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Tumor Necrosis Factor-α Convertase (ADAM17) Mediates Regulated Ectodomain Shedding of the Severe-acute Respiratory Syndrome-Coronavirus (SARS-CoV) Receptor, Angiotensin-converting Enzyme-2 (ACE2)*

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Angiotensin-converting enzyme-2 (ACE2) is a critical regulator of heart function and a cellular receptor for the causative agent of severe-acute respiratory syndrome (SARS), SARS-CoV (coronavirus). ACE2 is a type I transmembrane protein, with an extracellular N-terminal domain containing the active site and a short intracellular C-terminal tail. A soluble form of ACE2, lacking its cytosolic and transmembrane domains, has been shown to block binding of the SARS-CoV spike protein to its receptor. In this study, we examined the ability of ACE2 to undergo proteolytic shedding and investigated the mechanisms responsible for this shedding event. We demonstrated that ACE2, heterologously expressed in HEK293 cells and endogenously expressed in Huh7 cells, undergoes metalloproteinase-mediated, phorbol ester-inducible ectodomain shedding. By using inhibitors with differing potency toward different members of the ADAM (a disintegrin and metalloproteinase) family of proteinases, we identified ADAM17 as a candidate mediator of stimulated ACE2 shedding. Furthermore, ablation of ADAM17 expression using specific small interfering RNA duplexes reduced regulated ACE2 shedding, whereas overexpression of ADAM17 significantly increased shedding. Taken together, these data provided direct evidence for the involvement of ADAM17 in the regulated ectodomain shedding of ACE2. The identification of ADAM17 as the protease responsible for ACE2 shedding may provide new insight into the physiological roles of ACE2.

Angiotensin-converting enzyme-2 (ACE2)† is the only known mammalian homologue of the zinc-metalloproteinase angiotensin-converting enzyme (ACE), a critical regulator of the renin-angiotensin system (1, 2). Unlike ACE, which functions as a peptidyl dipeptidase, ACE2 acts as a carboxypeptidase, able to cleave a single C-terminal residue from a number of physiologically significant peptides (3, 4). Although the precise physiological role of ACE2 remains to be identified, studies in vivo have revealed that ACE2 is an essential regulator of heart function (5). ACE2 is also thought to have an important regulatory role in the renin-angiotensin system through its ability to convert the potent vasoconstrictor angiotensin II to the vasodilatory peptide angiotensin-1-7 (6). In addition, ACE2 has been recently identified as a functional receptor for the causative agent of severe-acute respiratory syndrome (SARS), the SARS coronavirus (7, 8).

ACE2 is an 805-amino-acid glycoprotein with an apparent molecular mass of 120 kDa (3, 9). Like ACE, ACE2 is a type I transmembrane protein comprising a short C-terminal cytoplasmic tail, a hydrophobic transmembrane region, and a heavily N-glycosylated N-terminal ectodomain containing the active site (3). ACE2 has a more restricted tissue distribution than ACE, being found predominantly in the heart, kidneys, and testes (3, 4), although low levels have been detected in a variety of tissues (10).

It is becoming increasingly apparent that the proteolytic shedding of cell surface proteins is an important mechanism regulating their expression and function. This ectodomain shedding event has been observed for a variety of membrane proteins with distinct functions, including cytokines (tumor necrosis factor-α (TNF-α), fractalkine), enzymes (ACE, β-site amyloid cleaving enzyme (BACE)), adhesion molecules (L-selectin), and proteins associated with neuropathological disorders (amyloid precursor protein (APP), cellular prion protein) (11–17). Although many of these proteins are shed in vitro under normal cell culture conditions, shedding is often stimulated by phorbol esters such as PMA (phorbol-12-myristate-13-acetate) and a number of other factors (18, 19). Both constitutive and PMA-stimulated ectodomain shedding appear to be mediated largely by members of the ADAM family (a disintegrin and metalloproteinase) of zinc-metalloproteinases (reviewed in Ref. 20). The best characterized ADAM protease to date is ADAM17, or TNF-α-converting enzyme, which was first identified as the sheddase for TNF-α but has subsequently been implicated in the shedding of other cell surface proteins (21–23). Other members of the ADAM family of proteinases, particularly ADAM9, ADAM10, and ADAM12, have been implicated as candidate sheddases for a wide range of proteins including APP, CXCL16, and heparin-binding epidermal growth factor (24–27).
In this study, we have sought to determine whether ACE2 undergoes proteolytic cleavage resulting in the release of its ectodomain. We provided evidence that a catalytically active soluble form of ACE2 is cleaved from the membrane by two distinct mechanisms: a low level constitutive shedding and a regulated shedding stimulated by phorbol ester. We further used a variety of strategies to establish that ADAM17 is responsible for the regulated shedding of ACE2. The implications of these findings for the role of ACE2 in vivo are also discussed.

