The Cytoplasmic Domain of A Disintegrin and Metalloproteinase 10 (ADAM10) Regulates its Constitutive Activity but is Dispensable for Stimulated ADAM10-dependent Shedding

Thorsten Maretzky1,*, Astrid Evers1,2,*, Sylvain Le Gall1,3,*, Rolake O. Alabi1,4, Nancy Speck2, Karina Reiss2, Carl P. Blobel1,5,

1Arthritis and Tissue Degeneration Program, Hospital for Special Surgery at Weill Cornell Medical College, New York 10021. 2Department of Dermatology and Allergology, University Hospital Schleswig Holstein, Campus Kiel, Kiel D-24105, Germany. 4Tri-Institutional MD/PhD Program, Rockefeller University/Memorial Sloan-Kettering Cancer Center/Weill Cornell Medical College, New York, NY, 10021. 5Departments of Medicine and of Physiology, Biophysics and Systems Biology, Weill Cornell Medical College, New York, New York 10021.

Running Title: Regulation of ADAM10 by its cytoplasmic domain

3 Current address: INSERM U970, Paris, Cardiovascular Research Centre, Paris, France.

To whom correspondence should be addressed: Dr. Carl P. Blobel, Arthritis and Tissue Degeneration Program, Caspary Research Building, Room 426, Hospital for Special Surgery
535 East 70th Street, New York, NY, 10021, Tel: 212-606-1429, Fax: 212-774-2301
E-mail: blobelc@hss.edu. * These authors contributed equally to this work.

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Background: Release of membrane proteins from the cell surface by the metalloproteinase ADAM10 is post-translationally regulated.

Results: The cytoplasmic domain of ADAM10 negatively controls its constitutive activity, but is dispensable for its stimulation.

Conclusion: Posttranslational regulation of ADAM10 requires its transmembrane and/or extracellular domain.

Significance: Elucidating the regulation of ADAM10 is crucial for understanding the control of ADAM10-dependent cell surface proteolysis.

ABSTRACT

The membrane-anchored metalloproteinase ADAM10 (a disintegrin and metalloprotease 10) is required for shedding of membrane proteins such as EGF, betacellulin, the amyloid precursor protein and CD23 from cells. ADAM10 is constitutively active and can be rapidly and post-translationally enhanced by several stimuli, yet little is known about the underlying mechanism. Here, we use ADAM10-deficient cells transfected with wild-type or mutant ADAM10 to address the role of its cytoplasmic- and transmembrane domain in regulating ADAM10-dependent protein ectodomain shedding. We report that the cytoplasmic domain of ADAM10 negatively regulates its constitutive activity through an ER-retention motif, but is dispensable for its stimulated activity. However, chimeras with the extracellular domain of ADAM10 and the transmembrane-domain of ADAM17 with or without the cytoplasmic domain of ADAM17 show reduced stimulated shedding of the ADAM10 substrate betacellulin, whereas the ionomycin-stimulated shedding of the ADAM17 substrates CD62-L and TGFα is not affected. Moreover, we show that influx of extracellular Calcium activates ADAM10, but is not essential for its activation by APMA and BzATP. Finally, the rapid stimulation of ADAM10 is not significantly affected by incubation with pro-protein convertase inhibitors for up to eight hours, arguing against a major role of increased pro-domain removal in the rapid stimulation of...
ADAM10. Thus, the cytoplasmic domain of ADAM10 negatively influences constitutive shedding through an ER retention motif, whereas the cytoplasmic domain and pro-domain processing are not required for the rapid activation of ADAM10-dependent shedding events.

INTRODUCTION

The cell surface metalloproteinase a disintegrin and metalloprotease 10 (ADAM10) is required for the proteolytic release of membrane proteins such as the epidermal growth factor (EGF) (1), betacellulin (BTC) (1,2), the amyloid precursor protein (3) and the low affinity IgE receptor CD23 from cells (4), and also has a critical function in regulating physiological ligand-induced Notch signaling (5-7). ADAM10 is constitutively active in cell-based assays that measure ADAM10-dependent substrate release (1), and its activity can be rapidly upregulated by treatment of cells with the calcium ionophore ionomycin, and by activation of the P2X7 receptor (8,9). ADAM10 is closely related to the protein ectodomain sheddase ADAM17 (also referred to as the TNFα convertase, or TACE), which can also be rapidly activated by several different stimuli (1,8,9). The rapid activation of ADAM17 does not require its cytoplasmic domain and is not regulated by removal of its inhibitory pro-domain by pro-protein convertases (8,10), but instead depends on the presence of its transmembrane domain (11). Little is currently known about the domains of ADAM10 that are important for its constitutive activity or rapid posttranslational activation. The goal of the current study was to learn more about the posttranslational regulation of ADAM10 and to determine what role, if any, the cytoplasmic- and the transmembrane domains of ADAM10 have in the constitutive and regulated shedding of its substrates. In addition, we explored whether pro-domain removal by pro-protein convertases as well as influx of calcium or other ions is important for the stimulation of ADAM10-dependent shedding.

EXPERIMENTAL PROCEDURES

Cell lines and reagents - Embryonic fibroblasts from Adam10/17−/− mice (mEF) were described previously (9). Cos7 cells were from ATCC (Manassas, VA). Cells were grown in DMEM, with antibiotics and 10% FCS. All reagents were from Sigma-Aldrich (St Louis, MO) unless otherwise indicated. The pro-protein convertase inhibitor Decanoyl-RVKR-CMK (RVKR), the chloride channel inhibitor diphenylamine-2-carboxylate and ionomycin (IO) were from Calbiochem (San Diego, CA); monoclonal mouse anti-HA antibodies were from Covance (Emeryville, CA). Marimastat was a gift from Ouathek Ouerfelli (Sloan-Kettering Institute, New York, NY).

