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

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Summary

Protein ectodomain shedding is critical for cell-cell interactions because it controls the bioavailability of soluble TNFα and EGFR-ligands 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 ADAM17, but not ADAM10, is the sheddase that rapidly responds to the physiological signaling pathways stimulated by Thrombin, EGF, LPA, 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, ruling out 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 novel concept that activation of ADAM17 involves a rapid and reversible exposure of its catalytic site.
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 critical mechanism to regulate 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 EGF-receptor, and cytokines, such as 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 Ca-influx, activation of ERK, PKC, 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 pre-requisite 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 the phorbol ester PMA and the Calcium ionophore ionomycin (IM), whereas ADAM10 only responds to IM, 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 lacking its cytoplasmic domain responds normally to PMA stimulation (Horiuchi et al., 2007b; Reddy et al., 2000). However, since 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 its 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 where 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, 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 BzATP (Le Gall et al., 2009), so to better understand their substrate selectivity under conditions where 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 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 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 TGFα by ADAM17 or of Betacellulin (BTC) by ADAM10 to distinguish between the response of these two enzymes to treatment of cells with lysophosphatidic acid (LPA), Thrombin (Thr), TNFα, EGF or the P2X7R agonist dibenzoyl-ATP (BzATP). Each of these compounds significantly increased shedding of TGFα in wild type mouse embryonic fibroblasts (mEF) compared to 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 ADAM17E>A mutant, 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 a mutant form of ADAM17 lacking its cytoplasmic domain (ADAM17Δ-cyto, Fig. 1E, see Suppl. Fig. 1A 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 (Suppl. Fig. 1B, C), even though Western blot analysis showed lower expression of ADAM17Δ-cyto than wild type ADAM17 in Adam17-/- cells (Suppl. Fig. 1D).

Since ionomycin (IM), 4-aminophenylmercuric acetate (APMA) and dibenzoyl-ATP (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 IM 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 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, 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 Suppl. Fig. 1A). Co-transfection with either chimera increased constitutive shedding of TGFα in Adam17-/- cells compared to the inactive ADAM17E>A control, but no stimulation was seen upon addition of LPA, Thr, TNF or PMA (Fig. 1 H-J, wild
type ADAM17 is shown as a positive control in K). 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 (Suppl. Fig. 1D), which responds normally to various stimuli (see above). Even though only relatively small amounts of mature ADAM17 are produced in all transient transfections compared to 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 critical for the ability of ADAM17 to respond to the stimuli of ectodomain shedding used here.

Since both ADAM10 and ADAM17 can cleave TGFα and CD62L when activated by IM, 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 critical role of ADAM17 in constitutive shedding of this substrate. The BzATP-stimulated shedding of CD62L from ADAM17-deficient B cells, which most likely 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 (Suppl. Fig. 2A). 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. 2 C, D). Moreover, flow cytometry did not uncover significant differences in the levels of ADAM10 in ADAM17-deficient B cells compared to controls (Suppl. Fig. 2B). 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%, 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 where 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 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 TIMP-3, 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-RVKR-CMK (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 transfected soluble ADAM17-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 since 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 to continuous incubation with PMA, consistent with the irreversible activation of PKC signaling by PMA. In order to determine if ADAM17 remains active for an extended period of time after PMA stimulation, we pre-incubated 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 hours pre-incubation period, MM blocked constitutive and PMA-stimulated AR shedding (Fig. 4B, top 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 pre-treated sample, and even more pronounced stimulation of shedding was seen in the sample pre-treated 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, or 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 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, since a 5 min treatment with the PKC inhibitor reduced AR shedding to constitutive levels after initial pre-treatment with MM + PMA for 5min. The inactivation of PKCs by BimI after PMA pre-treatment did not prevent subsequent activation of ADAM17 by a different pathway, since shedding could be re-activated by the PKC-independent ADAM17 activator pervanadate (PV, 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 where 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 to cells pretreated with MM and PMA (Fig. 5A). Interestingly, in cells pre-treated with DPC333 and PMA, addition of PV for 20-minutes after the washout did not increase shedding, suggesting that the majority of ADAM17 molecules were inhibited. Likewise, addition of PMA during the 20 minutes incubation following pre-treatment with DPC333 + PV did not activate shedding of AR (Fig. 5A). Since cells that we pre-incubated 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 pre-treatment for 40min.

