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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 proteases, 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)[1] is the only known mammalian homologue of the zinc-metallopeptidase 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).

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[1] The abbreviations used are: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2; APP, amyloid precursor protein; ADAM, a disintegrin and metalloproteinase; CoV, coronavirus; Dnp, (2,4-dinitrophenol)-OH; HEK, human embryonic kidney; Mca, (7-methoxycoumarin-4-yl)acetyl; PMA, phorbol myristate acetate; SARS, severe acute respiratory syndrome; CoV, coronavirus; TNF-α, tumor necrosis factor-α; TIMP, tissue inhibitor of metalloproteases; NTIMP, N-terminal TIMP; siRNA, small interfering RNA; PNGase F, *N*-glycosidase F.

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*R*,3*S*)-3-(formyl-hydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid ((1*S*)-5-benzyloxycarbamoylamino-1-(1,3-thiazol-2-ylcarbamoyl)-1-pentyl) amide) and GI254023X ((2*R*,3*S*)-3-(formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid ((1*S*)-2,2-dimethyl-1-methylcarbamoyl-1-propyl) amide) (12, 14) were kindly provided by Dr. I. Hussain (GlaxoSmithKline, Harlow, UK). The purified N-terminal domains of TIMP-1 and TIMP-3 were a gift of Prof. H. Nagase (Imperial College, London). Antibodies raised to C-terminal ACE2 peptide sequences were obtained from Pepceuticals 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 ADAM10 was obtained from Calbiochem, and 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 Amersham Biosciences, respectively. Genejuice transfection reagent was obtained from Novagen, and Lipofectamine and Oligofectamine were obtained from Invitrogen. RNA duplexes for siRNA were obtained from Eurogentec or Dharmacon, as indicated.

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 μg per transfection; see below for construction details) using Genejuice transfection reagent (Novagen). Recombinant clones were selected using 100 μg/ml gentamycin sulfate (G418; PAA Laboratories GmbH), and expression of ACE2 was confirmed by Western blotting.

Plasmid Construction—cDNA encoding full-length human ACE2 (GenBank™ accession number AB046569) was amplified from a human kidney cDNA library (Clontech) using the primer pair 5′-ACGTCCATGTCAAGCTCTTCCTGGCTCCTTCTC-3′ (forward) and 5′-CTAGGCTAAAAGGAGGTCTGAACATCATCAGTGTTT-3′ (reverse), digested, and ligated into the XbaI and XhoI sites of the pCI-Neo expression vector (Promega). The sequence fidelity of the insert was confirmed by automated sequencing (Lark Technologies Inc.). Murine ADAM17 in pcDNA3.1 (kindly provided by Immunex Corp.) was reamplified using the forward primer 5′-CGGGATCCACCATGAGGCGGCGTCTCCT-3′ (containing the Kozak sequence) and the reverse primer 5′-CCGCTCGAGGCACTCTGTCTCTTTGCTGTCA-3′. The resulting amplicons were digested with BamHI and XhoI and ligated into the expression vector pcDNA3.1a (BD Biosciences). Murine ADAM9 was amplified using the forward primer 5′-CTGGATCCACCATGGGGCCGCGCGCGCT-3′ and the reverse primer 5′-ATAGTTTAGCGGCCGCGGTGAGGGAGCTATATAAAGGAGGTGC AGGAGCG-3′. The resulting amplicons were digested with BamHI and NotI and ligated into the corresponding sites in pcDNA3.1b (BD Biosciences). Bovine ADAM10 was amplified with the forward primer 5′-GGGGTACCGCCACCATGGTGTTGCTGAGAGTGTTAATTC-3′ and the reverse primer 5′-GCTCTAGAACGTCTCATGTGTCCCATCTG-3′. The resulting amplicons were digested with KpnI and XbaI and ligated into the corresponding sites in pcDNA3.1b.

Treatment of Cells and Protein Extraction—Cells were grown to 60% confluence in 75-cm^3 culture flasks and rinsed twice with serum-free medium prior to experimentation. All pharmacological agents were diluted in OptiMEM (5 ml; Invitrogen), and all incubations were carried out at 37 °C. Proteinase inhibitors were added to cells at the indicated concentrations alongside control flasks treated with an equal volume of Me_2SO vehicle. After a 20-min incubation, cells were supplemented with 0.1 μM PMA or an equal volume of Me_2SO, and incubation continued for a further 1 (in the case of HEK-ACE2 cells) or 4 h (in the case of Huh7 cells). Medium was harvested and concentrated by centrifugation in 10-kDa Centricon tubes (VivaScience) to a final volume of ~200 μl. Cells were scraped into ice-cold phosphate-buffered saline, harvested by centrifugation, and solubilized in 500 μl of radioimmune precipitation buffer (0.1 M Tris-HCl. pH 7.4, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P-40). Protein concentration was determined using bicinchoninic acid with bovine serum albumin as a standard.

