ADAM17 is regulated by a rapid and reversible mechanism that controls access to its catalytic site

Sylvain M. Le Gall[1], Thorsten Maretzky[1], Priya D. A. Issuree[1,2], Xiao-Da Niu[3], Karina Reiss[4], Paul Saftig[5], Rama Khokha[6], Daniel Lundell[3] and Carl P. Blobel[1,7,*]

[1]Arthritis and Tissue Degeneration Program, Hospital for Special Surgery, New York, NY 10021, USA
[2]Department of Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA
[3]Department of Respiratory Care and Immunology, Merck Research Laboratories, Kenilworth, NJ 07033, USA
[4]Clinical Research Unit, Department of Dermatology, Christian-Albrechts-University, 24098 Kiel, Germany
[5]Biochemical Institute, Christian-Albrechts-University, 24098 Kiel, Germany
[6]Ontario Cancer Institute, University of Toronto, Toronto, Ontario, M5G 2M9, Canada
[7]Departments of Medicine and of Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University, New York, NY 10021, USA

*Author for correspondence (blobelc@hss.edu)

Summary

Protein ectodomain shedding is crucial for cell–cell interactions because it controls the bioavailability of soluble tumor necrosis factor-α (TNFα) and ligands of the epidermal growth factor (EGF) receptor, and the release of many other membrane proteins. Various stimuli can rapidly trigger ectodomain shedding, yet much remains to be learned about the identity of the enzymes that respond to these stimuli and the mechanisms underlying their activation. Here, we demonstrate that the membrane-anchored metalloproteinase ADAM17, but not ADAM10, is the sheddase that rapidly responds to the physiological signaling pathways stimulated by thrombin, EGF, lysophosphatidic acid and TNFα. Stimulation of ADAM17 is swift and quickly reversible, and does not depend on removal of its inhibitory pro-domain by pro-protein convertases, or on dissociation of an endogenous inhibitor, TIMP3. Moreover, activation of ADAM17 by physiological stimuli requires its transmembrane domain, but not its cytoplasmic domain, arguing against inside–out signaling via cytoplasmic phosphorylation as the underlying mechanism. Finally, experiments with the tight binding hydroxamate inhibitor DPC333, used here to probe the accessibility of the active site of ADAM17, demonstrate that this inhibitor can quickly bind to ADAM17 in stimulated, but not quiescent cells. These findings support the concept that activation of ADAM17 involves a rapid and reversible exposure of its catalytic site.

Key words: ADAM17, Cell–cell interactions, EGF-receptor

Introduction

Cell–cell interactions are vital to the development and maintenance of multicellular organisms. Proteolysis of cell surface molecules and the extracellular matrix has emerged as a crucial mechanism for regulating cell–cell communications (Blobel, 2005; López-Otin and Overall, 2002; Murphy, 2008; Page-McCaw et al., 2007). Protein ectodomain shedding regulates signaling through membrane-anchored growth factors, such as ligands of the epidermal growth factor receptor (EGFR), and cytokines such as tumor necrosis factor-α (TNFα) by controlling the release of the soluble form of these molecules (Blobel, 2005; Murphy, 2008). Shedding of EGFR ligands is required for their functional activation (Blobel, 2005; Peschon et al., 1998), and TNFα must be processed to engage in paracrine signaling in septic shock and rheumatoid arthritis (Black et al., 1997; Horiuchi et al., 2007a; Moss et al., 1997; Ruuls et al., 2001). Protein ectodomain processing also affects the signaling function of other membrane proteins, including Notch (Bozkulak and Weinmaster, 2009), CD23 (Weskamp et al., 2006) and LAG-3 (Li et al., 2007a).

Ectodomain shedding is a regulated process that can be rapidly stimulated, for example by calcium influx, activation of extracellular signal-regulated kinase, protein kinase C (PKC) and tyrosine kinases such as VEGFR2, and also via G-protein-coupled receptors (Arribas and Massague, 1995; Fan and Derynck, 1999;

Horiuchi et al., 2007b; Le Gall et al., 2009; Prenzel et al., 1999; Sahin et al., 2004; Swendeman et al., 2008). However, the underlying mechanism remains poorly understood. An important prerequisite for understanding how ectodomain shedding is regulated is to define which enzyme(s) is/are required for stimulated ectodomain shedding when specific signaling pathways are activated (Overall and Blobel, 2007). To date, several members of the ADAM (a disintegrin and metalloproteinase) family of membrane-anchored metalloproteinases have been implicated in the activated shedding of EGFR ligands, including ADAM9 (Izumi et al., 1998), ADAM10 (Yan et al., 2002), ADAM12 (Asakura et al., 2002; Kurisaki et al., 2003), ADAM15 (Schafer et al., 2004) and ADAM17 (Gschwind et al., 2003; Horiuchi et al., 2007b; Jackson et al., 2003; Le Gall et al., 2009; Peschon et al., 1998; Sahin et al., 2004; Sunnarborg et al., 2002). ADAM17 responds rapidly to stimulation with phorbol-12-myristate-13-acetate (PMA) and the calcium ionophore ionomycin, whereas ADAM10 only responds to ionomycin, but not PMA, in short-term assays of two hours or less (Horiuchi et al., 2007b; Le Gall et al., 2009; Sahin et al., 2004). However, questions remain about which of these ADAMs, or other enzymes, responds to activation by specific physiological signaling pathways. Moreover, it remains to be resolved how a membrane-anchored proteinase with an extracellular catalytic domain can be activated by inside–out signaling. A mutant

form of ADAM17 that lacks its cytoplasmic domain responds normally to PMA stimulation (Horiuchi et al., 2007b; Reddy et al., 2000). However, because PMA is a strong and pleiotropic stimulus, the cytoplasmic domain of ADAM17 could still be required for stimulation by physiological signaling pathways, and several studies have implicated phosphorylation of the cytoplasmic domain as a key step in ADAM17 activation (Diaz-Rodriguez et al., 2002; Fan and Derynck, 1999; Fan et al., 2003; Soond et al., 2005; Xu and Derynck, 2010). Moreover, rapid transport of ADAM17 to the cell surface has been correlated with stimulation of ectodomain shedding (Killock and Ivetic, 2010; Soond et al., 2005; Xu and Derynck, 2010), yet stimulation of ADAM17-dependent shedding is also observed under conditions in which its expression on the cell surface is not detectably increased (Horiuchi et al., 2007b; Killock and Ivetic, 2010; Willems et al., 2010). Finally, processing of ADAM17 by pro-protein convertases has been suggested to be important in its rapid activation by PMA (Nagano et al., 2004; Soond et al., 2005).

The goal of the current study was to provide new mechanistic insights into the regulation of stimulated ectodomain shedding. The first step was to identify which ADAM responds to various physiological stimuli [thrombin (Thr), lysophosphatidic acid (LPA), TNFα and EGF]. We found that ADAM17, but not ADAM10, responds to these signaling pathways. Previous studies have shown that ADAM10 and ADAM17 both respond to stimulation of cells with dibenzoyl-ATP (BzATP) (Le Gall et al., 2009), so to better understand their substrate selectivity under conditions in which both enzymes are activated, we compared the relative ability of

ADAM10 and ADAM17 to shed the endogenous substrate proteins CD62L and CD23 from BzATP-stimulated primary B cells. Moreover, we dissected the mechanism of ADAM17 activation by determining whether its cytoplasmic domain or its transmembrane domain is required to respond to physiological stimuli, and tested how rapidly ADAM17 is activated and whether its activation is reversible and requires pro-domain processing or removal of an endogenous inhibitor, TIMP3. Finally, we used a tight-binding small molecule inhibitor to probe the accessibility of the catalytic site of ADAM17 in quiescent cells, where shedding is relatively low, versus accessibility in stimulated cells, where ADAM17 is activated.

Results

Evaluation of the activation of ADAM10 and ADAM17 by physiological signaling pathways

To address whether ADAM10 or ADAM17 or both are activated by various physiological signaling pathways, we monitored shedding of transforming growth factor-α (TGFα) by ADAM17 or of betacellulin (BTC) by ADAM10 to distinguish between the response of these two enzymes to treatment of cells with LPA, Thr, TNFα, EGF or the P2X7R agonist BzATP. Each of these compounds significantly increased shedding of TGFα in wild-type mouse embryonic fibroblasts (mEF) compared with unstimulated conditions (Fig. 1A). However, only BzATP (Le Gall et al., 2009), but none of the other stimuli tested here, activated shedding of the ADAM10 substrate BTC (Fig. 1B). When similar experiments were performed in *Adam17–/–* cells expressing the catalytically inactive ADAM17