**MATERIALS AND METHODS**

PMA and all other routinely used reagents were purchased from Sigma unless otherwise stated. The hydroxamate-based inhibitors TAPI-1 and GM6001 were purchased from Calbiochem and Chemicon, respectively. The ADAM-specific inhibitors GW280264X (2,3R-TAPI-1 and GM6001 were purchased from Calbiochem and Chemicon, respectively. Genejuice transfection reagent was obtained from Novagen, and Lipofectamine and Oligofectamine were obtained from Invitrogen. RNA duplexes for siRNA were obtained from Eurogenetics, according to the manufacturer's instructions.

**Cell Culture and Transfection—**HEK293 cells (kindly provided by Prof. N. Buckley, University of Leeds) and HuH7 cells (kindly provided by Dr. M. Harris, University of Leeds) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stable expression of human ACE2 in HEK293 cells was achieved by transfecting pCI-Neo expression plasmids (Promega) encoding full-length ACE2 and neomycin phosphotransferase (5-materials and methods).

**Plasmid Construction—**DNA encoding full-length human ACE2 (GenBank™ accession number AB046569) was amplified from a human kidney cDNA library (Clontech) using the primer pair 5'-ACG-TCCATGCTCAATTCCTGTCATCTCACTCATTGTTT-3' (forward) and 5'-CT-AGGCTAAAGGAGGCTCAATGACTGCTGTTT-3' (reverse), digested, and ligated into the XbaI and Xhol sites of the pCI-Neo/H11032 plasmid (kindly provided by Immunex Corp.) was reamplified by automated sequencing (Lark Technologies Inc.). Murine ADAM17 was obtained from Calbiochem, and a polyclonal antibody to human ADAM9 from Ongenece. A polyclonal antibody to β-actin was purchased from Sigma. Donkey anti-goat and sheep anti-rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma and Amershams Bio-Sciences, respectively. Genejuice transfection reagent was obtained from Invitrogen, and Lipofectamine and Oligofectamine were obtained from Invitrogen. C-terminal ACE2 peptide sequences were obtained from Peptecuals and Santa Cruz Biotechnology. Additionally, a polyclonal antibody raised to the ectodomain of human ACE2 was obtained from R&D Systems. The ACE2-specific fluorescent substrate Mca-APK(Dnp) was synthesized by Dr. G. Knight (Cambridge University, Cambridge, UK). A polyclonal antibody to human ADAM17 was obtained from Chemicon, a polyclonal antibody to human ADAM9 from Oncogene. A polyclonal antibody to β-actin was purchased from Sigma. Donkey anti-goat and sheep anti-rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma and Amershams Biosciences, respectively. Genejuice transfection reagent was obtained from Novagen, and Lipofectamine and Oligofectamine were obtained from Invitrogen. DNA duplexes for siRNA were obtained from Eurogentec or Dharmaco, as indicated.

**Fluorogenic Assay of ACE2—**The enzymatic activity of recombinant ACE2 was measured using the specific fluorogenic substrate Mca-APK(Dnp). The reaction product was quantified using a standard curve generated from known concentrations of Mca.

| Target | Sense strand sequence |
|--------|-----------------------|
| ADAM9a | 5'-UCACCGGAGAAGCUUCCGAGC99-3' |
| ADAM9b | 5'-ACUCUCCAGGUGUAGAAUCCGAGC99-3' |
| ADAM10a | 5'-UGAAAGGAGGACACUCUCCG99-3' |
| ADAM10b | 5'-GUGGGCCUCCCUCUCCUCCAC99-3' |
| ADAM17a | 5'-CUGAGGCCCGUUCCCGUUCGGG99-3' |
| ADAM17b | 5'-AUUUCUGUUGGUUCUCGCUUC99-3' |
| ADAM17c | 5'-AUGAAGGAGGAAAGCACAGUG99-3' |
| GL-2 | 5'-CGGACGCGGAAUACUCUGGAGC99-3' |