Expression vectors - Expression vectors for wild type (WT) mouse ADAM10, the catalytically inactive mouse ADAM9E>A (A9E>A), the human P2X7R and alkaline phosphatase (AP)-tagged substrate proteins human betacellulin (BTC), human TGFα and human CD62L were described previously (1,4,8,9). The mouse ADAM10Δcyto mutant and the chimera between mouse ADAM10 and mouse ADAM17 were generated by fusion PCRs with murine ADAM10 and ADAM17 cDNAs as a template (see Table 1 for the sequences of the chimera and other mutants used in this study) (8).

Cell culture, transfection, ectodomain shedding assays - Cells were transiently transfected with the indicated plasmids using Genjet (SignaGen, Ijamsville, MD), Turbofect (ThermoFisher Scientific, Bremen, Germany) or Lipofectamine 2000 (Life Technologies, Carlsbad, CA) with essentially identical results. Shedding assays were performed the day after transfection (1,8,9,12). For shedding experiments including inhibitors, cells were pre-incubated with or without inhibitors for 2 to 12 hours, as indicated. For stimulation experiments, the cells were washed briefly 3 times followed by incubations with the indicated stimulus for 45 minutes. Constitutive shedding was measured after 2 hours of incubation. In experiments in which BzATP was used to stimulate ADAM10, its receptor, the human P2X7R was co-transfected with the ADAM10 substrate BTC-AP. Experiments to test the requirement for extracellular calcium ions were performed in MinimalMedium (MgSO₄ 7H₂O (0.814 mM), KCl (5.33 mM), NaHCO₃ (44.05 mM), NaCl (81.9 mM), NaH₂PO₄ H₂O (0.9 mM), Hepes (25.03 mM)) with or without added CaCl₂.
AP activity in the supernatants and cell lysates was measured by colorimetry (12). The ratio between the supernatant AP activity and the total AP activity in the cell lysate plus supernatant was calculated from three identically prepared wells, and averaged. This value can be used for side-by-side comparisons of the activity of a given sheddase towards a given AP-tagged protein for the indicated stimulus in a specific cell type. Evaluation of AP activity in the supernatants and cell lysates by SDS–PAGE or by colorimetric assays was performed as described previously (12).

**Western blot analysis** - Western blot analysis was performed as described (13). Briefly, for detection of endogenous ADAM10 or transfected hemagglutinin (HA)-tagged mouse ADAM10 and the mouse ADAM10/17 mutants, cells were lysed in PBS, 1% Triton X-100, 5 mM 1,10 phenantroline, protease inhibitor cocktail (14). The lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes (13), which were probed with the appropriate antibodies as indicated. Equal sample loading was confirmed by Ponceau S staining of the nitrocellulose membrane after transfer.

**Cell Surface Biotinylation** – For surface biotinylation experiments, cells were washed twice in ice cold PBS, and then incubated in PBS with 1 mg/ml of the non-membrane permeable biotinylation reagent NHS-LC-Biotin (Pierce, Rockford, IL) for 45 minutes at 4°C. To stop the biotinylation reaction, the cells were washed twice in ice cold PBS, 50 mM glycine, and then incubated in PBS, 50 mM glycine for 10 minutes. Following lysis in PBS, 1% Triton X-100, 5 mM 1,10 phenantroline, protease inhibitor cocktail (14), the cell surface biotinylated molecules were bound to Streptavidin beads, which were washed 3x in cell lysis buffer, and then boiled in SDS-sample buffer to remove bound proteins. The eluted proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes, and the biotinylated ADAM10 WT or ADAM10Δcyto were detected with antibodies against their C-terminal HA-tag.

**In-gel alkaline phosphatase assay** - The in gel detection of alkaline phosphatase-labeled membrane proteins was performed as previously described (12,15). Briefly, lysates of cells expressing AP-tagged human BTC were separated by SDS-PAGE, and the AP was re-natured by incubating the gel for 2 x 30 minutes in 2.5% Triton X-100, and visualized by adding the AP-substrates Nitro-Blue Tetrizolium (NBT) and (5-Bromo-4-Chloro-3'-Indolylphosphate (BCIP).

**Quantitative real-time-PCR** - RNA was isolated with the SV Total RNA Isolation Kit (Promega, Madison, WI) and reverse transcribed using PrimeScript™ RT Master Mix (TaKaRa, Mountain View, CA) according to the manufacturer’s protocols. Quantitative real-time-PCR (qPCR) was performed in a StepOnePlus real time PCR instrument (Applied Biosystems, Grand Island, NY). The reaction mixture consisted of 10 ng cDNA, 0.25 µM of each ADAM10 primer (CGCACACCTCTGGCTGAAAG; CCGAGAAGTCTGTAGTCTG) and 5 µl SYBR Premix Ex Taq II Master Mix (TaKaRa Mountain View, CA) per 10 µl mixture.

**Statistical analysis** - All data are representative of at least three separate experiments. Statistical analyses were performed using an unpaired 2-tailed Student’s t-test, with p<0.05 considered statistically significant (indicated by an asterisk). In figure 7A, non-parametric Two-way ANOVA was performed along with Tukey’s multiple comparison post-hoc tests to assess statistical significance with a 95% confidence interval. All calculations were performed using SigmaSTAT 3.1 software.