Fig. 1. Response of ADAM10 and ADAM17 to physiological stimuli of protein ectodomain shedding. (**A**) Wild-type (wt) mEF cells were transfected with TGFα-AP to monitor the activity of ADAM17, and stimulated for 30 minutes with LPA (10 μM), Thr (2 units/ml), TNFα (10 ng/ml), EGF (100 ng/ml) or BzATP (300 μM), as indicated. All stimuli tested here activated ADAM17, as evidenced by the significantly increased shedding of TGFα. (**B**) Identical experiments were performed with wild-type mEF cells transfected with the ADAM10 substrate BTC. Stimulation for 30 minutes with LPA, Thr, TNFα or EGF did not increase the shedding of BTC, whereas stimulation with BzATP did. Cells treated with BzATP in A and B were also co-transfected with its receptor, P2X7R. (**C**) In *Adam17–/–* mEFs transfected with TGFα and the inactive mutant ADAM17E>A (AD17E>A), shedding of TGFα was not stimulated above background levels by 30 minutes of treatment with LPA, Thr, TNF or EGF. (**D,E**) In *Adam17–/–* cells rescued with wild-type ADAM17 (AD17wt) (D) or ADAM17Δ-cyto (AD17Δ-cyto) (E) the stimulated shedding of TGFα by LPA, Thr, TNFα and EGF was restored to an equal extent. (**F**) To assess whether ADAM17 requires its cytoplasmic domain to respond to ionomycin or APMA, we performed similar rescue experiments in cells deficient in both ADAM10 and ADAM17 (*Adam10/17–/–*). Both ADAM17 and ADAM17Δ-cyto rescued IM- or APMA-stimulated shedding of ICAM-1 from these cells (30 minutes incubation) equally well. (**G**) To evaluate the role of the ADAM17 cytoplasmic domain in its response to stimulation with BzATP, *Adam10/17–/–* mEFs were transfected with P2X7R, ICAM-1 and wild-type ADAM17, ADAM17Δ-cyto or inactive ADAM17E>A There was no difference in the response of wild-type ADAM17 or ADAM17Δ-cyto to BzATP. (**H–K**) The role of the transmembrane domain of ADAM17 in its response to different stimuli was evaluated by generating chimera of the ADAM17 extracellular domain with the transmembrane domain of BTC (AD17-BTC) or CD62L (AD17-CD62L, see supplementary material Fig. S1A for details). Stimulated shedding of TGFα by LPA, Thr, TNFα or 25 ng/ml PMA was not restored by co-transfection of ADAM17-BTC (I) or ADAM17-CD62L (J) in *Adam17–/–* cells. ADAM17E>A (H) and ADAM17wt (K) served as negative and positive controls, respectively. Data are the average + s.e.m. of at least three separate experiments performed in duplicate. o indicates no statistical significance, *P<0.05, **P<0.01.
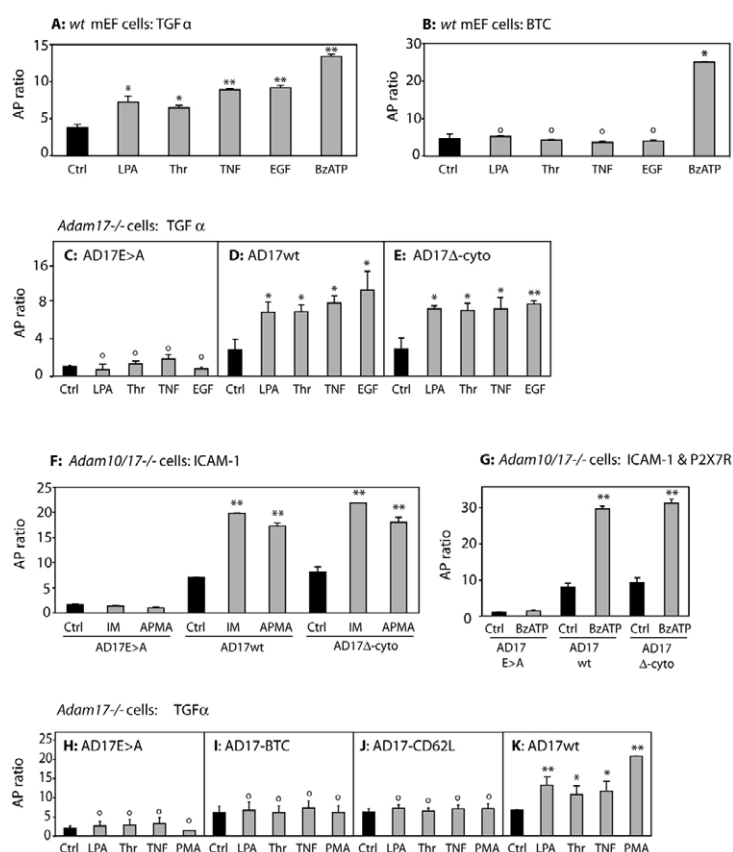
mutant having glutamic acid replaced by alanine (ADAM17E>A), the stimulation of TGFα shedding by LPA, Thr, TNFα and EGF was abolished (Fig. 1C). However, stimulation of TGFα shedding from *Adam17–/–* mEFs could be rescued with wild-type ADAM17 (Fig. 1D) and by a mutant form of ADAM17 lacking its cytoplasmic domain (ADAM17Δ-cyto) (Fig. 1E; see supplementary material Fig. S1A for sequence information). Constitutive shedding of TGFα from *Adam17–/–* cells over 4 hours was also rescued equally well by wild-type ADAM17 or ADAM17Δ-cyto, as was the reduction of cell-associated TGFα (supplementary material Fig. S1B,C), even though western blot analysis showed lower expression of ADAM17Δ-cyto than of wild-type ADAM17 in *Adam17–/–* cells (supplementary material Fig. S1D).

Because ionomycin, 4-aminophenylmercuric acetate (APMA) and BzATP can activate both ADAM10 and ADAM17 (Le Gall et al., 2009), we performed rescue experiments in *Adam10/17–/–* double knockout cells to determine whether ADAM17 requires its cytoplasmic domain to respond to these stimuli. Stimulated shedding of the ADAM17 substrate ICAM-1 by ionomycin and APMA in *Adam10/17–/–* cells could be rescued by wild-type ADAM17 and ADAM17Δ-cyto (Fig. 1F). Similarly, when *Adam10/17–/–* cells were transfected with P2X7R so that they would respond to BzATP, the BzATP-stimulated shedding of ICAM-1 was restored by wild-type ADAM17 and ADAM17Δ-cyto, but not by ADAM17E>A (Fig. 1G). Note that ICAM-1 was used as an ADAM17 substrate in all experiments with *Adam10/17–/–* cells because its expression is better than that of TGFα, which was especially important in triple transfections (ICAM-1 +

P2X7R + ADAM17 or ADAM17Δ-cyto). Taken together, these results demonstrate that the cytoplasmic domain of ADAM17 is not required for its constitutive activity or its response to any of the physiological stimuli listed above.

To assess whether the transmembrane domain of ADAM17 is required for its response to physiological stimuli or PMA, we generated chimera between the extracellular domain of ADAM17 and the transmembrane domain and cytoplasmic domain of the ADAM17 substrate CD62L (AD17-CD62L) or the ADAM10 substrate BTC (AD17-BTC) (for details, see supplementary material Fig. S1A). Co-transfection with either chimera increased constitutive shedding of TGFα in *Adam17–/–* cells compared with the inactive ADAM17E>A control, but no stimulation was seen upon addition of LPA, Thr, TNF or PMA (Fig. 1H–J, wild-type ADAM17 is shown as a positive control in Fig. 1K). Western blot analysis demonstrated comparable expression of AD17-BTC and wild-type ADAM17, and lower expression of A17-CD62L, but this was comparable to the expression of ADAM17Δ-cyto (supplementary material Fig. S1D), which responded normally to various stimuli (see above). Even though only relatively small amounts of mature ADAM17 are produced in all transient transfections compared with endogenous wild-type ADAM17, this nevertheless completely suffices for functional rescue of *Adam17–/–* cells (see also Horiuchi et al., 2007b). These results suggest that the transmembrane domain of ADAM17, which was previously implicated in constitutive shedding of TGFα (Li et al., 2007b), is crucial for the ability of ADAM17 to respond to the stimuli of ectodomain shedding used here.
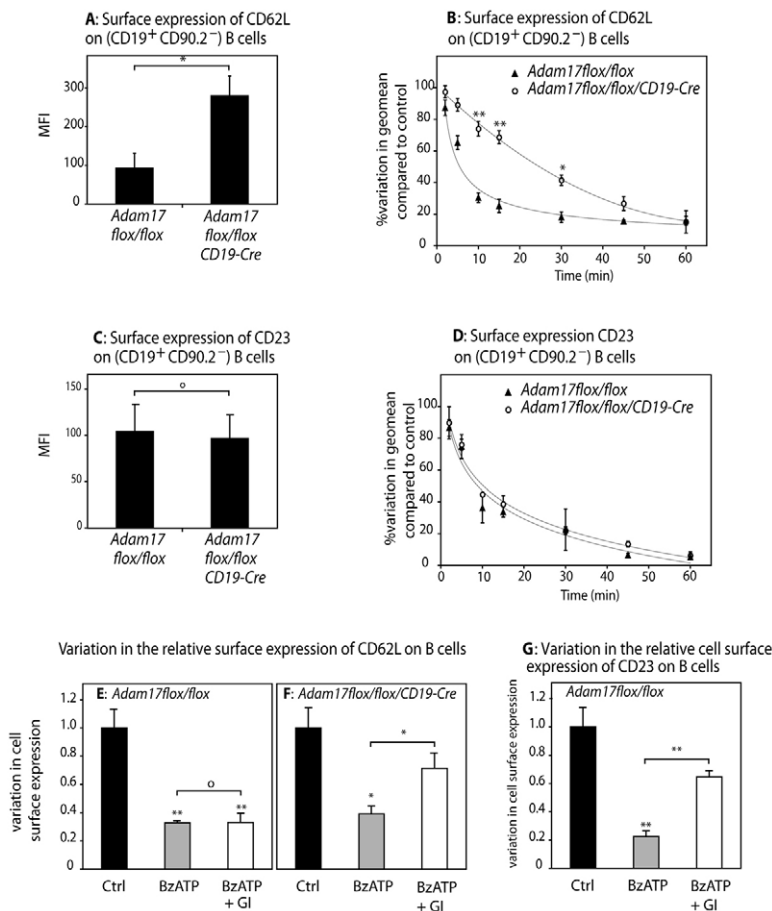

