10 and 17 can be stimulated by the P2X7R in adherent fibroblasts and nonadherent primary B cells. Moreover, ADAM10 stimulated via P2X7R was able to shed substrates such as TNF-α, ICAM, and L-Selectin. Nevertheless, selective ADAM inhibitors confirmed that ADAM17 is the major sheddase for TNF-α, TGF-α, HB-EGF, and ICAM in P2X7R-stimulated CHO cells, where both ADAMs 10 and 17 are present. So, the ability of ADAM10 to shed substrates such as TNF-α or TGF-α after activation of P2X7R is also only evident in the absence of ADAM17.

Interestingly, acute inhibition of ADAM17 with an ADAM17-selective inhibitor blocked stimulated shedding of

ADAM17 substrates from *wt* mEFs or CHO cells, whereas chronic inhibition of ADAM17 generated conditions that mimicked those in *Adam17−/−* cells, i.e., ADAM10 could take over shedding of ADAM17 substrates. These observations could be relevant for chronic and specific inhibition of ADAM17 in patients. A compensatory up-regulation of ADAM10 activity during chronic treatment with SP26 is unlikely, because there was no detectably increase in shedding of the ADAM10-substrate BTC. Perhaps chronic inactivation of ADAM17 leads to an accumulation of its substrates, which then become more accessible to ADAM10, possibly by "spilling over" into a compartment where ADAM10 is most active. The results obtained in primary B cells with endogenously expressed L-Selectin are consistent with this interpretation, because higher levels of L-Selectin are seen in the absence of ADAM17. Moreover, activation of these cells with IM or ATP does not lead to complete consumption of L-Selectin in *Adam17−/−* cells, suggesting that a subpopulation of L-Selectin is not accessible to ADAM10, even in the absence of ADAM17.

We predict that the results obtained with TGF-α, TNF-α, and several other membrane proteins (Supplemental Figure 1) are likely representative for many, if not most or all, proteins whose constitutive and PMA-stimulated shedding depends on ADAM17. However, although ADAM10 can, in principle, process substrates of ADAM17, it nevertheless normally does not when both enzymes are present, and a role for ADAM10 as a secondary sheddase has yet to be demonstrated in vivo. For example, ADAM10 cannot efficiently compensate for the loss of ADAM17 with respect to activating the EGFR during mouse development (Peschon *et al*., 1998; Jackson *et al*., 2003; Sternlicht *et al*., 2005), or in terms of generating soluble TNF-α in a mouse model for endotoxin shock (Bell *et al*., 2007; Horiuchi *et al*., 2007a). Therefore, we predict that ADAM17 will also emerge as the physiologically or pathologically more relevant sheddase of other membrane proteins that can be shed by both ADAM10 and 17, such as the amyloid precursor protein (Buxbaum *et al*., 1990; Lammich *et al*., 1999) and Klotho (Chen *et al*., 2007). In contrast, we found no evidence that ADAM17 can substitute as a sheddase for substrates of ADAM10 in *Adam10−/−* cells, at least in the presence of the stimuli used here. Finally, it should be noted that other ADAMs or non-ADAM metalloproteinases may also play significant roles as sheddases in other cell types or under different conditions than those tested here.

In summary, loss of function studies with cells lacking ADAM10 or ADAM17 or both have provided new insight into the principal components of a general, yet differentially regulated cellular shedding machinery for TGF-α, HB-EGF, TNF-α, L-Selectin, and several other membrane proteins. Because ectodomain shedding is increasingly being recognized as a critical signaling switch that affects the function of a large number of membrane proteins, these results are likely to provide a framework for understanding the regulation of processing of other membrane proteins by ADAMs 10 and 17. Based on our findings, we hypothesize that the substrate repertoire of ADAM10 can, in principle, overlap with that of ADAM17 in cells activated with IM, APMA or via the P2X7R. Nevertheless, identifying ADAM10 as an alternative sheddase for ADAM17 substrates is only relevant in the case of the deletion or the chronic inhibition of ADAM17, and for stimuli that will normally activate ADAM10. Clearly, defining the individual fingerprints of these two major sheddases under various conditions is a prerequisite for probing the mechanism underlying their regulation under specific physiological and pathological conditions. Moreover, it will be important to consider the implications of these results for the use of selective ADAM inhibitors to treat human diseases.

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REFERENCES

Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Docherty, A. J., and Murphy, G. (2000). The in vitro activity of ADAM-10 is inhibited by TIMP-1 and TIMP-3. FEBS Lett. *473*, 275–279.

Bell, J., Herrera, A.H.Li.Y., and Walcheck, B. (2007). Role of ADAM17 in the ectodomain shedding of TNF-α and its receptors by neutrophils and macrophages. J. Leukoc. Biol. *82*, 173–176.

Blobel, C. P. (2005). ADAMs: key players in EGFR-signaling, development and disease. Nat. Rev. Mol. Cell Biol. *6*, 32–43.

Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernick, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J., and Greengard, P. (1990). Processing of Alzheimer β/A4 amyloid precursor protein: modulation by reagents that regulate protein phosphorylation. Proc. Natl. Acad. Sci. USA *87*, 4489–4493.

Chen, C. D., Podvin, S., Gillespie, E., Leeman, S. E., and Abraham, C. R. (2007). Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. Proc. Natl. Acad. Sci. USA *104*, 19796–19801.