**RESULTS**

The cytoplasmic domain of ADAM10 exerts a negative control on its constitutive activity. To learn about the role of the cytoplasmic domain of ADAM10 in regulating its shedding activities, we transfected Adam10/17/-/- mouse embryonic fibroblasts (mEFs) with the ADAM10 substrate betacellulin (BTC) together with either wild type ADAM10 (ADAM10 WT, A10 WT) or ADAM10 lacking its cytoplasmic domain (ADAM10Δcyto, A10Δcyto). The catalytically inactive ADAM9E>A (A9E>A) mutant was used as a negative control instead of ADAM10E>A, because the latter is known to have dominant negative properties (16-19). The use of
Adam10/17/-/ double knockout cells transfected with ADAM10 in these experiments ensured that the BTC shedding was dependent on ADAM10 (9), whereas co-transfection with the inactive ADAM9E>A mutant allowed us to determine baseline shedding levels for BTC in these cells in the presence of an over-expressed membrane protein of similar domain structure as ADAM10. When we analyzed the levels of membrane-anchored alkaline phosphatase-tagged BTC (BTC-AP) in Adam10/17/-/ cells by using an in-gel alkaline phosphatase detection assay (see materials and methods for details), we found significantly lower levels of BTC-AP in the lysates of cells that were co-transfected with ADAM10 WT or ADAM10Δcyto compared to cells expressing the inactive ADAM9E>A. Prolonged incubation of transfected cells (12 hours) with 5 µM of the hydroxamate-type metalloprotease inhibitor Marimastat (MM) resulted in similar levels of BTC-AP in all samples. This suggested that the decrease in BTC-AP in the ADAM10 and ADAM10Δcyto-transfected cells was caused by the catalytic activity of the overexpressed enzymes (Fig. 1A, quantification shown in B). Thus, both ADAM10 WT and ADAM10Δcyto appear to have significant constitutive activity, enough to reduce the levels of BTC-AP compared to cells transfected with the inactive ADAM9E>A.

Next we analyzed how the cytoplasmic domain of ADAM10 affected the constitutive shedding of BTC-AP into the supernatant. For this purpose, we pre-treated Adam10/17/-/- mEFs expressing BTC-AP together with ADAM9E>A, ADAM10 WT or ADAM10Δcyto with 5 µM MM for 12 hours to prevent substrate depletion by the active forms of ADAM10, and then rapidly washed the inhibitor out to initiate constitutive shedding for 2 hours. Under these conditions, which ensured that the substrate levels were comparable at the outset of the experiment (as shown in the MM-treated samples in Fig. 1A and B), it became clear that the ADAM10Δcyto mutant had increased catalytic activity compared to ADAM10 WT (Fig. 1C), despite substantially lower levels of the pro-form and somewhat reduced levels of the mature form (Fig. 1D, expression of the ADAM9E>A mutant is shown in Fig. 1E). It should be noted that the ADAM10Δcyto mutant also consumed slightly more of the substrate in the 2 hours after the MM was washed out than ADAM10 WT, yet presumably sufficient amounts of substrate remained to ensure that the substrate levels were not limiting in samples pre-treated with MM (Fig. 1C, lower panel). Finally, when we performed a cell surface biotinylation, we found stronger labeling of the mature form of ADAM10Δcyto compared to ADAM10 WT. Taken together, these results suggest that the cytoplasmic domain of ADAM10 is a negative regulator of its ability to shed BTC-AP from cells.

The Western blot analysis of ADAM10 WT and ADAM10Δcyto suggested that the cytoplasmic domain of ADAM10 affects the levels of transfected ADAM10 in mEFs, since lower levels of pro-ADAM10Δcyto were expressed compared to the pro-ADAM10 WT, despite using identical amounts of expression plasmids for transfection experiments (Fig. 1D). This was further corroborated by transfecting Adam10/17/-/- mEFs with different amounts of expression plasmids (0.1 – 1 µg/well of a 6-well plate), which resulted in comparable mRNA expression of ADAM10 WT and ADAM10Δcyto, as determined by qPCR (Fig. 1A). Nevertheless, the protein expression levels of pro-ADAM10Δcyto were lower than for pro-ADAM10 WT at all three plasmid concentrations (Fig. 2B), whereas the sheddase activity of ADAM10Δcyto was higher at all three plasmid concentrations over 2 hours (Fig. 2C).

An ER-retention motif in the cytoplasmic domain of ADAM10 controls its constitutive activity. Previous studies have identified an ER-retention motif at position 723 in the cytoplasmic domain of ADAM10 (20). Mutation of this motif from ADAM10723RRR to ADAM10723RAR or its deletion through a truncation after residue 721 (ADAM10Δ721) has been shown to increase transport of the mutant proteins to the cell surface (20). Here, we demonstrate that both mutants have a similarly increased constitutive activity over 2 hours as ADAM10Δcyto (Fig. 3), suggesting that the increased activity is caused by a decrease in retention of the mutant proteins in the ER, thus providing a likely explanation for their enhanced constitutive activity.
The cytoplasmic domain of ADAM10 is not required for stimulated shedding. Next, we took a similar approach to determine whether the cytoplasmic domain of ADAM10 is required for its stimulation by various known activators of ADAM10. There was very little shedding of BTC-AP from Adam10/17-/- mEFs co-transfected with ADAM9E>A under unstimulated conditions or after stimulation with 250 µM 4-aminophenylmercuric acetate (APMA), 2.5 µM of the calcium ionophore ionomycin (IO), 600 µM of the alkylating agent N-ethylmaleimide (NEM), or 300 µM of the nucleotide receptor agonist Benzoyl-ATP (BzATP) for 45 minutes (Figure 4A-D). All four stimuli activated BTC-AP shedding in the presence of ADAM10 WT, and there was also an increase in constitutive shedding compared to ADAM10 WT-transfected cells, as described above. Moreover, the shedding in ADAM10Δcyto-transfected cells was significantly enhanced by all four stimuli, with an approximately comparable amount of increase over constitutive shedding as seen in ADAM10 WT-transfected cells (although minor effects of the cytoplasmic domain on stimulated shedding cannot be ruled out).