Fig. 2. Downregulation of CD62L by ADAM10 or ADAM17 in primary B cells. (**A**,**C**) The cell surface expression of CD62L (A) or CD23 (C) was quantified by flow cytometry on freshly isolated control (*Adam17flox/flox*) and ADAM17-deficient (*Adam17flox/flox/CD19-Cre*) B cells. CD62L levels were higher in ADAM17-deficient cells, whereas the CD23 geomean showed no significant difference in the presence or absence of ADAM17 in unstimulated B cells. (**B**,**D**) The cell surface expression (geomean) of the ADAM17 substrate CD62L (B) and the ADAM10 substrate CD23 (D) was determined by flow cytometry in control and ADAM17-deficient B cells at different times after treatment with 300 μM BzATP compared with untreated controls. The change in CD62L geomean following BzATP treatment is expressed as percentage compared with untreated cells. The downregulation of CD62L on control B cells was significantly faster than in *Adam17–/–* cells (B). The shedding of the ADAM10 substrate CD23 was similar for both cell types (D). (**E–G**) To assess the relative contribution of ADAM10 to CD62L shedding in control versus *Adam17–/–* B cells, we tested how the ADAM10-selective inhibitor GI affected the decrease in CD62L levels following stimulation with BzATP. 1 μM GI did not affect the downregulation of CD62L on control cells (E), but reduced CD62L release by ~50% from *Adam17–/–* cells (F). 1 μM GI also reduced BzATP-stimulated shedding of CD23 by ~50% (G), providing a control for the effect of GI on ADAM10 under these conditions. Data are the average + s.e.m. of at least three separate experiments performed in duplicate. o signifies no statistical significance, *P<0.05, **P<0.01.
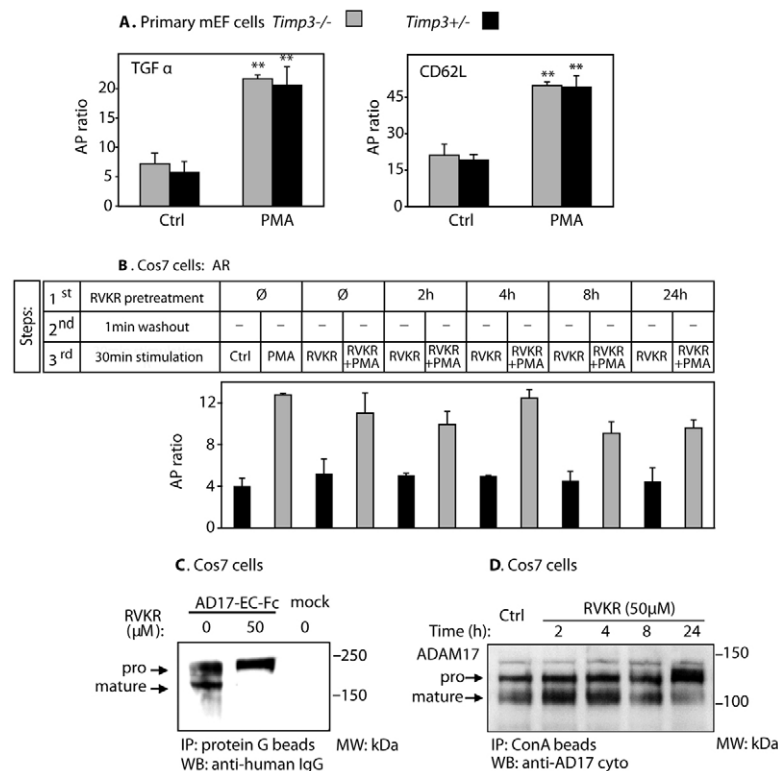
Fig. 3. Stimulation of ADAM17 does not require removal of TIMP3 or pro-domain processing. (**A**) Primary mEF cells isolated from *Timp3–/–* and *Timp3+/–* embryos were transfected with the ADAM17 substrates TGFα or CD62L and tested for their ability to respond to stimulation with 25 ng/ml PMA for 30 minutes. No significant difference was observed between *Timp3–/–* mEFs and *Timp3+/–* controls. (**B**) Cos7 cells transfected with the ADAM17 substrate AR were preincubated for different times (2–24 hours) with 50 μM RVKR, and then stimulated with 25 ng/ml PMA for 30 minutes with or without RVKR. No significant effect of RVKR on shedding of AR was observed. (**C**) As a positive control for inhibition of pro-protein convertases by 50 μM RVKR, Cos7 cells expressing AD17-EC-Fc were preincubated for 4 hours with 50 μM RVKR, and then fresh medium with or without 50 μM RVKR was added for 1hour. Processing of AD17-EC-Fc to the mature form was blocked by 50 μM RVKR. (**D**) Western blot of endogenous ADAM17 in Cos7 cells treated with 50 μM RVKR for 2–24 hours (as in B) and probed with anti-ADAM17 cytoplasmic domain antibodies. There was no major decrease in the ratio of mature to pro-ADAM17 in treated versus untreated cells up to 8 hours, whereas an accumulation of pro-ADAM17 accompanied by a decrease of mature ADAM17 became apparent after treatment for 24 hours. Data represent the average + s.e.m. of at least three separate experiments performed in duplicate, **P<0.01.

Because both ADAM10 and ADAM17 can cleave TGFα and CD62L when activated by ionomycin, APMA or BzATP treatment, this raised the question of why ADAM17 is nevertheless the principal sheddase for TGFα or CD62L when both enzymes are present (Le Gall et al., 2009). To address this question, we used ADAM17-deficient primary B cells (from *Adam17flox/flox/CD19-Cre* mice) and control B cells (from *Adam17flox/flox* mice) to establish the time course of BzATP-stimulated shedding of an endogenous substrate, CD62L, in the presence or absence of ADAM17. The cell surface levels of CD62L on freshly isolated B cells lacking ADAM17 were higher than on control B cells (Fig. 2A), consistent with a crucial role of ADAM17 in constitutive shedding of this substrate. The BzATP-stimulated shedding of CD62L from ADAM17-deficient B cells, which most probably depends on ADAM10 (Le Gall et al., 2009), was slower than from control B cells (Fig. 2B). When B cells were cultured overnight, the surface levels of CD62L on unstimulated cells were more variable than in freshly isolated cells, yet the BzATP-stimulated decrease in CD62L levels was always faster in controls than in ADAM17-deficient B cells, regardless of the initial CD62L surface expression (supplementary material Fig. S2A). By comparison, the initial expression level of the ADAM10 substrate CD23 and its time course of shedding was similar in both cell types, arguing against an increase in ADAM10 activity in the absence of ADAM17 (Fig. 2C,D). Moreover, flow cytometry did not uncover significant differences in the levels of ADAM10 in ADAM17-deficient B cells compared with controls (supplementary material Fig. S2B). To confirm that ADAM10 is the CD62L sheddase in B cells lacking ADAM17, we tested how CD62L shedding is affected by the metalloproteinase inhibitor GI254023X (GI), which is selective for ADAM10 over ADAM17 at 1 μM (Le Gall et al., 2009; Weskamp et al., 2006). We found that 1 μM GI had no effect on CD62L shedding from control B cells, but that it inhibited CD62L shedding

from ADAM17-deficient B cells (Fig. 2F) to the same extent (~50%; see Fig. 2F) as it blocked shedding of the ADAM10 substrate CD23 from control B cells (Fig. 2G). GI was used at 1 μM in these experiments because it does not block ADAM17 at this concentration, and because using GI at a concentration at which it is selective for ADAM10 was more informative in this context than using it at higher concentrations that would have completely blocked ADAM10. These results demonstrate that the BzATP-stimulated downregulation of CD62L by ADAM17 is significantly more rapid than CD62L processing by ADAM10, further corroborating the suggestion that ADAM17 is the principal sheddase for CD62L in cells where both ADAMs are present, even though both ADAMs can process CD62L (Le Gall et al., 2009).

TIMP3 and pro-domain processing are dispensable for activation of ADAM17

To assess whether removal of a bound inhibitor is required for stimulation of ADAM17, we tested whether TIMP3, an endogenous inhibitor of ADAM17 and other metalloproteinases (Mahmoodi et al., 2005; Mohammed et al., 2004; Smookler et al., 2006), could be required for this process. We found that PMA-stimulated shedding of TGFα and CD62L was similar in primary *Timp3–/–* mEFs and *Timp3+/–* controls (Fig. 3A) and in wild-type cells (Fig. 1A). Another mechanism that has been proposed for the quick stimulation of ADAM17 is the removal of its pro-domain by pro-protein convertases (Nagano et al., 2004; Soond et al., 2005). However, 50 μM of the pro-protein convertase inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (RVKR) (Jean et al., 1998; Peiretti et al., 2003) had no significant effect on shedding of the ADAM17 substrate amphiregulin (AR) from Cos7 cells at different time points (Fig. 3B), even though 50 μM RVKR effectively blocked processing of a transfected soluble Fc-fusion protein with the ADAM17 extracellular domain (AD17-EC-Fc) (Fig. 3C). AR

was used in these experiments because its membrane-anchored form accumulates to higher levels than TGFα (Horiuchi et al., 2007b), and is therefore more suitable for experiments lasting more than 30 minutes because it is not readily depleted by shedding or turnover. The levels of mature endogenous membrane-anchored ADAM17 in Cos7 cells were not strongly affected by treatment with 50 μM RVKR for up to 8 hours, with a decrease only becoming evident after 24 hours (Fig. 3D). The relatively long half-life of mature ADAM17 [over 24 hours, (Schlöndorff et al., 2000)] provides an explanation for why inhibitors of pro-protein convertases do not significantly affect its constitutive and stimulated activity, even when added for up to 24 hours.