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 pre-incubation time of 5 minutes (Suppl. Fig. 3A). 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-minutes pre-treatment (Suppl. Fig. 3A).

When we tested whether DPC333 added to unstimulated cells can block subsequent stimulated shedding, we found that PMA-stimulated shedding of AR (Fig. 5B) or PV-stimulated shedding (not shown) was not affected by pre-treatment 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 the majority of ADAM17 molecules in unstimulated cells during a 40 minute pre-incubation, as 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 pre-incubated 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, pre-incubation of RAW cells with PMA + DPC333 strongly reduced the subsequent TNFα shedding compared to cells that were pre-incubated with PMA alone (Fig. 5C), whereas pre-incubation 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 TNF shedding if added during LPS stimulation of RAW cells (Fig. 5 C, D)
Discussion

The ectodomain shedding of membrane proteins such as TNFα and TGFα is a highly regulated process with critical 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 likely 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 Thrombin clearly established that ADAM17, and not ADAM10, is the enzyme that is posttranslationally 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 the P2X7R (Le Gall et al., 2009). However, even under conditions where both ADAM10 and 17 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 ability of either enzyme 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, compared to Adam17-/- cells, where it depends on ADAM10, so 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 17 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, since the activation state and local concentration of enzymes and their substrates cannot be accurately determined. Nevertheless, these experiments help understand 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 (Thrombin, LPA, TNFα, EGF, BzATP). The ADAM17Δ-cyto construct used here is truncated after Asp699, so all cytoplasmic Threonine, Serine and Tyrosine residues are lacking from this mutant, effectively ruling out 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 critical for its activation. Recently, Xu et al. 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. Possible explanations include 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 et al.) cannot be ruled out despite the 89.5% identity of their cytoplasmic tail.

The rapid activation of ADAM17 most likely does not depend on processing of its inhibitory pro-domain, since 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, since 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 by 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, pre-incubation 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 the majority of ADAM17 molecules on quiescent cells. Although we cannot rule out 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 pre-incubation. Moreover, no increase in transport of ADAM17 to the cell surface has been 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 corroborate 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 DPC333 + PV pre-treated cells. This suggests that the majority of potentially activeable 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 a 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 has identical properties to DPC333 in experiments in RAW cells like those shown in figure 5 D (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 that 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 likely 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 (wt), 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. 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. Bisindolylmaleimide I (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), monoclonal mouse anti-HA antibodies from Covance, and polyclonal rabbit anti-ADAM17 cytoplasmic domain antibodies were described previously (Schlöndorff et al., 2000). LPS was from E. coli (L-2889 Sigma-Aldrich). Marimastat was a gift from Dr. Ouathek Ouerfelli (Sloan-Kettering Institute, New York, NY), and DPC333 was kindly provided by Dr. Robert Waltermire (Brystol-Myers Squibb, New Brunswick, NJ).