**RESULTS**

To determine whether ACE2 undergoes ectodomain shedding, we first created a stable line of HER293 cells expressing human ACE2, designated HEK-ACE2. Western blot analysis of detergent extracts collected from HEK-ACE2 cells (Fig. 1, indicated by C) revealed the presence of a polypeptide of ~120
ADAM17-mediated Regulated ACE2 Shedding

Fig. 1. Shedding of ACE2 involves loss of its cytoplasmic tail. HEK293 cells were stably transfected with an expression vector encoding full-length ACE2 as described under “Materials and Methods.” OptiMEM containing 0.1 µM PMA or an equal volume of Me2SO carrier was added to exponentially growing cells and collected after 1 h. Following sedimentation of cells, the media were concentrated 10-fold, and 40 µg of media proteins (M) were separated by SDS-PAGE (6% v/v) alongside 20 µg of corresponding detergent cell extract (C) and immunoblotted with an antibody raised to the ectodomain of ACE2 (left panel, ectodomain) or the cytosolic tail of ACE2 (right panel, cytosolic). Immunoreactive bands were visualized with enhanced chemiluminescence as described under “Materials and Methods.”

Fig. 2. Shed ACE2 occurs as two distinct glycoforms. HEK-ACE2 cells were incubated in OptiMEM containing 0.1 µM PMA or an equal volume of Me2SO carrier for 1 h, and the media were collected and concentrated as described. Media proteins (M: 40 µg) or detergent cell extracts (C: 20 µg) were incubated at 37 °C for 16 h in the presence or absence of endoglycosidase H (Endo H) or PNGase F and subsequently separated by SDS-PAGE. Following electrotransfer, immunoblotting was carried out using an antibody to the ectodomain of ACE2 as described under “Materials and Methods.”

Reduced potency toward ADAM17 (12, 14). Immunoblotting revealed that the mixed ADAM10/ADAM17 inhibitor (GW280264X) effectively blocked stimulated ACE2 shedding at low concentrations, whereas the ADAM10-selective inhibitor (GI254023X) had a much lower potency (Fig. 4).

We next sought to determine whether the two polypeptides of 95 and 105 kDa, corresponding to shed forms of ACE2, represented different glycosylation states. Treatment of the cellular and media proteins with endoglycosidase H did not affect the electrophoretic mobility of the cell-associated or shed protein; treatment with PNGase F, however, produced bands of ~85 and 80 kDa for membrane-bound and secreted ACE2, respectively (Fig. 2). Soluble ACE2 released under basal and stimulated conditions had indistinguishable electrophoretic mobility following deglycosylation with PNGase F. These results indicated that the two forms of ACE2 released by cleavage-secretion represent different glycoforms, both containing mature carbohydrate groups. Similar results were obtained in Chinese hamster ovary cells stably transfected with ACE2 (data not shown).

The shedding of a number of membrane proteins, such as TNFα, APP, and ACE, is inhibited by certain hydroxamic acid-derived metalloproteinase inhibitors. Thus, we next examined whether the PMA-stimulated and basal shedding of ACE2 was sensitive to such inhibitors. Both TAPI-1 and GM6001 inhibited the PMA-stimulated release of ACE2 in a dose-dependent manner, as demonstrated by Western blotting (Fig. 3). Basal shedding of ACE2 was largely insensitive to both inhibitors in this system, with a maximal decrease in shedding of ~25% observed (Fig. 3).

Having established that PMA-stimulated ACE2 shedding is inhibited by non-selective secretase inhibitors, we next sought to identify the secretase(s) involved. To this end, we used two synthetic compounds displaying differential potency toward two members of the ADAM family of metalloproteinases, ADAM10 and ADAM17. Compound GW280264X is a potent inhibitor of both ADAM10 and ADAM17, whereas compound GI254023X is a potent inhibitor of ADAM10 but has >100-fold reduced potency toward ADAM17 (12, 14). Immunoblotting revealed that the mixed ADAM10/ADAM17 inhibitor (GW280264X) effectively blocked stimulated ACE2 shedding at low concentrations, whereas the ADAM10-selective inhibitor (GI254023X) had a much lower potency (Fig. 4).

We next tested the ability of the natural inhibitors of AD-AMs, TIMP-1 and TIMP-3, to inhibit ACE2 shedding. The purified N-terminal domains of TIMP-1 and TIMP-3 (NTIMP-1 and NTIMP-3) were used as these have greater potency toward their respective proteases (28). TIMP-3 is a potent inhibitor of ADAM10 and ADAM17, whereas TIMP-1 only inhibits ADAM10 (29, 30). As demonstrated by Western blotting, NTIMP-3 inhibited PMA-stimulated ACE2 shedding in a dose-dependent manner, reducing shedding to below basal levels at 50 nM (Fig. 5). NTIMP-1, in contrast, had little effect on stimulated ACE2 shedding. These data were consistent with ADAM17 being involved in stimulated ACE2 shedding.