Stimulated shedding by ADAM17 can be rapidly switched ON and OFF

If ADAM17 were regulated by a bound factor that is released upon stimulation, then the activation of ADAM17 should not be rapidly reversible. To test this, we investigated the shedding of the ADAM17 substrate AR from Cos7 cells after washing out activators and inhibitors of ectodomain shedding. Shedding of AR was induced within 10 minutes of PMA treatment, and stimulation persisted for up to 110 minutes after addition of PMA (Fig. 4A). When the PMA was removed after 10 minutes and the cells were then incubated in control medium, there was no significant reduction in stimulated shedding compared with continuous incubation with PMA, consistent with the irreversible activation of PKC signaling by PMA. In order to determine whether ADAM17 remains active for an extended period of time after PMA stimulation, we preincubated Cos7 cells expressing AR for 4 hours with or without the metalloproteinase inhibitor marimastat (MM) in the presence or absence of PMA, and then washed out all compounds. As expected, during the 4-hour preincubation period, MM blocked constitutive and PMA-stimulated AR shedding (Fig. 4B, upper graph). After washout, all wells were incubated for 1 hour in control medium without activators or inhibitors. Constitutive shedding of AR from MM-pretreated cells was increased after removing MM, presumably because MM treatment resulted in accumulation of the substrate (not shown). After washout, the continued PKC activation was evident in the PMA pretreated sample, and even more pronounced stimulation of shedding was seen in the sample pretreated with MM + PMA, where substrate depletion was prevented by MM (Fig. 4B, lower graph).

In order to distinguish between the possibilities that (a) ADAM17 remains active once switched 'on' by PKCs, (b) that its persistent activity is caused by constant activation of PKCs by PMA, or (c) that MM kept ADAM17 in an active conformation, we tested whether the stimulation of ADAM17 by PMA was quickly reversed by bisindolylmaleimide I (BimI), an inhibitor of PKCs. Cos7 cells expressing AR were stimulated with PMA for 5 minutes with MM, and then washed and incubated in MM with or without BimI for 5 minutes, with two sequential mock treatments used as control. BimI treatment blocked the PMA-dependent increase in AR shedding that is observed after washing out MM (Fig. 4C). Thus, the activated state of ADAM17 was rapidly reversed by blocking PKC signaling because a 5-minute treatment with the PKC inhibitor reduced AR shedding to constitutive levels after initial pretreatment with MM + PMA for 5 minutes. The inactivation of PKCs by BimI after PMA pretreatment did not prevent subsequent activation of ADAM17 by a different pathway, because shedding could be re-activated by the PKC-independent ADAM17 activator pervanadate (PV)
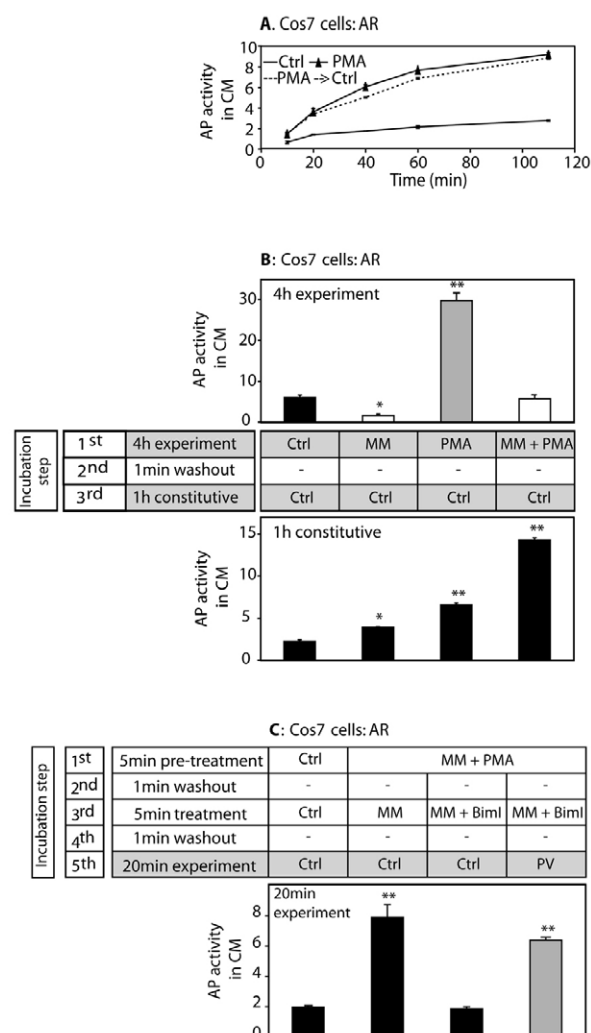

Fig. 4. PMA stimulation of ADAM17 is rapidly reversible. (**A**) Cos7 cells transfected with AR were stimulated with 25 ng/ml PMA continuously for 110 minutes, or for 10 minutes followed by washing out of excess PMA. Removal of PMA had no significant effect on the shedding of AR compared with continuously stimulated cells. (**B**) Cos7 cells transfected with AR were sequentially incubated with or without MM (4 μM), or 25 ng/ml PMA, or MM and PMA for 4 hours. Then the cells were washed, and incubated in conditioned medium (CM) for 1 hour. MM blocked both constitutive and PMA-stimulated shedding in the first 4 hours, and could be readily washed out, resulting in increased AR shedding from unstimulated cells treated with MM compared with cells not incubated with MM. Moreover, PMA increased shedding over constitutive levels even after being washed out, and shedding from cells treated with MM + PMA was further increased after removing MM. (**C**) To test whether the activation of ADAM17 is reversible, we performed similar experiments as in B; however, following stimulation of cells with MM + PMA for 5 minutes, the cells were washed and then treated with MM only, or with MM and BimI (4 μM) for an additional 5 minutes, before these compounds were washed out. The final treatment consisted of incubation in normal medium for 20 minutes with no additions or addition of PV (50 μM), as indicated. The increase in shedding from cells treated with MM + PMA following washout was blocked by treatment with BimI for 5 minutes, demonstrating that the activation of ADAM17 is reversible. When cells that were pretreated with MM + PMA and then with BimI were incubated with 50 μM PV, this strongly stimulated AR shedding. Data are representative of experiments performed at least three times, and are shown as average + s.e.m. of duplicates. Statistical significance was determined relative to the control, *P<0.05, **P<0.01.

(Fig. 4C). Essentially identical results were obtained when the first incubation step (control or MM + PMA) was for 60 minutes (data not shown), demonstrating that the activation state of ADAM17 could also be reversed after prolonged stimulation.

A tight-binding active site metalloproteinase inhibitor rapidly gains access to ADAM17 on stimulated, but not on quiescent cells

Sequential treatments with the rapidly reversible inhibitor MM allowed us to demonstrate the fast activation and inactivation of ADAM17. As a next step, we tested whether the activation of ADAM17 affects access to its catalytic site using the tight-binding active site inhibitor DPC333 (Qian et al., 2007) as a probe. To corroborate that DPC333 acts as a tight-binding inhibitor of ADAM17, we performed sequential incubation experiments in which cells were first treated with or without PMA or PV for 40 minutes in the presence of DPC333 or MM (Fig. 5A). We then quantified the shedding of AR into the supernatant for 20 minutes after the unbound compounds were washed out. Consistent with the mode of action of DPC333, cells pretreated with DPC333 and PMA had very low shedding activity during the 20 minutes following the washout compared with cells pretreated with MM and PMA (Fig. 5A). Interestingly, in cells pretreated with DPC333 and PMA, addition of PV for 20 minutes after the washout did not increase shedding, suggesting that most ADAM17 molecules were inhibited. Likewise, addition of PMA during the 20 minutes of
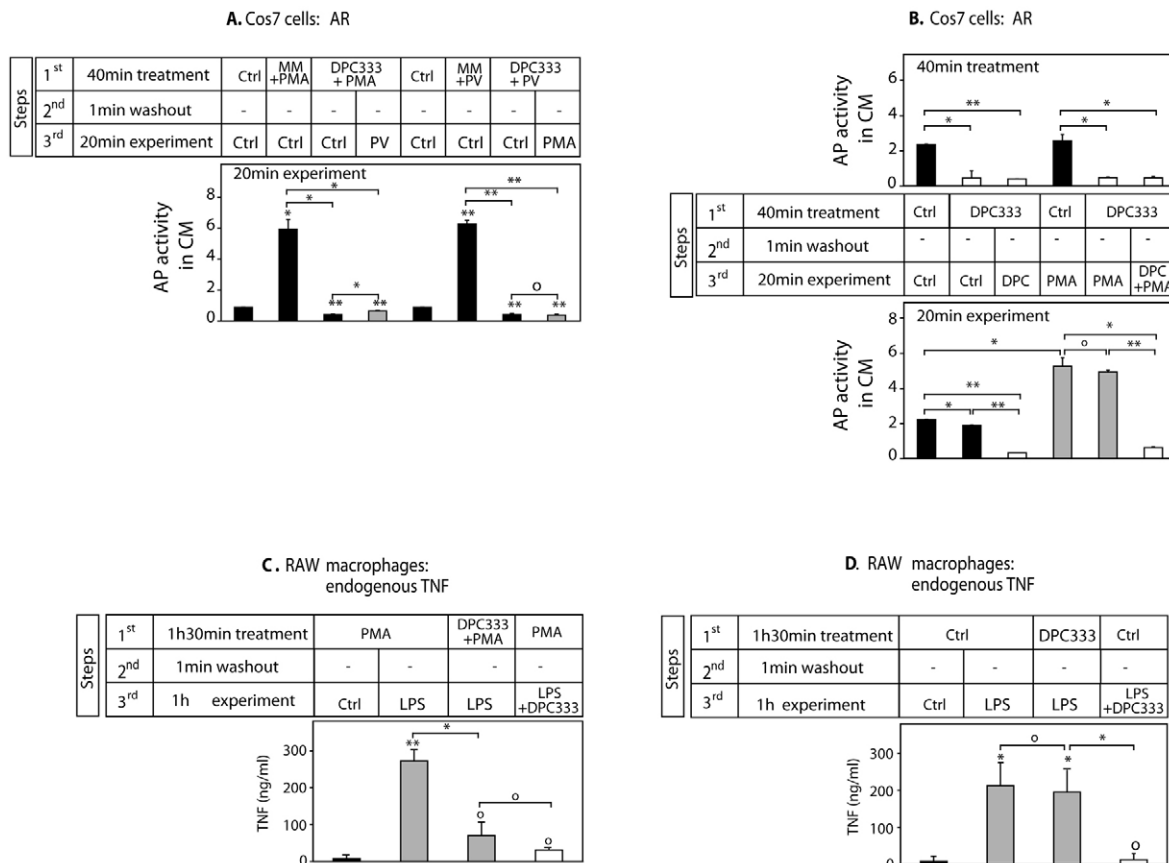