TABLE I
Sequences of sense strands of double-stranded RNA used to ablate specific protein expression in HEK-ACE2 and Huh7 cells

Target	Sense strand sequence
ADAM9a	5′-UCACUGUGGAGACAUUUGC99-3′
ADAM9b	5′-ACUUCCAGUGUGUAGAUGC99-3′
ADAM10a	5′-UGAAGAGGGACACUUCCCU99-3′
ADAM10b	5′-GUUGCCUCCUCCUAAACCA99-3′
ADAM17a	5′-CAUAGAGCCACUUUGGAGA99-3′
ADAM17b	5′-AGUUUGCUUGGCACACCUU99-3′
ADAM17c	5′-AGUAAGGCCCAGGAGUGUU99-3′
GL-2	5′-CGUACGCGGAAUACUUCGA99-3′

SDS-PAGE and Immunoblotting—Media samples and cell lysates (amounts loaded are indicated in individual figure legends) were separated by SDS-PAGE and proteins electroblotted to polyvinylidene difluoride membranes (Hybaid). Following blocking of nonspecific protein binding sites using 5% (w/v) dried milk in Tris-buffered saline containing 0.5% (v/v) Tween 20, the membranes were incubated with antibodies directed to the ACE2 ectodomain (1:500), the cytoplasmic tail of ACE2 (1:500), ADAM17, ADAM9, or ADAM10 (all diluted 1:1000). Donkey anti-goat and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies were diluted 1:5000. All antibodies were diluted in 5% milk in Tris-buffered saline containing 0.5% (v/v) Tween 20. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Biosciences) according to the manufacturer's instructions.

Fluorogenic Assay of ACE2—The enzymic activity of recombinant ACE2 was measured using the specific fluorogenic substrate Mca-APK(Dnp). Protein (20 μg) was incubated with 50 μM Mca-APK(Dnp) at room temperature, and the fluorescence resulting from substrate hydrolysis was monitored over 1 h using a Victor^2 fluorescence plate reader (Wallac). Specific activity was determined using the ACE2 inhibitor C16, a gift from Dr. Natalie Dales (Millennium Pharmaceuticals). The reaction product was quantified using a standard curve generated from known concentrations of Mca.

Specific mRNA Silencing (siRNA)—HEK-ACE2 or Huh7 cells were grown to ~40% confluence in antibiotic-free growth medium and transfected with siRNA duplexes (Eurogentech and Dharmacon) using Oligofectamine (Invitrogen), according to the manufacturer's instructions. Cells were transfected with 100 nM duplexes targeted to ADAM10, ADAM17, or control duplexes targeted to a scramble sequence or a protein not expressed in mammalian cells (GL2; see Table I for sequence information). Control flasks were subjected to mock transfection with double distilled H_2O. Twenty-four hours after transfection, cell lysates were prepared and assayed for specific gene silencing by Western blotting.

Transient overexpression of ADAM9, ADAM10, and ADAM17—HEK-ACE2 or Huh7 cells (~30–40% confluence) were transfected with 10 μg of ADAM9, ADAM10, or ADAM17 expression plasmids using Lipofectamine (Invitrogen), according to the manufacturer's instructions. Twenty-four hours after transfection, cells were rinsed with OptiMEM and incubated in OptiMEM containing 0.1 μM PMA or an equal volume of Me_2SO carrier for 1 (HEK-ACE2) or 6 h (Huh7). The medium was harvested and concentrated as described above.

RESULTS

To determine whether ACE2 undergoes ectodomain shedding, we first created a stable line of HEK293 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

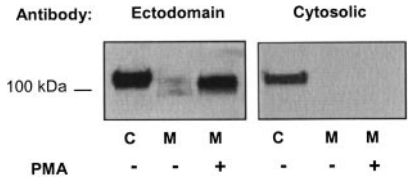

FIG. 1. **Shedding of ACE2 involves loss of its cytoplasmic tail.** HEK293 cells were stably transfected with an expression vector encoding full-length ACE2 as described under "Materials and Methods." OptiMEM containing 0.1 μM PMA or an equal volume of Me_2SO carrier was added to exponentially growing 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."