To confirm the involvement of ADAM17 in stimulated ACE2 shedding, we next used siRNA to reduce the expression of ADAM17, alongside ADAM10, in HEK-ACE2 cells. Western blotting of cell lysates collected from cells transfected with specific siRNAs revealed a significant reduction in the level of both ADAM10 and ADAM17 protein (~64 and 78%, respectively; Fig. 6A) as compared with cells transfected with siRNA to a control sequence and mock transfected cells. Analysis of media proteins collected following incubation of transfected cells showed an associated decrease in stimulated ACE2 shedding (Fig. 6, A and C). Cells treated with control double-stranded RNA showed no alteration in stimulated ADAM10 or ADAM17 expression or ACE2 shedding.

To further examine the importance of ADAM17 in stimulated ACE2 shedding, we next transiently overexpressed ADAM17 and its homologues ADAM9 and ADAM10 in HEK-ACE2 cells. Successful overexpression was confirmed by immunoblotting of cell lysates (Fig. 7A). Analysis of media proteins collected from transfected cells incubated in the presence of PMA revealed that overexpression of ADAM17 increased stimulated ACE2 shedding ~2-fold as compared with the mock transfected cells (Fig. 7, A and B). Overexpression of both ADAM9 and ADAM10 had no significant effect.

Having established a role for ADAM17 in the regulated shedding of ACE2 heterologously expressed in HEK293 cells, we next sought to determine whether ACE2 is similarly processed in an endogenously expressing system. For these studies, we utilized Huh7 cells, a cell line derived from a hepatocellular carcinoma that expresses significant levels of ACE2 (Fig. 8A) (31). Analysis of medium harvested from Huh7 cells by immunoblotting with an antibody raised to the ectodomain of ACE2 revealed the presence of two bands, of apparent molecular mass of 95 and 105 kDa, which could not be detected by an antibody directed to the intracellular domain (Fig. 8A). This
was in keeping with the results obtained with HEK-ACE2 cells and indicates an ectodomain shedding event. The abundance of the soluble species was elevated by treatment with PMA, resulting in a corresponding increase in ACE2 activity in the media (Fig. 8B). This increase was significantly ablated by the broad range metalloproteinase inhibitor GM6001 and by the mixed ADAM10/ADAM17 inhibitor GW280264X but was unaffected by the ADAM10 inhibitor GI254023X (Fig. 8B). The constitutive shedding of ACE2 was not significantly altered by any of the inhibitors tested, suggesting a distinct mechanism, as shown in HEK-ACE2 cells. These results implied a role for ADAM17 in the regulated ectodomain shedding of endogenously expressed ACE2, a finding corroborated by the results of experiments in which the cellular levels of both ADAM17 and ADAM10 were ablated by RNA. Reduction of the expression of ADAM17 significantly decreased the PMA-stimulated...
sheding of ACE2 in Huh7 cells (Fig. 9, A and B). Ablation of ADAM10 expression did not reduce the stimulated shedding of ACE2 in the same system. Furthermore, overexpression of ADAM17 resulted in an increase in the regulated shedding of ACE2 in Huh7 cells (Fig. 9, A and B). Ablation of ADAM10 expression did not reduce the stimulated shedding of ACE2 in the same system. Furthermore, overexpression of ADAM17 resulted in an increase in the regulated shedding of ACE2 from Huh7, an effect not seen following overexpression of either ADAM9 or ADAM10 (Fig. 10, A and B).

**DISCUSSION**

Many physiologically significant cell surface proteins are subject to a proteolytic cleavage event resulting in ectodomain shedding. Often this shedding event is an important regulatory mechanism (11, 12) or results in the release of a protein with a distinct physiological function (14, 16). In this study, we demonstrated that the SARS-CoV receptor, ACE2, undergoes proteolytic shedding, releasing an enzymically active ectodomain.