Fig. 5. Use of a tight-binding active site inhibitor of ADAM17, DPC333, as a probe for the accessibility of the catalytic site of ADAM17 in unstimulated and stimulated cells. (**A**) Cos7 cells transfected with AR were treated with conditioned medium (CM), or medium containing MM ($4\,\mu M$) + PMA (25 ng/ml), DPC333 ($0.25\,\mu M$) + PMA, or MM + PV ($50\,\mu M$) or DPC333 + PV. After 40 minutes, all preincubated cells were washed, and then incubated in control medium without further additions, or in medium with PV or PMA, as indicated, for an additional 20 minutes. The results show that DPC333 cannot be washed out after PMA treatment, as the rebounding activity seen after washing out the reversible inhibitor MM in PMA treated samples is not observed in samples treated with DPC333 + PMA. Moreover, in samples treated with DPC333 + PMA for 40 minutes, then washed and treated with PV, no activation of ADAM17 was observed. A similar absence of stimulated shedding was found for samples preincubated with DPC333 + PV and then treated with PMA as a second stimulus. (**B**) AR-transfected Cos7 cells were incubated for 40 minutes in the presence or absence of DPC333, then washed and incubated for 20 minutes in the presence or absence of PMA or DPC333 or DPC333 + PMA, as indicated. DPC333 reduced constitutive shedding of AR in the first 40 minutes of treatment. However, after washing out of DPC333, the constitutive activity recovered to almost normal levels. PMA stimulation of cells that had been preincubated with DPC333 for 40 minutes under non-stimulated conditions resulted in almost identical stimulation as in cells that were not preincubated with DPC333. (**C,D**) RAW cells were cultured in six-well plates and preincubated for 90 minutes with or without $0.25\,\mu M$ DPC333 together with 25 ng/ml PMA (C) or without PMA (D). Then, all compounds were washed out and the cells treated for 1 hour with fresh medium with or without 100 ng/ml LPS in the presence or absence of $0.25\,\mu M$ DPC333. The conditioned culture supernatants were then used to quantify the amount of shed TNF using ELISA. Pretreatment of RAW cells with PMA and DPC333 significantly decreased the subsequent stimulation of TNF shedding by LPS, whereas pretreatment with DPC333 alone had no effect on LPS-stimulated TNF shedding compared with controls that were not pretreated. The results in A and B are representative of experiments performed at least three times, and the data in C and D represent the average + s.e.m. of three separate experiments performed in duplicates. o indicates not statistically significant, *$P<0.05$, **$P<0.01$.

incubation following pretreatment with DPC333 + PV did not activate shedding of AR (Fig. 5A). Because cells that we preincubated with MM and PMA or MM and PV recovered full shedding activity after washing out the inhibitor (Fig. 5A), both stimuli were still able to activate ADAM17-mediated shedding after pretreatment for 40 minutes.

In order to determine how rapidly DPC333 gains access to ADAM17 molecules after stimulation, we performed an essentially identical experiment as described above, but with a shorter preincubation time of 5 minutes (supplementary material Fig. S3A). Under these conditions, the secondary stimulation of cells (PV after DPC333 + PMA, or PMA after DPC333 + PV) resulted in increased shedding, indicating that some ADAM17 molecules had not been inhibited by DPC333 during the initial 5-minute pretreatment (supplementary material Fig. S3A).

When we tested whether DPC333 added to unstimulated cells can block subsequent stimulated shedding, we found that PMA-stimulated shedding of AR (Fig. 5B) and PV-stimulated shedding (not shown) were not affected by pretreatment of quiescent cells with this inhibitor for up to 40 minutes, so long as it was washed out prior to addition of the stimulus (Fig. 5B). Thus, even though DPC333 can irreversibly inactivate stimulated forms of ADAM17, and can block constitutive shedding of AR (Fig. 5B), it is evidently unable to bind to most ADAM17 molecules in unstimulated cells during a 40-minute preincubation because it does not prevent subsequent constitutive or stimulated shedding.

To further corroborate these findings using an endogenously expressed substrate of ADAM17, we performed similar experiments with RAW cells, a murine macrophage cell line that expresses the ADAM17 substrate TNFα (Black et al., 1997; Horiuchi et al., 2007a; Moss et al., 1997). RAW cells were preincubated with or without PMA to activate ADAM17 in the presence or absence of DPC333, and subsequently stimulated with LPS to induce production of TNFα, with or without added DPC333 (Fig. 5C). Consistent with the results in Cos7 cells, preincubation of RAW cells with PMA + DPC333 strongly reduced the subsequent TNFα shedding compared with cells that were preincubated with PMA alone (Fig. 5C), whereas preincubation with DPC333 in the absence of PMA did not affect the subsequent LPS-stimulated TNFα shedding after DPC333 had been washed out (Fig. 5D). In both cases, DPC333 efficiently blocked TNFα shedding if added during LPS stimulation of RAW cells (Fig. 5C,D).

Discussion

The ectodomain shedding of membrane proteins such as TNFα and TGFα is a highly regulated process with crucial roles in cell–cell interactions, yet little is known about the underlying regulatory mechanisms. Here, we show that ADAM17 is the principal enzyme that responds to stimulation of various physiological signaling pathways, and that its rapid response to these pathways requires its transmembrane domain, but not its cytoplasmic tail. Moreover, we demonstrate that ADAM17 can be readily switched 'on' and 'off', and that access of a tight-binding inhibitor to its catalytic site is strongly enhanced when ADAM17 is switched on, most probably via a conformational change in its extracellular domain.

The side-by-side comparison of how ADAM10 and ADAM17 respond to stimulation of cells with TNFα, EGF, LPA or Thr clearly established that ADAM17, and not ADAM10, is the enzyme that is post-translationally activated by the corresponding signaling pathways. The different sensitivity to these stimuli can explain, at least in part, why ADAM17 is the principal inducible sheddase for

molecules such as TNFα or TGFα, even though these can also be shed by ADAM10 in the absence of ADAM17 (Le Gall et al., 2009). By comparison, the activity of ADAM10 can only be enhanced by very few stimuli, including ionomycin, APMA, and activation of P2X7R (Le Gall et al., 2009). However, even under conditions in which both ADAM10 and ADAM17 are present and activated, such as in wild-type mEF cells stimulated with ionomycin, ADAM17 nevertheless emerged as the principal sheddase for substrates such as CD62L (L-selectin), TGFα or TNFα (Le Gall et al., 2009). To better understand the basis for this substrate selectivity, we compared the abilities of ADAM10 and ADAM17 to downregulate endogenous CD62L at different time points in BzATP-stimulated primary mouse B cells. We observed a more rapid depletion of CD62L from wild-type cells, where CD62L shedding depends on ADAM17, than from *Adam17–/–* cells, where it depends on ADAM10. Thus, the ADAM17-dependent downregulation of CD62L is faster than the ADAM10-dependent release in primary B cells. This interpretation is also supported by a recent biochemical study that identified distinct peptide substrate preferences for ADAM10 and ADAM17 in vitro (Caescu et al., 2009). In this context, it is important to emphasize that cell-based assays do not lend themselves to direct kinetic studies because the activation state and the local concentration of enzymes and their substrates cannot be accurately determined. Nevertheless, these experiments help in understanding the basis of the observed substrate selectivity of ADAM17 over ADAM10 for substrates such as CD62L in cell-based assays. Apparently, it is achieved by a combination of distinct sensitivities to physiological stimuli (ADAM17 responds to more stimuli than ADAM10), and more rapid processing of ADAM17 substrates by ADAM17 than by ADAM10 when both enzymes are present and activated by a stimulus such as BzATP.