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 ADAM17∆-cyto mutant and the chimera between the extracellular domain of ADAM17 and the transmembrane- and cytoplasmic domains of BTC or CD62L (see Suppl. Figure 1A for the sequences of the chimera) or IgG Fc were generated by PCR using wt 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 min to 4 hours, as indicated. For sequential stimulation experiments the cells were washed 1hr in OptiMEM followed by different incubations as indicated, from 5 min to 4 hours. Washing out the compounds between incubations took 1 min 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 HA-tagged ADAM17 and ADAM17 mutants, cells were lysed in PBS, 1% TritonX100, 5mM 1,10 Phenantroline, 1x protease inhibitors cocktail, and 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 HRP antibody. Each experiment was repeated at least 3 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 non-specific 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 “control” and “300 µM BzATP treatment” groups. Cytometry was performed with a FACSCalibur and data were analyzed with CellQuest software (Becton Dickinson, 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 GI254023X (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 1µM GI for 15 min 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 (Becton Dickinson Bioscience 51-26731E, Franklin Lakes, NJ) 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 37C. After washing, biotinylated anti-mouse TNFα detection antibody (BD Bioscience 51-26732E) was added for 1 hour, followed by washes and addition of streptavidin-HRP (BD Bioscience 51-9002813) for 30 min. After washing, the peroxidase substrate 3,3′,5,5′-Tetramethylbenzidine was added for 15 minutes, and the reaction was stopped with 1M phosphoric acid before reading the OD at 450nm (all steps were performed at room temperature).

**Statistical analysis**

All data are representative of at least 3 separate experiments. Statistical analysis was performed using the Students t-test.
Acknowledgements. This study was supported by NIH GM64750 (CPB), the Emerald Foundation (TM), the DFG SFB 415, IUAP P6/58, Belgian Federal Science Policy Office, DeZnit (EU-FP VI), and the Center of Excellence “Inflammation at Interfaces” (PS, KR).
References


Figure Legends

Figure 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 (10µM), Thrombin (Thr, 2units/ml), TNFα (10ng/ml), EGF (100ng/ml) or BzATP (300µM), as indicated. All stimuli tested here activated ADAM17, as evidenced by the significantly increased shedding of TGFα. B) Identical experiments performed with wild type mEF cells transfected with the ADAM10-substrate betacellulin (BTC). Stimulation for 30 minutes with LPA, Thr, TNFα or EGF did not increase the shedding of BTC, whereas stimulation with BzATP did, corroborating that ADAM10 can be activated in these cells (Le Gall et al., 2009). 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, shedding of TGFα was not stimulated above background levels by 30 minutes treatment with LPA, Thr, TNFα or EGF. D, E) In Adam17/-/- cells rescued with wild type ADAM17 (D) or an ADAM17 mutant lacking its cytoplasmic domain (E, ADAM17Δ-cyto) the stimulated shedding of TGFα by LPA, Thr, TNFα and EGF was restored to an equal extent, so the cytoplasmic domain of ADAM17 is not essential for its response to these physiological stimuli. F) To assess whether ADAM17 requires its cytoplasmic domain to respond to ionomycin (IM) or APMA, both of which also activate shedding of TGFα or ICAM-1 by ADAM10 in Adam17/-/- mEFs (Le Gall et al., 2009), we performed similar rescue experiments in cells deficient in both ADAM10 and ADAM17 (Adam10/17/-/- double knockout cells), since these cells provide a selective readout for the activity of transfected ADAM17 or ADAM17Δ-cyto. 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/-/- double knockout mEFs were transfected with the BzATP receptor (P2X7R), ICAM-1 and either wt ADAM17 or ADAM17Δ-cyto or inactive ADAM17E>A. There was no difference in the response of wt ADAM17 or ADAM17Δ-cyto to BzATP, so ADAM17 does not require its cytoplasmic domain to respond to any of the stimuli used here. 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 Suppl. Figure 1A for details). Stimulated shedding of TGFα by LPA, Thr, TNFα or 25ng/ml PMA was not restored by co-transfection of AD17-BTC (I) or AD17-CD62L (J) in Adam17/-/- cells, with AD17E>A (H) and AD17wt (K) serving as negative and positive controls, respectively. Data are the average + SEM of at least 3 separate experiments performed in duplicate. o: indicates no statistical significance, * and ** indicate P-values of <0.05 and <0.01 respectively.