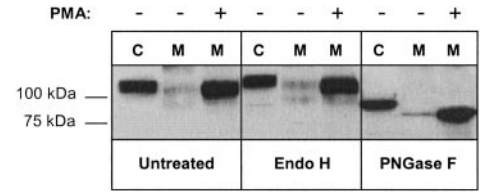

FIG. 2. **Shed ACE2 occurs as two distinct glycoforms.** HEK-ACE2 cells were incubated in OptiMEM containing 0.1 μM PMA or an equal volume of Me_2SO carrier for 1 h, and the media were collected and concentrated as described. Media proteins (*M*; 40 μg) or detergent cell extracts (*C*; 20 μg) were incubated 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."

kDa, corresponding to the mature ACE2 protein (Fig. 1). Immunoblotting of media proteins (*M*) collected following incubation of HEK-ACE2 cells in OptiMEM for 1 h, using an antibody to the ectodomain of ACE2, revealed two bands of ~105 and 95 kDa (Fig. 1, *left panel*). The abundance of the 105-kDa form of ACE2 was significantly enhanced by treatment of the cells with the phorbol ester PMA for 1 h, whereas the abundance of the lower molecular weight species remained unaffected (Fig. 1). Neither form of ACE2, secreted under basal or stimulated conditions, could be detected in the medium by an antibody raised to the cytosolic domain of ACE2 (Fig. 1, *right panel*), implicating a proteolytic cleavage event, most likely occurring in the extracellular juxtamembrane region. The shed ectodomain of ACE2 was enzymically active as determined by an assay using the fluorogenic peptide Mca-APK(Dnp) (data not shown).

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 metalloprotease 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 ADAMs, 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. 6*A*) 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 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. 7*A*). 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. 8*A*) (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. 8*A*). This