Evaluation of the role of the transmembrane domain of ADAM10 in regulating its sheddase activity. Since the transmembrane domain of ADAM17 is required for its response to a variety of stimuli (11), we tested whether the transmembrane domain of ADAM10 is important for its activation by ionomycin (IO). For this purpose, we generated chimeric constructs containing the extracellular domain of ADAM10 fused to the transmembrane- and cytoplasmic-domain of ADAM17 or only the transmembrane domain of ADAM17, without its cytoplasmic domain (KKTT or KKT, where K stands for Kuzbanian, an alternative name for ADAM10 (6), and T stands for TACE, or TNFα convertase (21), an alternative name for ADAM17, see Table 1 and the diagram in Figure 5A for details). We observed slightly increased shedding of BTC from Adam10/17-/- mEFs co-transfected with KKTT or KKT compared to ADAM10 WT under unstimulated conditions. However, addition of IO only stimulated BTC-AP shedding in the presence of ADAM10 WT, but not in the presence of the KKTT and KKT mutants (Fig. 5B). A Western blot analysis confirmed comparable expression levels of the pro- and mature forms of ADAM10 WT, KKTT and KKT (Fig. 5C, mature forms marked by arrowheads). Previous studies have shown that ADAM10 can cleave substrates that are primarily ADAM17 substrates, but only when ADAM17 is inactivated and ADAM10 is stimulated to enhance its activity (9). Interestingly, when we co-transfected KKTT or KKT with CD62-L (L-selectin) or TGFα, two substrates whose primary sheddase is ADAM17 (1,9,22), we found a similar increase in shedding following stimulation with IO compared to ADAM10 WT (Fig. 5D). None of the constructs tested here was able to support PMA-stimulated shedding of CD62L or TGFα, which is a hallmark feature of ADAM17 (Fig. 5E). Finally, KKTT and KKT promoted slightly higher levels of constitutive shedding of CD62-L and TGFα than ADAM10 WT (Fig. 5E).

The cytoplasmic domain of ADAM10 does not affect the catalytic activity of ADAM17 in cell-based assays. Next we tested whether the cytoplasmic domain of ADAM10 could negatively affect the activity of ADAM17. We found that replacement of the cytoplasmic domain of ADAM17 with that of ADAM10 had no detectable effect on the sheddase activity of ADAM17, just like removal of the cytoplasmic domain of ADAM17 (Fig. 6A, B). However, when the transmembrane domain of ADAM10 was used to replaced that transmembrane domain of ADAM17, then the ability of ADAM17 to respond to PMA was lost, regardless of whether the cytoplasmic domain was present or not (Fig. 6A, B). None of these constructs could promote the PMA-dependent shedding of the ADAM10 substrate BTC (Fig. 6C), even though the expression levels were approximately comparable by Western blot analysis (Fig. 6D).

Inhibition of pro-protein convertases does not significantly reduce the rapid activation of ADAM10 by ionomycin. Next we focused on the pro-domain of ADAM10, which is removed by pro-protein convertases in the
secretory pathway (23). Processing of the pro-domain of ADAM10 in the secretory pathway is thought to be a pre-requisite for its ability to acquire catalytic activity. In order to test whether pro-protein convertases (PC) are also required for the rapid response of ADAM10 to stimulation with ionomycin, we pre-incubated Cos7 cells expressing BTC-AP with the PC inhibitor RVKR for up to 8 hours, and then stimulated them for 45 minutes in the continued presence or absence of RVKR. There was no significant difference in the release of BTC-AP from Cos7 cells under constitutive conditions and following IO stimulation in the presence or absence of the PC inhibitor at the different time points tested, although we cannot rule out a minor decrease in the presence of the inhibitor (Fig. 7A). Moreover, the amount of mature endogenous ADAM10 was not significantly affected by treatment with the PC inhibitor for up to 4 hours, but was significantly reduced after 8 hours (Fig. 7B, quantification shown in C). As a positive control for the inhibitory effect of RVKR, we expressed the recombinant ADAM17 metalloprotease and pro-domain fused to an Fc-protein (11), and found that pro-domain removal was completely blocked in the presence of the PC inhibitor (data not shown, see also (11)).

**Evaluation of the contribution of ion flux across cell membranes to the activation of ADAM10.** The next point we addressed was whether stimulation of ADAM10 by IO depends entirely on influx of extracellular calcium, or whether other major ions such as sodium, potassium or chloride could be involved in this process. When Cos7 cells were incubated with different concentrations of IO, only the relatively high concentration of 2.5 μM IO activated ADAM10 strongly, whereas concentrations of 1 μM or lower did not (Fig. 8A). This concentration of IO is likely to promote a strong influx of extracellular Ca++ (24), which could also lead to a change in membrane potential and subsequent activation of cation and anion fluxes for normalization of the potential, so that secondary ion fluxes could be responsible for the stimulation of ADAM10. However, when we added the Na⁺-ionophores Monensin (200 μM) or Nystatin (300 μg/ml) or the K⁺-ionophore Valinomycin (10 μM, Fig 8B) or the Cl⁻-ionophore I (25) (10 μM, Fig. 8C) to Cos7 cells expressing BTC-AP, we saw no activation of BTC-AP shedding. Moreover, IO-stimulated shedding of BTC-AP was not blocked by the Chloride channel blocker, diphenylamine-2-carboxylate (200 μM, Fig. 8C).

To determine how crucial influx of extracellular Ca++ is for all four stimuli of ADAM10 used in this study, we stimulated Cos7 cells transfected with the purinergic receptor P2X7R and BTC-AP with IO (2.5 μM), APMA (250 μM), BzATP (300 μM) or NEM (600 μM) in the presence or absence of Ca++ in the medium (Fig. 9). We found that Ca++ was required for the stimulation of ADAM10 by IO and NEM, but not for its stimulation by APMA and BzATP. Taken together, these results demonstrate that influx of extracellular Ca++ is sufficient to activate ADAM10, but not required for its activation.