Figure 2. Downregulation of CD62L (L-selectin) 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 (Adam17floxflo) and ADAM17-deficient (Adam17floxflox/CD19-Cre) B cells. As previously determined (Le Gall et al., 2009), CD62L levels are higher in ADAM17-deficient cells, whereas the
CD23 geomean showed no significant difference in the presence or absence of ADAM17 in unstimulated B cells, arguing against a compensatory upregulation of ADAM10 activity in Adam17-/− 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 to untreated controls. The change in CD62L geomean following BzATP treatment is expressed as percentage compared to untreated cells. The downregulation of CD62L on control B cells, which depends on ADAM17, is significantly faster than in Adam17-/− cells, where it depends on ADAM10 (Le Gall et al., 2009) (B). The shedding of the ADAM10 substrate CD23 is 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 GI254023X (GI) affected the decrease in CD62L levels following stimulation with BzATP. At 1µM, GI is selective for ADAM10 over ADAM17 and inhibits the activity of ADAM10 by about 50 – 75% (Weskamp et al., 2006). We found that 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 + SEM of at least 3 separate experiments performed in duplicate. o: no statistical significance, * and **: statistical significance, P-values of <0.05 or <0.01 respectively.

Figure 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 25ng/ml PMA for 30 minutes. No significant difference was observed between Timp3-/− mEFs and Timp3+/− controls. B) Cos7 cells transfected with the ADAM17 substrate amphiregulin (AR) were pre-incubated for different times (2 – 24 hours) with 50µM of the pro-protein convertase inhibitor Decanoyl-RVKR-CMK (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, arguing against the role of pro-domain removal by pro-protein convertases in the rapid activation of ADAM17. C) As a positive control for inhibition of pro-protein convertases by 50 µM RVKR, Cos7 cells expressing a soluble Fc-fusion protein with the ADAM17 extracellular domain (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) A Western blot of endogenous ADAM17 in Cos7 cells treated with 50 µM RVKR for 2 – 24 hours (as in panel B), 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 + SEM of at least 3 separate experiments performed in duplicate. ** indicates statistical significance with a P-value of <0.01.
**Figure 4. PMA stimulation of ADAM17 is rapidly reversible.** A) Cos7 cells transfected with the ADAM17 substrate amphiregulin (AR) were stimulated with 25 ng/ml PMA continuously for 110 minutes, or for 10 minutes followed by washing out excess PMA. Removal of PMA had no significant effect on the shedding of AR compared to continuously stimulated cells. B) Cos7 cells transfected with AR were sequentially incubated with or without the metalloprotease-inhibitor marimastat (MM, 4µM), or 25 ng/ml PMA, or MM and PMA for 4 hours. Then the cells were washed, and incubated in medium without additions 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 to 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, most likely because MM preserved the substrate levels in cells treated with PMA. 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 the PKC inhibitor 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 (50µM), as indicated. The increase in shedding from MM + PMA treated cells 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 50µM PV, this strongly stimulated AR shedding, demonstrating that ADAM17 can be switched ON and OFF and back ON again. Data are representative of experiments performed at least 3 times, and are shown as average + SEM of duplicates. Statistical significance was determined relative to the control, * and ** indicate P values of <0.05 and <0.01, respectively.