Chen, L., and Brosnan, C. F. (2006). Regulation of Immune Response by P2X7 Receptor. Crit. Rev. Immunol. *26*, 499–513.

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.

Gu, B., Bendall, L. J., and Wiley, J. S. (1998). Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. Blood *92*, 946–951.

Haro, H., Crawford, H. C., Fingleton, B., Shinomiya, K., Spengler, D. M., and Matrisian, L. M. (2000). Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption. J. Clin. Invest. *105*, 143–150.

Harris, R. C., Chung, E., and Coffey, R. J. (2003). EGF receptor ligands. Exp. Cell Res. *284*, 2–13.

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lubke, T., Lena Illert, A., von Figura, K., and Saftig, P. (2002). The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum. Mol. Genet. *11*, 2615–2624.

Hinkle, C. L., Sunnarborg, S. W., Loiselle, D., Parker, C. E., Stevenson, M., Russell, W. E., and Lee, D. C. (2004). Selective roles for tumor necrosis factor alpha-converting enzyme/ADAM17 in the shedding of the epidermal growth factor receptor ligand family: the juxtamembrane stalk determines cleavage efficiency. J. Biol. Chem. *279*, 24179–24188.

Hooper, N. M., Karran, E. H., and Turner, A. J. (1997). Membrane protein secretases. Biochem. J. *321*, 265–279.

Horiuchi, K., Kimura, T., Miyamoto, T., Takaishi, H., Okada, Y., Toyama, Y., and Blobel, C. P. (2007a). Cutting edge: TNF-α-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock. J. Immunol. *179*, 2686–2689.

Horiuchi, K., Le Gall, S., Schulte, M., Yamaguchi, T., Reiss, K., Murphy, G., Toyama, Y., Hartmann, D., Saftig, P., and Blobel, C. (2007b). Substrate selectivity of EGF-receptor ligand sheddases and their regulation by phorbol esters and calcium influx. Mol. Biol. Cell *18*, 176–188.

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.

Hundhausen, C., *et al*. (2003). The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. Blood *102*, 1186–1195.

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.

Kawaguchi, N., Horiuchi, K., Becherer, J. D., Toyama, Y., Besmer, P., and Blobel, C. P. (2007). Different ADAMs have distinct influences on Kit ligand processing: phorbol-ester-stimulated ectodomain shedding of Kitl1 by ADAM17 is reduced by ADAM19. J. Cell Sci. *120*, 943–952.

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., and Fahrenholz, F. (1999). Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc. Natl. Acad. Sci. USA *96*, 3922–3927.

Li, N., *et al*. (2007). Metalloproteases regulate T-cell proliferation and effector function via LAG-3. EMBO J. *26*, 494–504.

Li, Q., Park, P. W., Wilson, C. L., and Parks, W. C. (2002). Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell *111*, 635–646.

Li, Y., Brazzell, J., Herrera, A., and Walcheck, B. (2006). ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. Blood *108*, 2275–2279.

Lynch, C. C., *et al*. (2005). MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. Cancer Cell *7*, 485–496.

Mazzola, R. J., *et al*. (2008) Discovery of novel hydroxamates as highly potent tumor necrosis factor-α converting enzyme inhibitors: part II: optimization of the S3′ pocket. Bioorg. Med. Chem. Lett. (*in press*).

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-alpha-converting enzyme. J. Biol. Chem. *276*, 48510–48517.

Michel, A. D., Chessell, I. P., Hibell, A. D., Simon, J., and Humphrey, P. P. (1998). Identification and characterization of an endogenous P2X7 (P2Z) receptor in CHO-K1 cells. Br. J. Pharmacol. *125*, 1194–1201.

Moore, S. F., and MacKenzie, A. B. (2007). Murine macrophage P2X7 receptors support rapid prothrombotic responses. Cell Signal. *19*, 855–866.

Overall, C. M., and Blobel, C. P. (2007). In search of partners: linking extracellular proteases to substrates. Nat. Rev. Mol. Cell Biol. *8*, 245–257.

Pan, D., and Rubin, J. (1997). KUZBANIAN controls proteolytic processing of NOTCH and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. Cell *90*, 271–280.

Peschon, J. J., *et al*. (1998). An essential role for ectodomain shedding in mammalian development. Science *282*, 1281–1284.

Powell, W. C., Fingleton, B., Wilson, C. L., Boothby, M., and Matrisian, L. M. (1999). The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr. Biol. *9*, 1441–1447.

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.

Sahin, U., and Blobel, C. P. (2007). Ectodomain shedding of the EGF-receptor ligand epigen is mediated by ADAM17. FEBS Lett. *581*, 41–44.

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.

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.

Tsakadze, N. L., Sithu, S. D., Sen, U., English, W. R., Murphy, G., and D'Souza, S. E. (2006). Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM-17) mediates the ectodomain cleavage of intercellular adhesion molecule-1 (ICAM-1). J. Biol. Chem. *281*, 3157–3164.

Weskamp, G., *et al*. (2006). ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. Nat. Immunol. *7*, 1293–1298.

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 TNFα shedding and of the function of the TNFα ectodomain in ensuring selective stimulated shedding by the TNFα convertase (TACE/ADAM17). J. Biol. Chem. *279*, 42898–42906.

Zhou, B. B., *et al*. (2006). Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. Cancer Cell *10*, 39–50.