The response of ADAM17 to various physiological signaling pathways raises questions about the underlying mechanism. Previous studies showed that PMA can activate ADAM17 lacking a cytoplasmic domain (Horiuchi et al., 2007b; Reddy et al., 2000). However, PMA is a highly pleiotropic activator of signaling pathways, so it remained to be determined whether the cytoplasmic domain of ADAM17 is required for its response to physiological stimuli. We show that the ADAM17 cytoplasmic domain is dispensable for its response to stimulation by several physiological signaling pathways (Thr, LPA, TNFα, EGF, BzATP). The ADAM17Δ-cyto construct used here is truncated after aspartate 699, so all cytoplasmic threonine, serine and tyrosine residues are lacking from this mutant, effectively ruling out the possibility that cytoplasmic phosphorylation of ADAM17 is required for its rapid stimulation. However, when the transmembrane domain of ADAM17 was replaced with that of the ADAM17 substrate CD62L or of the ADAM10 substrate BTC, the resulting chimera no longer responded to stimuli of shedding, suggesting that the transmembrane domain of ADAM17 is crucial for its activation. Recently, Xu and Derynck reported that phosphorylation of threonine 735 (T735) in the cytoplasmic domain of ADAM17 is essential for its response to p38MAPK (Xu and Derynck, 2010), so future studies will be necessary to understand the basis of this apparent discrepancy. One possible explanation is that T735 functions as an inhibitory residue that must be phosphorylated as a prerequisite for activation of ADAM17, although differences between cell types or the properties of mouse ADAM17 (used here) and human ADAM17 (used by Xu and Derynck) cannot be ruled out, despite the 89.5% identity of their cytoplasmic tail.

The rapid activation of ADAM17 most probably does not depend on processing of its inhibitory pro-domain because blocking the responsible pro-protein convertases for up to 24 hours did not affect the stimulation of ADAM17. Moreover, the rapid reversibility of the activation of ADAM17 argues against the possibility that a portion of its pro-domain remains associated after processing by pro-protein convertases because any dissociated non-covalently attached pro-domain fragment should be washed away (see Fig. 4C). We cannot rule out a tight, but non-covalent, association of the processed pro-domain, although we could not detect pro-domain fragments that co-immunoprecipitate with mature ADAM17 using western blot with antibodies against its pro-domain (data not shown). Finally, even though ADAM17 is considered a main target of TIMP3 in vivo, (Mahmoodi et al., 2005; Mohammed et al., 2004; Smookler et al., 2006), the PMA-stimulated shedding of ADAM17 was not affected in *Timp3*–/– cells, ruling out a model in which removal of pre-bound TIMP3 is essential for the rapid activation of ADAM17.

Our experiments with a tight-binding inhibitor of ADAM17, DPC333 (Qian et al., 2007), provide new conceptual insights into the regulation of ADAM17. Remarkably, preincubation of unstimulated cells with DPC333 had no effect on the subsequent activation of ADAM17 by PMA or PV after washing out this inhibitor, indicating that it could not gain access to the catalytic site of most of the ADAM17 molecules on quiescent cells. Although we cannot rule out the possibility that ADAM17 is sequestered in an intracellular compartment where it is not accessible to DPC333, it appears unlikely that small molecule inhibitors could not gain access to ADAM17 in such a compartment during 90 minutes of preincubation. Moreover, no increase in transport of ADAM17 to the cell surface was detected under the experimental conditions used in our study (Horiuchi et al., 2007b). Therefore, it is tempting to speculate that ADAM17 assumes a conformation in unstimulated cells in which its catalytic site is not accessible, such that ADAM17 can exist in a 'closed/off' and 'open/on' conformation in cellular membranes. When cells were stimulated with DPC333 present, then this tight-binding inhibitor gained access to the activated ADAM17 molecules and could not be washed out, thereby preventing subsequent re-activation of ADAM17. This is in contrast to the reversible inhibitor MM, which could be readily washed out and did not prevent re-activation of ADAM17 under otherwise identical conditions.

These findings confirm that DPC333 is indeed a potent and tight-binding inhibitor of ADAM17 in the context of intact cells. Moreover, they provide information on the time course of activation of ADAM17, in that incubation of PMA-treated Cos7 cells for 40 minutes with DPC333 precluded the subsequent activation of ADAM17 by PV after washing out this inhibitor, and vice versa for PMA on cells pretreated with DPC333 + PV. This suggests that most potentially activatable ADAM17 molecules had become accessible and were blocked after 40 minutes of stimulation, whereas relatively fewer ADAM17 molecules were blocked when cells were only stimulated for 5 minutes with DPC333 present. Taken together, these results are consistent with a model in which activation of ADAM17 requires a conformational change in its catalytic site that affects its accessibility to DPC333. This model is further supported by the crystal structure of the catalytic domain of ADAM17 with bound IK682, which is almost identical to DPC333. In this structure, a conformational change in the metalloproteinase domain of ADAM17 was observed in the presence of bound IK682 (Niu et al., 2006). IK682 also had

identical properties to DPC333 in experiments in RAW cells, similar to the experiments shown in Fig. 5D (data not shown). Finally, our results are consistent with recently reported conformation-specific antibodies that can distinguish between active and inactive ADAM17 (Willems et al., 2010).

In summary, this study demonstrates that ADAM17 is the principal TGFα sheddase in mEF cells and rapidly responds to a variety of different signaling pathways. The activation of ADAM17 by several physiological stimuli is swift and readily reversible, and requires its transmembrane domain, but not its cytoplasmic domain. Moreover, our findings support the novel concept that controlling access to the catalytic site of ADAM17 is a fundamental aspect of its regulation, most probably through a conformational change in the extracellular catalytic domain. The 'off' conformation of ADAM17 must be sufficiently rigid to prevent access of the tight-binding small molecular inhibitor DPC333 to the active site in quiescent cells. However, ADAM17 is quickly switched 'on' when cells are activated, as evidenced by the ability of DPC333 to bind and block its activity. These findings provide new insights into the substrate selectivity and regulation of ADAM17, and highlight its function as a principal stimulated sheddase in cells.

Materials and Methods
Cell lines and reagents
Embryonic fibroblasts from wild-type *Adam17*–/– and *Adam10/17*–/– mice (mEF) were described previously (Horiuchi et al., 2007b; Le Gall et al., 2009; Sahin et al., 2004). Primary mEFs were prepared from E13.5 *Timp3*–/– embryos (Mohammed et al., 2004) as described (Sahin et al., 2006). Cos7 cells and RAW cells were from ATCC (Manassas, VA). Cells were grown in DMEM or RPMI, with or without antibiotics and 5% FCS. All reagents were from Sigma-Aldrich (St Louis, MO) unless otherwise indicated. BimI, Furin inhibitor I (Decanoyl-RVKR-CMK), and ionomycin were from Calbiochem (San Diego, CA); recombinant mouse TNFα and rat anti-mouse ADAM10-PE antibody from R&D Systems (Minneapolis, MN); flow cytometry antibodies from PharMingen, BD Biosciences (San Diego, CA); and monoclonal mouse anti-HA antibodies from Covance. Polyclonal rabbit anti-ADAM17 cytoplasmic domain antibodies were described previously (Schlöndorff et al., 2000). LPS was from *Escherichia coli* (L-2889 Sigma-Aldrich). Marimastat was a gift from Ouathek Ouerfelli (Sloan-Kettering Institute, New York, NY), and DPC333 was kindly provided by Robert Waltermire (Brystol-Myers Squibb, New Brunswick, NJ). GI1254023X, referred to as GI, was provided by Andreas Ludwig (Rhein-Westphalian Technical University, Aachen, Germany).

Expression vectors
Expression vectors for ADAMs and alkaline phosphatase (AP)-tagged proteins were described previously (Horiuchi et al., 2007b; Le Gall et al., 2009; Sahin et al., 2004; Weskamp et al., 2006). The mouse ADAM17Δ-cyto mutant and the chimera between the extracellular domain of ADAM17 and the transmembrane and cytoplasmic domains of BTC or CD62L (see supplementary material Fig. S1A for the sequences of the chimera) or IgG Fc were generated by PCR using wild-type mouse ADAM17 as template.

Cell culture, transfection, ectodomain shedding assays and Western blot analysis
Cells were transiently transfected with Genjet (SignaGen, Ijamsville, MD) and the indicated plasmids. Shedding assays were performed the day after transfection (Horiuchi et al., 2007b; Le Gall et al., 2009; Sahin et al., 2006; Sahin et al., 2004). For shedding experiments, cells were washed with OptiMEM for 1 hour, then incubated in fresh OptiMEM with or without activators or inhibitors of shedding for 10 minutes to 4 hours, as indicated. For sequential stimulation experiments, the cells were washed for 1 hour in OptiMEM followed by different incubations as indicated, from 5 minutes to 4 hours. Washing out the compounds between incubations took 1 minute or less. The AP activity in the supernatant and cell lysates was measured by colorimetry (Sahin et al., 2006). The ratio between the supernatant AP activity 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 towards a given AP-tagged protein. Western blot analysis was performed as described (Schlöndorff et al., 2000). Briefly, for detection of endogenous ADAM17 or transfected hemagglutinin (HA)-tagged ADAM17 and ADAM17 mutants, cells were lysed in PBS, 1% Triton X-100, 5 mM 1,10 phenantroline, 1× protease inhibitors cocktail. Glycoproteins were concentrated using concanavalin A beads, separated by SDS-PAGE and transferred to nitrocellulose membranes, which were probed with the appropriate antibodies. The AD17-EC-Fc fusion protein was isolated

from cell lysates using protein-G beads, and the nitrocellulose membranes were probed with an anti-human horse radish peroxidase (HRP) antibody. Each experiment was repeated at least three times, with comparable outcomes.