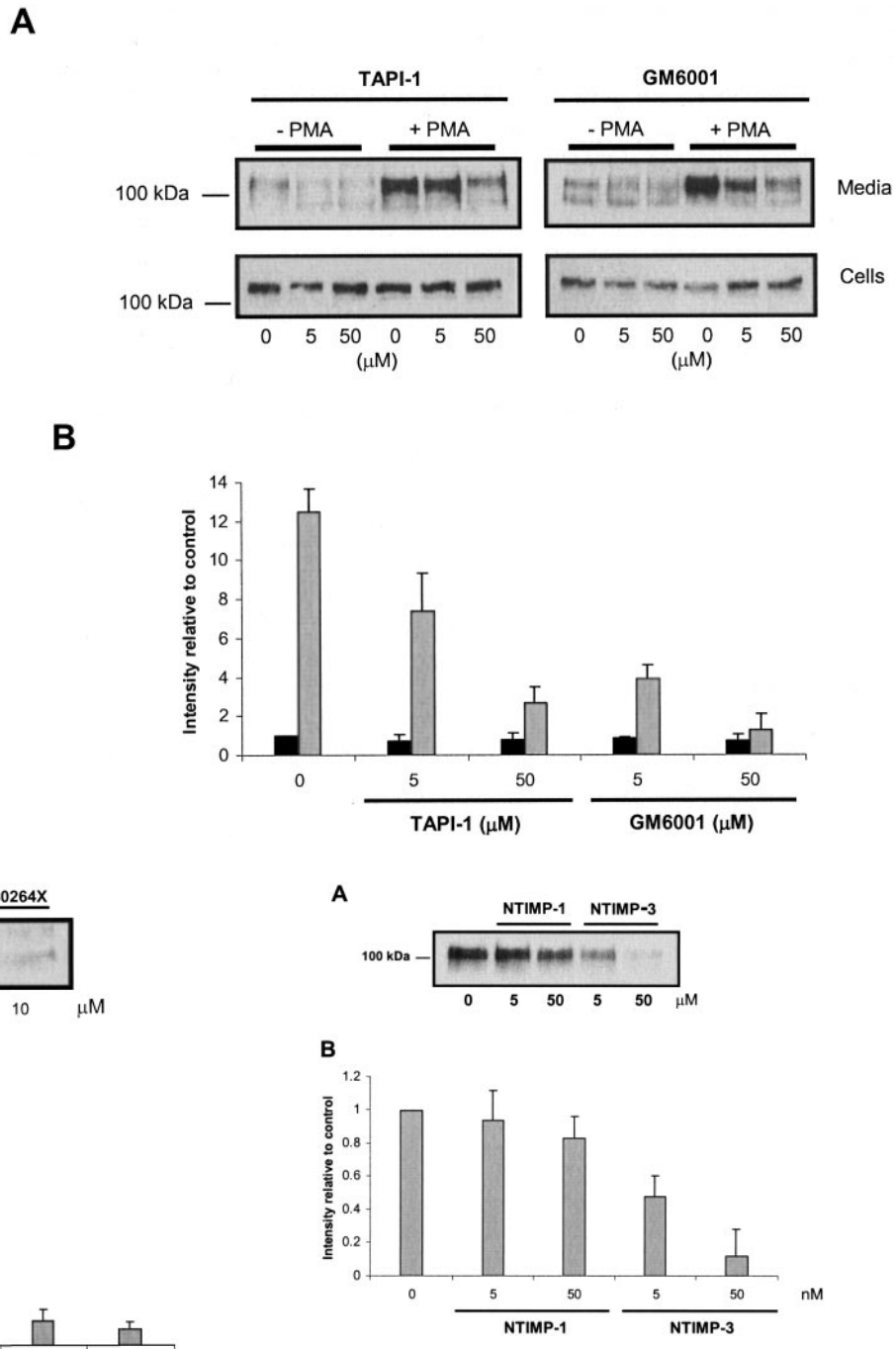

FIG. 3. **Shedding of ACE2 is inhibited by broad spectrum hydroxamate-based metalloprotease inhibitors.** HEK-ACE2 cells were incubated in OptiMEM containing various concentrations of the secretase inhibitors TAPI-1 or GM6001 or an equal volume of carrier (Me_2SO). After 15 min, PMA (0.1 μM) or an equal volume of Me_2SO was added, and incubation was continued for 1 h. The medium was subsequently harvested and concentrated as described; cells were pelleted and detergent extracts were collected as described under "Materials and Methods." *A*, media proteins (*upper panel*, 40 μg) and cell lysates (*lower panel*, 20 μg) were separated by SDS-PAGE and immunoblotted for ACE2. Immunoreactive bands were visualized by enhanced chemiluminescence. *B*, graphical representation of results of densitometric analysis of three such experiments, ± S.E. *Black shading*, −PMA; *gray shading*, +PMA.

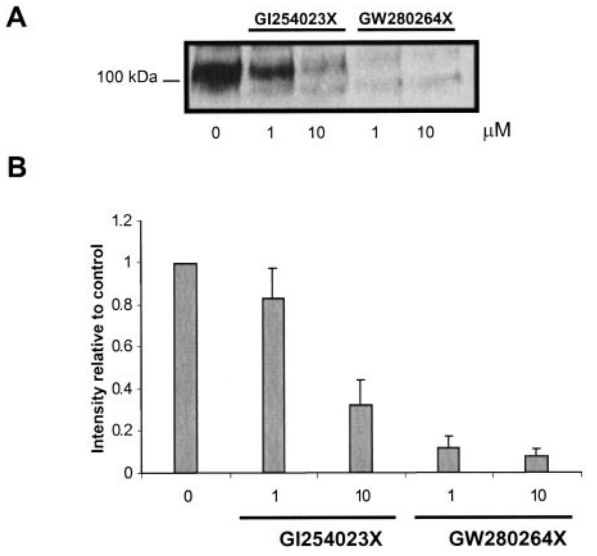

FIG. 4. **PMA-stimulated ACE2 shedding is sensitive to ADAM17 inhibition.** HEK-ACE2 cells were incubated for 15 min in the presence of the ADAM10 inhibitor GI254023X or the mixed ADAM10/ADAM17 inhibitor GW280264X or an equal volume of Me_2SO. Subsequently, incubation was continued in the presence of PMA (0.1 μM). Media were harvested and concentrated as described under "Materials and Methods." *A*, media proteins (40 μg) were separated by SDS-PAGE and immunoblotted for ACE2. *B*, graphical representation of results of densitometric analysis of three such experiments, ± S.E.