**DISCUSSION**

ADAM10 is required for the proteolytic release of several cell surface proteins with important roles in development and diseases such as Alzheimer’s and allergic responses, yet much remains to be learned about how ADAM10-dependent protein ectodomain shedding is regulated. Our results demonstrate that the cytoplasmic domain of ADAM10 functions as a negative regulator of the constitutive activity of ADAM10, but is dispensable for the rapid posttranslational stimulation of this enzyme. The cytoplasmic domain of ADAM10 is known to contain an ER-retention motif and basolateral sorting signals in polarized epithelial cells (26). We found that deletion of the ER-retention motif or a point mutation in this motif both had the same effect as deleting the cytoplasmic domain altogether. Both mutants have been shown to traffic to the cell surface more efficiently (26), and we show that this is also the case for the ADAM10Δcyto protein. The lack of an ER-retention motif also explains our finding that the levels of pro-ADAM10Δcyto are lower than those of the ADAM10 WT, despite transfection with comparable amounts of expression vector for either form. Thus, the increased sheddase activity of ADAM10Δcyto and the two mutants that affect the ER retention motif compared to
the ADAM10 WT indicates that the intracellular domain of ADAM10 functions as a negative regulator of its constitutive shedding activity by limiting the exit of ADAM10 from the ER. Targeting this ER retention motif could thus represent an attractive means to modulate the activity of ADAM10, which has been shown to function as a protective $\alpha$-secretase in the context of Alzheimer’s disease (27-30).

The observation that the cytoplasmic domain of ADAM10 is not required for stimulation of ADAM10-dependent shedding of BTC-AP shows for the first time that ADAM10 resembles the related metalloprotease ADAM17 in that a mutant form of ADAM17 lacking its cytoplasmic domain can be activated as efficiently as wild type ADAM17 by various stimulators of ectodomain shedding (11,31,32). Taken together, these results also help explain the previously reported observation that there is less stimulated shedding of BTC and EGF in Adam10--/ cells rescued with ADAM10Δcyto compared to cells rescued with ADAM10 WT (8). The increased constitutive activity of ADAM10Δcyto most likely depleted the membrane-anchored substrate to a point where any increase in stimulated shedding was not evident, unless the cells are pre-treated with MM to normalize the substrate levels at the outset of the experiment, as shown here.

Moreover, the finding that the transmembrane domain of ADAM10 is important for the ionomycin-stimulated shedding of BTC uncovers another similarity to ADAM17, which requires its transmembrane domain in order to respond to various stimuli of ectodomain shedding (11). This suggested the possibility that ADAM17 is regulated through an interaction with one or more other membrane proteins, which was later corroborated by the identification of the seven-membrane-spanning iRhoms1 and 2 as crucial regulators of ADAM17-dependent shedding events (33-36). Recently an interaction of ADAM10 with tetraspanins that regulates its function and transport to the cell surface was reported (37-40). However, in preliminary studies (performed in collaboration with Drs. Michael Tomlinson and Paul Saftig), we found that over expression of Tspan15 increased the activity of the KKTT and KKT to a similar degree as it did for ADAM10 WT and ADAM10Δcyto (data not shown). While these experiments do not rule out a functionally relevant interaction between tetraspanins and the transmembrane domain of ADAM10, it is clear that further studies will be necessary to understand the mechanism responsible for regulating the stimulation of ADAM10 through its transmembrane domain. Interestingly, the constitutive and ionomycin-stimulated shedding of CD62-L and TGF$\alpha$, two substrates of ADAM17 that are only shed by ADAM10 in cells lacking ADAM17 (9), still occurred in cells expressing the KKTT or KKT chimeras, and was similar to the shedding seen in ADAM10Δcyto-expressing cells. Thus the transmembrane domain of ADAM10 appears to have a role in the ionomycin-stimulated shedding of the ADAM10 substrate BTC, but not in the constitutive or ionomycin-stimulated shedding of the ADAM17-substrates CD62-L and TGF$\alpha$, suggesting that the transmembrane domain of ADAM10 also has a role in determining its substrate selectivity.

When the cytoplasmic domain of ADAM17 was replaced with that of ADAM10, this did not detectably reduce ADAM17-dependent shedding compared to full length ADAM17 or ADAM17 lacking its cytoplasmic domain. Thus the negative regulatory effect of the cytoplasmic domain of ADAM10 on the constitutive activity of ADAM10 apparently does not affect ADAM17. On the other hand, when the extracellular domain of ADAM17 was attached to the transmembrane domain of ADAM10, or to the transmembrane domain and cytoplasmic domain of ADAM10, then all PMA-stimulated activity was abolished, although constitutive activity was not affected. These results are consistent with a model in which the transmembrane domain of ADAM17 is important for its stimulation by various stimuli, most likely because of its interaction with iRhoms (34,35).

The experiments addressing the role of pro-protein convertases (PC) as regulators of ADAM10 argue against a major role for PCs in the stimulation of ADAM10-dependent shedding, although longer incubation with the PC inhibitor should eventually lead to depletion of mature ADAM10 and loss of its constitutive activity (23). Similar findings have been
reported for ADAM17, which does not require processing by PCs for its rapid activation by various signaling pathways (11). Additional information on the regulation of ADAM10 is provided by our observation that its activation by IO is regulated by an influx of extracellular Ca++ ions and not by Na+, K+ or Cl− fluxes. However, extracellular Ca++ is not absolutely required for the activation of ADAM10, at least by APMA and BzATP.