**Figure 5. Use of a tight-binding active site inhibitor of ADAM17, DPC333, as probe for the accessibility of the catalytic site of ADAM17 in unstimulated and stimulated cells.** A) Cos7 cells transfected with amphiregulin (AR) were treated with control medium, or medium containing MM (4µM) + PMA (25ng/ml), DPC333 (0.25µM) + PMA, or MM + PV (50µM) or DPC333 + PV. After 40 minutes, all pre-incubated 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 can not 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, suggesting that the pool of ADAM17 molecules that was available for stimulation was inhibited by DPC333. 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, suggesting that
ADAM17 molecules that are active in unstimulated cells can be blocked by DPC333. However, after washing out of DPC333, the constitutive activity recovered to almost normal levels, suggesting that there were still sufficient non-inhibited ADAM17 molecules available for constitutive shedding. PMA stimulation of cells that had been pre-incubated with DPC333 for 40 minutes under non-stimulated conditions resulted in almost identical stimulation as in cells that were not pre-incubated with DPC333. These results suggest that DPC333 is unable to bind to the majority of ADAM17 molecules in unstimulated cells, because pre-incubation of quiescent cells does not block the subsequent stimulation of AR shedding by PMA, whereas pre-incubation of PMA-stimulated cells with DPC333 blocks the subsequent activation of ADAM17 (see panel A). C, D) RAW cells were cultured in 6 well plates and pre-incubated for 90 minutes with or without 0.25µM DPC333 together with 25 ng/ml PMA (C) or without PMA (D). Then all compounds were washed out, and the cells were treated for 1 hour with fresh medium with or without 100ng/ml LPS in the presence or absence of 0.25 µM DPC333. The conditioned culture supernatants were then used to quantify the amount of shed TNF by 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 to controls that were not pre-treated. The results in A and B are representative of experiments performed at least 3 times, and the data in C and D represent the average + SEM of 3 separate experiments performed in duplicates. o: not statistically significant, * and **: statistically significant, P-values of <0.05 and <0.01, respectively.
**Figure 1:**

**A:** wt mEF cells: TGFα

**B:** wt mEF cells: BTC

**C:** AD17E>A

**D:** AD17wt

**E:** AD17Δ-cyto

**F:** Adam10/17-/- cells: ICAM-1

**G:** Adam10/17-/- cells: ICAM-1 & P2X7R

**H:** AD17E>A

**I:** AD17-BTC

**J:** AD17-CD62L

**K:** AD17wt

*Note: The figure includes bar graphs showing the expression levels of various proteins in different cell types under different conditions. The graphs are labeled with appropriate identifiers and show statistical significance markers.*
Figure 2:

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

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

C: Surface expression of CD23 on (CD19^+ CD90.2^-) B cells

D: Surface expression CD23 on (CD19^+ CD90.2^-) B cells

E: Variation in the relative surface expression of CD62L on B cells (Adam17^+/flox/flox)

F: Variation in the relative surface expression of CD62L on B cells (Adam17^+/flox/flox/CD19-Cre)

G: Variation in the relative cell surface expression of CD23 on B cells (Adam17^+/flox/flox)

Legend:
- △ Adam17^+/flox/flox
- ○ Adam17^+/flox/flox/CD19-Cre
A. Primary mEF cells *Timp3*^-/-*  vs  *Timp3*^+/-. 

![Bar chart for TGF α and CD62L AP ratio](image)

B. Cos7 cells: AR

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![Bar chart for AP ratio](image)

C. Cos7 cells

- RVKR (µM): 0, 50, 0
- AD17-EC-Fc, mock
- IP: protein G beads
- WB: anti-human IgG
- MW: kDa

![Western blot](image)

D. Cos7 cells

- RVKR (50µM): 2, 4, 8, 24
- IP: ConA beads
- WB: anti-AD17 cyto
- MW: kDa

![Western blot](image)
Figure 4:

A. Cos7 cells: AR

![Graph showing AP activity in CM over time.(min)](image)

B. Cos7 cells: AR

![Bar chart showing AP activity in CM during different experiments.](image)

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C. Cos7 cells: AR

![Bar chart showing AP activity in CM during different experiments.](image)

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** and * indicate statistical significance.
**Figure 5:**

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![Graph showing AP activity in CM for Cos7 cells](image)

**B. Cos7 cells:**

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![Graph showing AP activity in CM for Cos7 cells](image)

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![Graph showing TNF activity (ng/ml) for RAW macrophages](image)

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![Graph showing TNF activity (ng/ml) for RAW macrophages](image)