Constitutive shedding of both heterologously and endogenously expressed ACE2 resulted in the extracellular release of two forms of the enzyme with distinct electrophoretic mobilities (~105 and 95 kDa). Both species contain mature carbohydrate moieties, migrating at ~80 kDa following enzymatic deglycosylation with PNGase F. The presence of a single band following deglycosylation suggests that the difference in electrophoretic mobility is a result of differential glycosylation rather than the result of cleavage occurring at two distinct sites. Immunoblotting using antibodies targeted to different domains of ACE2 reveals the absence of cytoplasmic domains in the soluble species, indicating that they are generated by a proteolytic ectodomain shedding event.

The ectodomain shedding of ACE2 from HEK-ACE2 and Huh-7 cells was rapidly stimulated by the phorbol ester PMA, with a corresponding increase in ACE2 activity in the media. The broad spectrum hydroxamic acid-based metalloproteinase inhibitors, TAPI-1 and GM6001, both inhibited the PMA-stimulated shedding of ACE2 at low micromolar concentrations, with a concomitant reduction in ACE2 activity in the media. Neither inhibitor, however, significantly affected basal shedding, suggesting that distinct mechanisms are responsible for constitutive and phorbol ester-stimulated shedding of ACE2.

The sensitivity of stimulated ACE2 shedding to hydroxamic acid-based inhibitors is in keeping with results obtained with many other shed proteins, including TNF-α, fractalkine, and APP, all of which are known to be secreted by members of the ADAMs family of zinc metalloproteases (11, 12, 16). Evidence of a role for ADAMs proteases in ACE2 shedding was provided by the results of experiments using synthetic inhibitors of two members of this family of proteases, ADAM10 and ADAM17. Stimulated ACE2 shedding was strongly inhibited by the mixed ADAM10/ADAM17 inhibitor but was unaffected by the selective ADAM10 inhibitor. Neither inhibitor significantly abrogated basal shedding, providing further evidence for a distinct shedding mechanism. These data implicated ADAM17 as a candidate sheddase for stimulated ACE2 shedding, a conclusion supported by results gained in HEK-ACE2 cells using the N-terminal domains of two natural inhibitors of metalloproteases, TIMP-1 and TIMP-3. Although TIMP-1 did not inhibit constitutive or stimulated ACE2 shedding, TIMP-3 preferentially inhibited the stimulated shedding. These two members of the TIMP family of glycoproteins, which play an important role in matrix metalloprotease processing and regulation, have been shown to selectively inhibit distinct ADAMs proteases. TIMP-1 is a potent inhibitor of ADAM10 but is ineffective...
against ADAM8, ADAM9, ADAM12, and ADAM17 (32). TIMP-3, however, is a potent inhibitor of ADAM17 but also inhibits ADAM10.

Direct evidence for the involvement of ADAM17 in the regulated shedding of ACE2 was provided by the results of experiments in which the cellular level of ADAM17 was depleted by specific RNA interference. Abrogation of ADAM17 protein expression resulted in a proportional reduction in stimulated ACE2 shedding while having no significant effect on constitutive shedding. No change in ACE2 shedding was observed following reduction of ADAM10 expression. Furthermore, transient overexpression of ADAM17, but not ADAM9 or ADAM10, resulted in a significant increase in stimulated ACE2 shedding as compared with mock-transfected controls, providing strong evidence that ADAM17 is responsible for regulated shedding of ACE2.

Analysis of the amino acid sequence of the predicted juxtamembrane stalk region of ACE2 reveals no marked sequence conservation with the corresponding region of ACE. ACE is also subject to ectodomain shedding, but the secretase responsible for this still remains to be identified (33, 34). Interestingly, it has been reported that ADAM17 is not responsible for ACE ectodomain shedding (33, 34). Although it is tempting to con-
clude that this is due to the divergence of sequence in the juxtaglomerular region, it is important to note that ADAM17 appears to be able to cleave a diverse array of sequences (21–23). Indeed, it has been suggested that the structure of the stalk region may be a more important determinant of susceptibility to secretease cleavage than the amino acid sequence around the cleavage site (35, 36). In addition, it has been recently reported that differences observed between the shedding of single and dual domain ACE (with identical juxtaglomerular domain sequences) may be due to the occlusion of a sheddase “recognition motif” present within the ectodomain (37).

Although it is difficult to predict the physiological importance of ACE2 ectodomain shedding, evidence is available that such a shedding process occurs in vivo. Donoghue et al. (39) observed the presence of soluble ACE2 following cardiac-specific overexpression of membrane-bound ACE2 in mice. In addition, we and others have detected soluble ACE2 in human urine (39), where it may be responsible for interstitial formation, we and others have detected soluble ACE2 in human urine (39), where it may be responsible for interstitial formation.