Taken together, our findings demonstrate that the constitutive activity of ADAM10 is negatively regulated by an ER-retention motif in its cytoplasmic domain. We also show that the transmembrane domain of ADAM10 is required for stimulation of ADAM10-dependent shedding, whereas the cytoplasmic domain is not. Moreover, we show that pro-protein convertases or influx of extracellular Ca++ are not required for the rapid activation of ADAM10, although influx of extracellular Ca++ can activate ADAM10. Further studies will be necessary to explore how the transmembrane domain of ADAM10 controls stimulated ADAM10-dependent processing events.
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FIGURE LEGENDS

Figure 1. The cytoplasmic domain of ADAM10 is a negative regulator of constitutive shedding. (A) Mouse embryonic fibroblasts lacking ADAMs10 and 17 (Adam10/17-/- mEFs) were transfected with alkaline phosphatase-tagged betacellulin (BTC-AP) together with the inactive ADAM9E>A mutant (A9E>A), wild-type ADAM10 (A10WT), or ADAM10 lacking its cytoplasmic domain (A10Δcyto). An in-gel alkaline phosphatase (AP) assay was used to visualize the levels of membrane-anchored BTC-AP in the cell lysates of cells incubated with or without the metalloprotease inhibitor Marimastat (MM, 5 µM) for 12 hours, as indicated. The figure is representative of 3 experiments (densitometric quantification of the results of all 3 experiments is shown in panel (B)). (C) Adam10/17-/- mEFs transfected with BTC-AP were pre-incubated with or without MM (5µM) for 12 hours. Then the cells were washed, and incubated for 2 hours. MM blocked the constitutive shedding in the first 12 hours, and could be readily washed out, resulting in increased BTC shedding from cells transfected with A10WT or A10Δcyto treated with MM compared with cells not incubated with MM. The top panel shows the AP activity in the conditioned medium (CM), whereas the lower panel shows the AP activity in the cell lysate (CL) (n = 17; mean ± SD). (D) Western blot analysis of the expression of A10WT and A10Δcyto in Adam10/17-/- mEFs, using antibodies against the C-terminal HA-tag of the expressed proteins. (E) Western blot of the expression of ADAM9 in Adam10/17-/- mEFs that were not transfected or were transfected with A9E>A. (F) Detection of the biotinylated mature form of A10WT or A10Δcyto after precipitation of cell surface biotinylated materials with Streptavidin-sepharose followed by detection with antibodies against the HA tag attached to both ADAM10 constructs. The Western blots (D, E) and the detection of cell surface biotinylation (F) are representative of 3 experiments each. Mean ±/− SD. *P<0.05, unpaired 2-tailed Student’s t-test.

Figure 2. Analysis of the mRNA and protein expression of ADAM10 WT and ADAM10Δcyto in Adam10/17-/- mouse embryonic fibroblasts. Adam10/17-/- mEFs were co-transfected with increasing amounts of A9E>A, A10 WT or A10Δcyto (0.1, 0.5 and 1.0 µg/ well of a 6-well plate) together with BTC-AP. The wells were then treated with 5 µM of the hydroxamate-type metalloprotease inhibitor Marimastat (MM) for 12 hours, and then the MM was washed out and the cells incubated for 2 hours to monitor constitutive shedding. (A) qPCR confirmed comparable mRNA expression of A10 WT and A10Δcyto in Adam10/17-/- cells (representative of 3 experiments). (B) Western blot analysis of the expression of A10 WT and A10Δcyto in Adam10/17-/- mEFs transfected with different amounts of expression vector, as indicated. The Western blot is representative of 3 experiments. (C) Increasing amounts of transfected A10 WT or A10Δcyto increased the shedding of co-transfected BTC-AP from Adam10/17-/- cells (n=3). Mean ± SD; *P<0.05, unpaired 2-tailed Student’s t test.

Figure 3. Mutations in an ER-retention motif in the cytoplasmic domain of ADAM10 increase constitutive shedding. Adam10/17-/- mEFs expressing BTC-AP together with A9E>A, A10WT, A10Δcyto or ADAM10 that was truncated at cytoplasmic residue 721 to remove an ER retention motif at
position 723 (A10Δ721) or ADAM10 carrying a point mutation in the ER retention motif (RRR>RAR) were pre-incubated with or without MM (5µM) for 12 hours. After washing out the MM for 1 minute, the cells were incubated for 2 hours. We observed increased BTC shedding from Adam10/17−/− mEFs transfected with A10Δcyto, A10Δ721 and A10RRR>RAR compared to cell expressing A10 WT. AP activity in the conditioned medium (CM) in shown in the top panel, whereas the AP activity in the cell lysate (CL) is shown in the lower panel (n = 3; mean + SD). *P<0.05, unpaired 2-tailed Student’s t-test.

Figure 4. Response of wild type ADAM10 and ADAM10Δcyto to stimuli of protein ectodomain shedding. Adam10/17−/− cells were co-transfected with A9E>A, A10 WT or A10Δcyto and BTC-AP, treated with 5µM MM for 12 hours, and then the MM was washed out and the cells either not treated or stimulated for 45 minutes with (A) Aminophenylmercuric acetate (APMA, 250 µM), (B) ionomycin (IO, 2.5 µM), (C) N-ethyl-maleimide (NEM, 600 µM) or (D) benzyl-ATP (BzATP 300µM, cells treated with BzATP were also co-transfected with P2X7R). All stimuli tested here activated A10 WT and A10Δcyto, as evidenced by the significantly increased shedding of BTC compared to unstimulated controls. Arrows with numbers indicate the increase in the average AP-ratio following stimulation over constitutive shedding. Mean + SD, *P<0.05, unpaired 2-tailed Student’s t-test. 