Shedding of CD62L and CD23 from *Adam17flox/flox* or *Adam17flox/flox/CD19-Cre* B cells

Adam17flox/floxCD19-Cre mice were used as a source of ADAM17-deficient B cells, which were isolated from 2- to 4-month-old mice as described (Le Gall et al., 2009). Splenocytes were incubated with anti-mouse FcγR mAbs to reduce nonspecific antibody binding, and then with cell-type-specific fluorescent Abs (anti-CD90.1.2 APC for T cells, anti-CD19 FITC for B cells), and with PE-conjugated anti-CD62L or anti-CD23. Live stained cells were separated into a control group and a 300 μM BzATP treatment group. Cytometry was performed with a FACSCalibur and data analyzed with CellQuest software (BD Bioscience, Franklin Lakes, NJ). CD19+ CD90– cells were considered to represent B cells. The variation in the geomean (MFI, mean fluorescent intensity) of expression of CD62L or CD23 following BzATP treatment was expressed as a percentage of the unstimulated control at outset of the experiment, which was set to 100%. Experiments with the ADAM10 inhibitor GI were also performed on cells isolated in this manner. After staining, duplicate samples of live cells were left unstimulated or incubated with BzATP in the presence or absence of 1 μM GI for 15 minutes before being analyzed. The geomean of CD62L and ADAM10 were assessed on freshly isolated unstimulated cells or after overnight culture in 10% serum RPMI medium, as indicated.

TNFα ELISA

Plates were coated with anti-mouse TNFα capture antibody (51-26731E, BD Bioscience) following the manufacturer's instructions, washed with PBS + 0.05% Tween20, and then blocked with PBS + 5% FBS. The experimental samples and TNFα standards were added to the wells after dilution in PBS + 5%FBS and incubated for 2 hours at 37°C. After washing, biotinylated anti-mouse TNFα detection antibody (51-26732E, BD Bioscience) was added for 1 hour, followed by washes and addition of streptavidin-HRP (51-9002813, BD Bioscience) for 30 minutes. After washing, the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine was added for 15 minutes, and the reaction stopped with 1M phosphoric acid before reading the optical density at 450 nm (all steps were performed at room temperature).

Statistical analysis

All data are representative of at least three separate experiments. Statistical analyses were performed using the Students *t*-test.
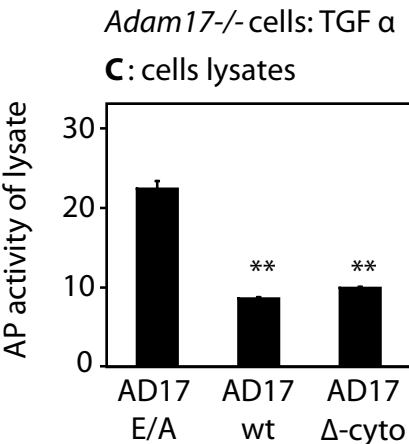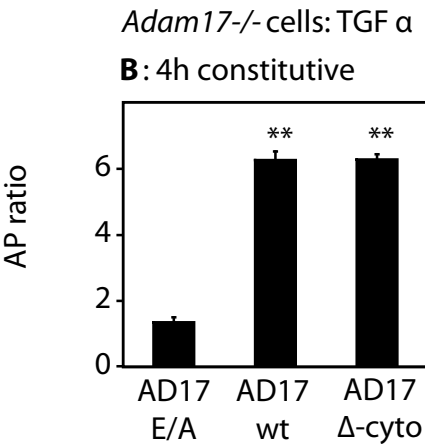
References

Arribas, J. and Massague, J. (1995). Transforming growth factor-alpha and beta-amyloid precursor share a secretory mechanism. *J. Cell Biol.* **128**, 433-441.

Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H. et al. (2002). Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med.* **8**, 35-40.

Black, R., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997). A metalloprotease disintegrin that releases tumour-necrosis factor-α from cells. *Nature* **385**, 729-733.

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

Bozkulak, E. C. and Weinmaster, G. (2009). Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. *Mol. Cell. Biol.* **29**, 5679-5695.

Caescu, C. I., Jeschke, G. R. and Turk, B. E. (2009). Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10. *Biochem. J.* **424**, 79-88.

Diaz-Rodriguez, E., Montero, J. C., Esparis-Ogando, A., Yuste, L. and Pandiella, A. (2002). Extracellular signal-regulated kinase phosphorylates tumor necrosis factor α-converting enzyme at threonine 735: a potential role in regulated shedding. *Mol. Biol. Cell* **13**, 2031-2044.

Fan, H. and Derynck, R. (1999). Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *EMBO J.* **18**, 6962-6972.

Fan, H., Turck, C. W. and Derynck, R. (2003). Characterization of growth factor-induced serine phosphorylation of tumor necrosis factor-alpha-converting enzyme and of an alternatively translated polypeptide. *J. Biol. Chem.* **278**, 18617-18627.

Gschwind, A., Hart, S., Fischer, O. M. and Ullrich, A. (2003). TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J.* **22**, 2411-2421.

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.

Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S. et al. (1998). A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* **17**, 7260-7272.

Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C. and Lee, D. C. (2003). Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J.* **22**, 2704-2716.

Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J. and Thomas, G. (1998). α1-Antitrypsin Portland, a bioengineered serpin highly selective for furin: application as an antipathogenic agent. *Proc. Natl. Acad. Sci. USA* **95**, 7293-7298.

Killock, D. J. and Ivetic, A. (2010). The cytoplasmic domains of TNFalpha-converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding. *Biochem. J.* **428**, 293-304.

Kurisaki, T., Masuda, A., Sudo, K., Sakagami, J., Higashiyama, S., Matsuda, Y., Nagabukuro, A., Tsuji, A., Nabeshima, Y., Asano, M. et al. (2003). Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: involvement of Meltrin alpha in adipogenesis and myogenesis. *Mol. Cell. Biol.* **23**, 55-61.

Le Gall, S., Bobe, P., Reiss, K., Horiuchi, K., Niu, X.-D., Lundell, D., Gibb, D., Conrad, D., Saftig, P. and Blobel, C. (2009). ADAMs 10 and 17 represent differentially regulated components of a general shedding machinery for membrane proteins such as TGFα, L-selectin and TNFα. *Mol. Biol. Cell* **20**, 1785-1794.

Li, N., Wang, Y., Forbes, K., Vignali, K. M., Heale, B. S., Saftig, P., Hartmann, D., Black, R. A., Rossi, J. J., Blobel, C. P. et al. (2007a). Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *EMBO J.* **26**, 494-504.

Li, X., Perez, L., Pan, Z. and Fan, H. (2007b). The transmembrane domain of TACE regulates protein ectodomain shedding. *Cell Res.* **17**, 985-998.

López-Otín, C. and Overall, C. M. (2002). Protease degradomics: a new challenge for proteomics. *Nat. Rev. Mol. Cell Biol.* **3**, 509-519.

Mahmoodi, M., Sahebjam, S., Smookler, D., Khokha, R. and Mort, J. S. (2005). Lack of tissue inhibitor of metalloproteinases-3 results in an enhanced inflammatory response in antigen-induced arthritis. *Am. J. Pathol.* **166**, 1733-1740.

Mohammed, F. F., Smookler, D. S., Taylor, S. E., Fingleton, B., Kassiri, Z., Sanchez, O. H., English, J. L., Matrisian, L. M., Au, B., Yeh, W. C. et al. (2004). Abnormal TNF activity in Timp3–/– mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat. Genet.* **36**, 969-977.

Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Cartner, H. L., Chen, W.-J., Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R. et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature* **385**, 733-736.

Murphy, G. (2008). The ADAMs: signalling scissors in the tumour microenvironment. *Nat. Rev. Cancer* **12**, 929-941.

Nagano, O., Murakami, D., Hartmann, D., De Strooper, B., Saftig, P., Iwatsubo, T., Nakajima, M., Shinohara, M. and Saya, H. (2004). Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. *J. Cell Biol.* **165**, 893-902.

Niu, X., Umland, S., Ingram, R., Beyer, B. M., Liu, Y. H., Sun, J., Lundell, D. and Orth, P. (2006). IK682, a tight binding inhibitor of TACE. *Arch. Biochem. Biophys.* **451**, 43-50.

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.

Page-McCaw, A., Ewald, A. J. and Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodelling. *Nat. Rev. Mol. Cell Biol.* **8**, 221-233.

Peiretti, F., Canault, M., Deprez-Beauclair, P., Berthet, V., Bonardo, B., Juhan-Vague, I. and Nalbone, G. (2003). Intracellular maturation and transport of tumor necrosis factor alpha converting enzyme. *Exp. Cell Res.* **285**, 278-285.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russel, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N. et al. (1998). An essential role for ectodomain shedding in mammalian development. *Science* **282**, 1281-1284.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884-888.

Qian, M., Bai, S. A., Brogdon, B., Wu, J. T., Liu, R. Q., Covington, M. B., Vaddi, K., Newton, R. C., Fossler, M. J., Garner, C. E. et al. (2007). Pharmacokinetics and pharmacodynamics of DPC 333 ((2R)-2-((3R)-3-amino-3{4-[2-methyl-4-quinolinyl] methoxy] phenyl}-2-oxopyrrolidinyl)-N-hydroxy-4-methylpentanamide)), a potent and selective inhibitor of tumor necrosis factor α-converting enzyme in rodents, dogs, chimpanzees, and humans. *Drug Metab. Dispos.* **35**, 1916-1925.