Figure 5. Rescue of constitutive and stimulated shedding of BTC in Adam10/17−/− cells by ADAM10/ADAM17 chimeras. (A) Diagram of the chimeras containing the extracellular domain of ADAM10 fused with the transmembrane- and cytoplasmic domain of ADAM17 (KKTT) or with only the transmembrane domain of ADAM17 (KKT). Domains of ADAM17 (TACE, T) or ADAM10 (KUZ, K) are represented by red bars and blue bars, respectively. (B) Constitutive and ionomycin (2.5 µM IO) stimulated shedding of BTC-AP from Adam10/17−/− mEFs transfected with A9E>A, A10 WT or KKTT or KKT (the supernatants were collected after 45 minutes incubation, n = 8; mean + SD). (C) Western blot analysis of A10 WT, KKTT and KKT expression, detected with antibodies against a C-terminal HA-tag, representative of n=3 experiments. Arrowsheads indicate the position of the mature form of each mutant (D, E) Constitutive and IO-stimulated (D) or PMA-stimulated (E) shedding of the ADAM17-substrates CD62-L-AP or TGFα-AP from Adam10/17−/− mEFs transfected with A9E>A, A10 WT, KKTT or KKT. In all experiments shown in panels B, D and E, Adam10/17−/− mEFs were pre-incubated with MM (5µM) for 12 hours, and then the MM was washed out and the cells were stimulated with 2.5 µM IO (B, D) or 25ng/ml PMA (E) or left untreated for 45 minutes (n=5). Mean + SD; *, #: P<0.05 for increase in stimulated shedding (*) of KKTT, KKT- or A10Δcyto-transfected cells over A9E>A transfected cells, unpaired 2-tailed Student’s t-test.

Figure 6. PMA-stimulated shedding of TGFα or BTC in Adam10/17−/− cells by ADAM17/ADAM10 chimeras. (A) Schematic representation of full length ADAM17 (TACE, T, red) and ADAM10 (Kuz, K, blue), as well as a chimera with the extracellular domain of ADAM17 attached to the cytoplasmic domain of ADAM10 (TTTK), ADAM17 lacking its cytoplasmic domain (TTT), or the extracellular domain of ADAM17 attached to the transmembrane domain and cytoplasmic domain of ADAM10 (TTK) or only the transmembrane domain of ADAM10 (TTK). (B,C) Constitutive and PMA-stimulated (25 ng/ml PMA) shedding of TGFα-AP (B) or BTC-AP (C) from Adam10/17−/− mEFs transfected with A9E>A, A17 WT, TTTK, TTT, TTKK or TTK, as indicated. All conditioned supernatants were collected after 45 minutes incubation, n = 3; mean + SD). (D) Western blot analysis of all constructs shown in (A), probed with antibodies against the C-terminal HA-tag on these constructs (except A9E>A, which is not tagged, see Fig. 1), representative of n=3 experiments. In the experiments shown in panels B and C, Adam10/17−/− mEFs were pre-incubated with MM (5µM) for 12 hours, then the MM was washed out and 25ng/ml PMA was added to stimulate the cell where indicated (n=3). Mean + SD; *: P<0.05 for increase in stimulated shedding (*) over constitutive shedding unpaired 2-tailed Student’s t-test.
Figure 7. Stimulation of ADAM10 does not depend on rapid pro-domain processing. (A) Cos7 cells were transfected with BTC-AP to monitor the activity of endogenous ADAM10, and were then pre-incubated for different times (2–8 hours) with 50 µM of the pro-protein convertase inhibitor RVKR, and then incubated with or without 2.5 µM IO for 45 minutes in the presence or absence of RVKR. No significant effect of RVKR on the shedding of BTC was observed at any of the time points analyzed here (n = 5; mean ± SD, 2-way ANOVA, followed by Tukey’s test, n.s. indicates no significant difference between the conditions indicated by the lines). (B) Western blot of endogenous ADAM10 in Cos7 cells treated with 50 µM RVKR for 2, 4 or 8 hours (as in A) and probed with anti-ADAM10 cytoplasmic domain antibodies. The densitometric quantification of Western blots of 3 separate experiments (C) shows that there was no significant reduction in the ratio of mature to pro-ADAM10 in RVKR-treated cells versus untreated controls up to 4 hours.

Figure 8. Ionomycin-activated shedding of BTC by ADAM10 depends on influx of calcium. (A) Cos7 cells transfected with BTC-AP were incubated with different concentrations of ionomycin (IO, 0.1 – 2.5 µM). Only the highest concentration of 2.5 µM strongly activated ADAM10. (B) BTC-AP-transfected Cos7 cells were incubated with the Na⁺-ionophores Monensin (200 µM) or Nystatin (300 µg/ml) or the K⁺-ionophore Valinomycin (10 µM) or IO (2.5 µM). ADAM10-dependent shedding was only stimulated by IO. (C) Cos7 cells transfected with BTC-AP were treated with Cl⁻-ionophore I (10 µM) or with IO (2.5 µM) alone or in the presence of the chloride channel blocker, di-phenylamine-2-carboxylate (200 µM). No stimulatory effect of the chloride ionophore on constitutive shedding was observed, and the chloride channel blocker did not affect IO stimulated shedding. n = 3; mean ± SD, *P<0.05, unpaired 2-tailed Student’s t test.

Figure 9. Influx of extracellular Ca⁺⁺ is crucial for the activation of ADAM10 by IO or NEM, but not by APMA or BzATP. Cos7 cells expressing BTC-AP were stimulated with NEM (600 µM), IO (2.5 µM), APMA (250 µM) or BzATP (300 µM, BzATP-treated cells were co-transfected with the P2X7R receptor) in MinimalMedium (see materials and methods for details) in the presence or absence of Ca⁺⁺ in the medium. Free calcium ions were required for the stimulation of ADAM10 by NEM and IO, but not for its stimulation by APMA and BzATP. n = 3; mean ± SD, *P<0.05, unpaired 2-tailed Student’s t test, n.s. indicates no significant difference between the conditions indicated by the lines.
| Extracellular domain | Predicted Transmembrane domain | Predicted cytoplasmic domain |
|----------------------|---------------------------------|------------------------------|
| A10wt                | ...YENIAE WIVAHWWAVLLMGIALIMLM | AGFIKICSVHTPSSNPKLPPPKP     |
| A10Δcyto             | ...YENIAE WIVAHWWAVLLMGIALIMLM | LPGTLKRRRPPQPIQQPRQRPR     |
| A17wt                | ...FLADNI VGSVLVFLIFWIPFSILVH | ESYQGHMR-R-HA-tag           |
| A17Δcyto             | ...FLADNI VGSVLVFLIFWIPFSILVH | CVDDKLD-HA-tag              |
| KKTT                 | ...YENIAE VGSVLVFLIFWIPFSILVH | CVDDKLD-HA-tag              |
| KKT                  | ...YENIAE VGSVLVFLIFWIPFSILVH | CVDDKLD-HA-tag              |
| TTTK                 | ...FLADNI VGSVLVFLIFWIPFSILVH | CVDDKLD-HA-tag              |
| TTKK                 | ...FLADNI WIVAHWWAVLLMGIALIMLM | AGFIKICSVHTPSSNPKLPPPKP     |
| TTK                  | ...FLADNI WIVAHWWAVLLMGIALIMLM | LPGTLKRRRPPQPIQQPRQRPR     |
| A10Δ721              | ...YENIAE WIVAHWWAVLLMGIALIMLM | ESYQGHMR-HA-tag             |
| A10RRR>RAR           | ...YENIAE WIVAHWWAVLLMGIALIMLM | AGFIKICSVHTPSSNPKLPPPKP     |

Table 1 Amino acid sequence of ADAM10/17 constructs
**Figure 1**

**A**

Marimastat

| A9 E>A | A10 WT | A10Δcyto | A9 E>A | A10 WT | A10Δcyto |
|--------|--------|----------|--------|--------|----------|
|        |        |          |        |        |          |

A10/17/-/- mEFs /BTC-AP, cell-lysates

MW: kDa

**B**

Densitometry arbitrary values

| A9 E>A | A10 WT | A10Δcyto | A9 E>A | A10 WT | A10Δcyto |
|--------|--------|----------|--------|--------|----------|
|        |        |          |        |        |          |

**C**

*Adam10/17/-/- mEFs/BTC-AP*

| 1st | 12h pre-treatment | Ctrl | Ctrl | Ctrl | MM | MM | MM |
|-----|-------------------|------|------|------|----|----|----|
| 2nd | 1min washout      | +    | +    | +    | +  | +  | +  |
| 3rd | 2h experiment     | Ctrl | Ctrl | Ctrl | Ctrl | Ctrl | Ctrl |

**D**

AP-activity in CM

- ■ A9 E>A
- □ A10 WT
- ▪ A10Δcyto

**E**

AP-activity in CL

- ■ A9 E>A
- □ A10 WT
- ▪ A10Δcyto

**F**

Biotinylation: anti-HA tag

MW: kDa
Figure 2

A

Adam10/17/-/- mEFs

| mRNA transcript/10ng cDNA | 0  | .1 | .5 | 1  | 0  | .1 | .5 | 1  |
|---------------------------|----|----|----|----|----|----|----|----|
| A10 WT                    |    |    |    |    |    |    |    |    |
| A10Δcyto                  |    |    |    |    |    |    |    |    |

μg/well

B

MW: kDa

| MW: kDa | 0 | .1 | .5 | 1 |
|---------|---|----|----|---|
| A10 WT  |   |    |    |   |
| A10Δcyto|   |    |    |   |

WB: anti-HA tag

C

Adam10/17/-/- mEFs + BTC-AP

| AP-ratio | .1 | .5 | 1  | .1 | .5 | 1  |
|----------|----|----|----|----|----|----|
| A9 E>A   |    |    |    |    |    |    |
| A10 WT   |    |    |    |    |    |    |
| A10Δcyto|    |    |    |    |    |    |

μg/well
Figure 3

![Graph showing AP activity in CM and CL for different conditions.](Attachment)

- **ADAM10/17-/- mEFs**
  - A9 E>A
  - A10 WT
  - A10Δcyto
  - A10Δ721
  - A10 RRR>RAR

*Note: Significant differences indicated by asterisks.*

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Figure 4

A

Adam10/17/-/ mEFs/BTC-AP

- A9 E>A
- A10 WT
- A10Δcyto

AP-ratio

APMA
- + - + - +

B

Adam10/17/-/ mEFs/BTC-AP

- A9 E>A
- A10 WT
- A10Δcyto

AP-ratio

IO
- + - + - +

C

Adam10/17/-/ mEFs/BTC-AP

- A9 E>A
- A10 WT
- A10Δcyto

AP-ratio

NEM
- + - + - +

D

Adam10/17/-/ mEFs/P2X7R/BTC-AP

- A9 E>A
- A10 WT
- A10Δcyto

AP-ratio

BzATP
- + - + - +
Figure 5

A

A17 (FACE)  A10 (KUZ)  KKTT  KKT

B

Adam10/17-/- mEFs + BTC-AP (A10 substrate)

C

untransfected  A10 WT  KKTT  KKT

WB: anti-HA tag

MW: kDa

100

60

D

Adam10/17-/- mEFs + A17 substrates

CD62-L-AP

TGFα-AP

AP-ratio

E

AP-ratio

PMA

AP-ratio

PMA
Figure 6

A

B

Adam10/17-/- mEFs + TGFα (A17 substrate)

C

Adam10/17-/- mEFs + BTC (A10 substrate)

D

untransfected A17 TTKK TTT TTK

WB: anti-HA tag
MW: kDa
Figure 7

A

Cos7/BTC-AP

AP-ratio

RVKR

0h 2h 4h 8h

Ctrl IO RVKR RVKR/IO

n.s n.s n.s

B

Cos7

RVKR (50μM)

Time (h): 0 2 4 8

proform

mature

MW: kDa

-100

-60

-50

WB: anti-A10

tubulin

C

Arbitrary units

pro mature

Time (h) 0 2 4 8

*
The Cytoplasmic Domain of A Disintegrin and Metalloproteinase 10 (ADAM10) Regulates its Constitutive Activity but is Dispensable for Stimulated ADAM10-dependent Shedding
Thorsten Maretzky, Astrid Evers, Sylvain Le Gall, Rolake O. Alabi, Nancy Speck, Karina Reiss and Carl P. Blobel

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