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-alpha converting enzyme. *J. Biol. Chem.* **275**, 14608-14614.

Ruuls, S. R., Hoek, R. M., Ngo, V. N., McNeil, T., Lucian, L. A., Janatpour, M. J., Korner, H., Scheerens, H., Hessel, E. M., Cyster, J. G. et al. (2001). Membrane-
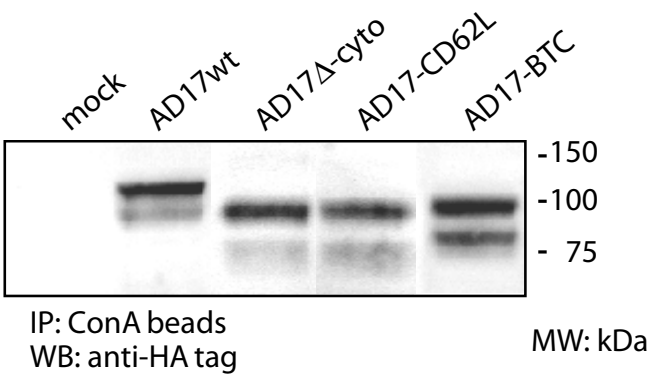
bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* **15**, 533-543.

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.

Sahin, U., Weskamp, G., Zheng, Y., Chesneau, V., Horiuchi, K. and Blobel, C. P. (2006). A sensitive method to monitor ectodomain shedding of ligands of the epidermal growth factor receptor. In *Epidermal Growth Factor: Methods and Protocols*, Vol. 327 (ed. T. B. Patel and P. J. Bertics), pp. 99-113. Totowa, NJ: Humana Press.

Schafer, B., Gschwind, A. and Ullrich, A. (2004). Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* **23**, 991-999.

Schlöndorff, J., Becherer, J. D. and Blobel, C. P. (2000). Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem. J.* **347**, 131-138.

Smookler, D. S., Mohammed, F. F., Kassiri, Z., Duncan, G. S., Mak, T. W. and Khokha, R. (2006). Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation. *J. Immunol.* **176**, 721-725.

Soond, S. M., Everson, B., Riches, D. W. and Murphy, G. (2005). ERK-mediated phosphorylation of Thr735 in TNFalpha-converting enzyme and its potential role in TACE protein trafficking. *J. Cell Sci.* **118**, 2371-2380.

Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Raska, C. S., Peschon, J. J., Castner, B. J., Gerhart, M. J., Paxton, R. J., Black, R. A. et al. (2002). Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J. Biol. Chem.* **277**, 12838-12845.

Swendeman, S., Mendelson, K., Weskamp, G., Horiuchi, K., Deutsch, U., Scherle, P., Hooper, A., Rafii, S. and Blobel, C. P. (2008). VEGF-A stimulates ADAM17-dependent shedding of VEGFR2 and crosstalk between VEGFR2 and ERK signaling. *Circ. Res.* **103**, 916-918.

Weskamp, G., Ford, J., Sturgill, J., Martin, S., Docherty, A., Swendeman, S., Broadway, N., Hartmann, D., Saftig, P., Umland, S. et al. (2006). ADAM10 is a principal 'sheddase' of the low-affinity immunoglobulin E receptor CD23. *Nat. Immunol.* **7**, 1293-1298.

Willems, S. H., Tape, C. J., Stanley, P. L., Taylor, N. A., Mills, I. G., Neal, D. E., McCafferty, J. and Murphy, G. (2010). Thiol isomerases negatively regulate the cellular shedding activity of ADAM17. *Biochem. J.* **428**, 439-450.

Xu, P. and Derynck, R. (2010). Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation. *Mol. Cell* **37**, 551-566.

Yan, Y., Shirakabe, K. and Werb, Z. (2002). The metalloprotease Kuzbanian (ADAM10) mediates the transactivation of EGF receptor by G protein-coupled receptors. *J. Cell Biol.* **158**, 221-226.
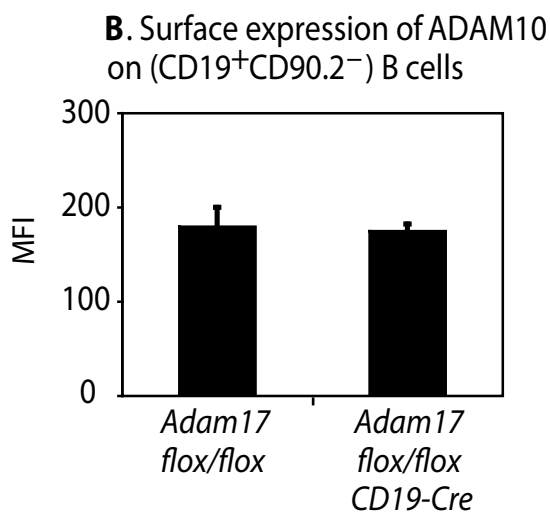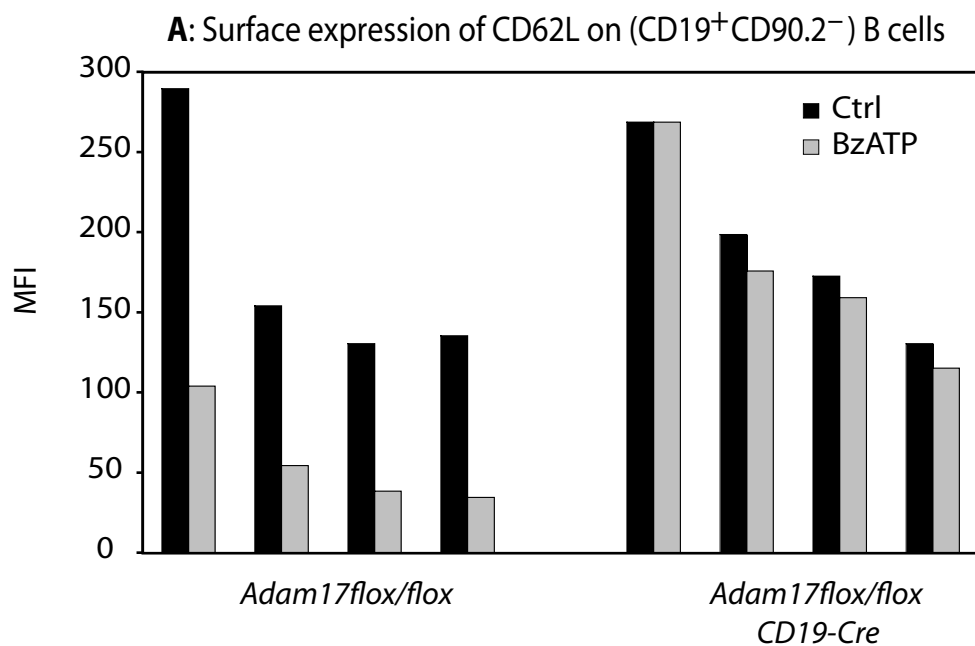
A. Amino acid sequence of AD17 constructs

	Extracellular domain	Predicted Transmembrane domain	Predicted cytoplasmic domain
AD17wt	...KFLADN	IVGSVLVFSLIFWIPFSILVHCV	DKKLDKQYESLSLFHHSNIEMLSSMDSASVRIIKPFPAPQT PGRLQALQPAAMMPPVSAAPKLDHQRMDTIQEDPSTDSHVD DDGFEKDPFPNSSTAAKSFEDLTDHPVTRSEKAASFKLQRQ SRVDSKETEC -HAtag
AD17Δ-cyto	...KFLADN	IVGSVLVFSLIFWIPFSILVHCV	DKKLD -HAtag
mBTC	..QQDRGQ	ILVVCLIVVMVVFIILVIGVCTCC	HPLRKHRKKKKEEKMETLDKDKTPISEDIQETNIA
AD17-BTC	...LADNGS	ILVVCLIVVMVVFIILVIGVCTCC	HPLRKHRKKKKEEKMETLDKDKTPISEDIQETNIA -HAtag
mCD62L	...KEGDYN	PLFIPVAVMVTAFSGLAFLIWLA	RRLKKGKKSQERMDDPY
AD17-CD62L	...KFLADN	PLFIPVAVMVTAFSGLAFLIWLA	RRLKKGKKSQERMDDPY -HAtag

Adam17-/- cells: TGF α

B: 4h constitutive

Adam17-/- cells: TGF α

C: cells lysates

D: WB of transfected ADAM17-HA clones in *Adam17-/-* cells

IP: ConA beads
WB: anti-HA tag

MW: kDa

A: Surface expression of CD62L on (CD19$^+$CD90.2$^-$) B cells

B. Surface expression of ADAM10 on (CD19$^+$CD90.2$^-$) B cells

Cos7 cells: AR

Steps			Ctrl	MM +PMA	DPC333 + PMA		Ctrl	MM +PV	DPC333 + PV	
	1st	5min treatment	Ctrl	MM +PMA	DPC333 + PMA		Ctrl	MM +PV	DPC333 + PV	
	2nd	1min washout	-	-	-	-	-	-	-	-
	3rd	20min experiment	Ctrl	Ctrl	Ctrl	PV	Ctrl	Ctrl	Ctrl	PMA

20min experiment

AP activity in CM