FIG. 5. **Stimulated ACE2 shedding is inhibited by NTIMP3 but not by NTIMP1.** HEK-ACE2 cells were incubated for 15 min in the presence of NTIMP1, NTIMP3, or an equal volume of phosphate-buffered saline. Subsequently, incubation was continued in the presence of PMA (0.1 μM). Media were harvested and concentrated as described. *A*, media proteins (40 μg) were separated by SDS-PAGE and immunoblotted for ACE2. *B*, graphical representation of results of densitometric analysis of the immunoblots of three such experiments, ± S.E.

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. 8*B*). 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. 8*B*). 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

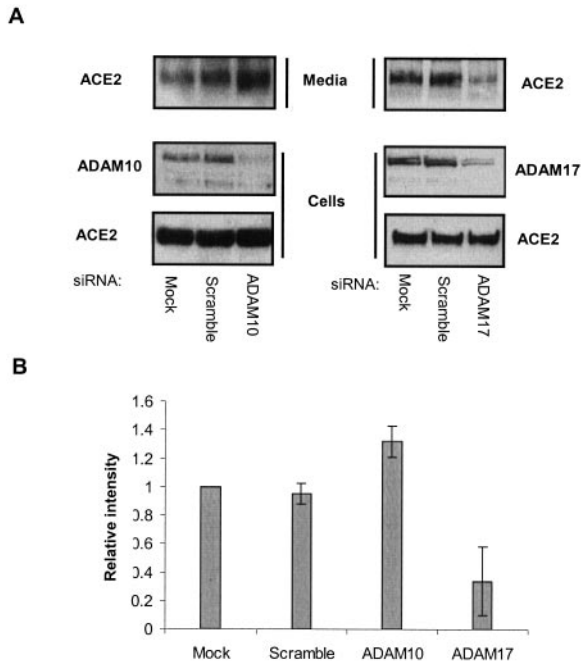

FIG. 6. **Ablation of ADAM17 expression by siRNA reduces stimulated ACE2 shedding.** HEK-ACE2 cells were transiently transfected with a mixture of double-stranded RNA derived from the coding sequence of ADAM10, ADAM17, or the control sequence GL2 as described under "Materials and Methods." Twenty-four hours after transfection, cells were incubated in OptiMEM containing 0.1 μM PMA for 1 h. Media were concentrated as described, and cell lysates were prepared. *A*, media proteins (40 μg) and cell lysates (50 μg) were separated by SDS-PAGE and immunoblotted for ACE2, ADAM10, and ADAM17, as indicated. *Mock*, mock transfection. *B*, graphical representation of densitometric analysis of immunoblots of media ACE2 from three such experiments, ± S.E.

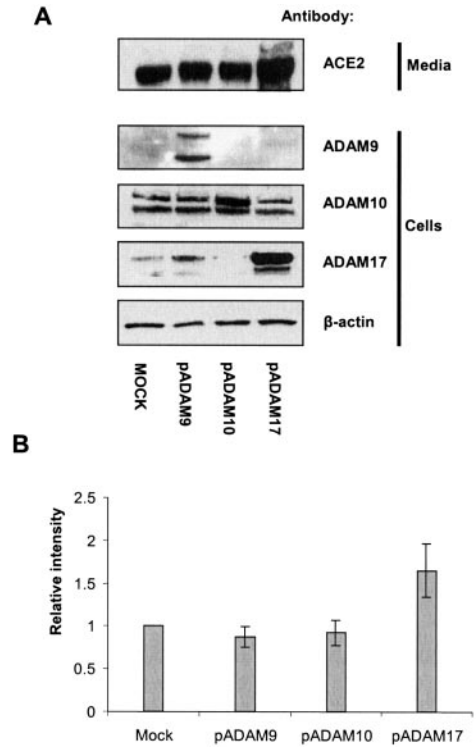

FIG. 7. **Overexpression of ADAM17 increases PMA-stimulated ACE2 shedding.** HEK-ACE2 cells were transiently transfected with an expression vector encoding ADAM9, ADAM10, or ADAM17, as described under "Materials and Methods." Thirty-six hours after transfection, cells were incubated in OptiMEM containing 0.1 μM PMA for 1 h. Media were concentrated as described, and detergent cell extracts were harvested. *A*, media proteins (40 μg) and detergent cell extracts (50 μg) were separated by SDS-PAGE and immunoblotted for ACE2 (*upper panel*, media), ADAM9, ADAM10, or ADAM17 (*lower panels*, lysates). Membranes were stripped and reprobed for β-actin as a loading control. *B*, graphical representation of densitometric analysis of immunoblots of media ACE2 from three such experiments, ± S.E.

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 enzymic 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

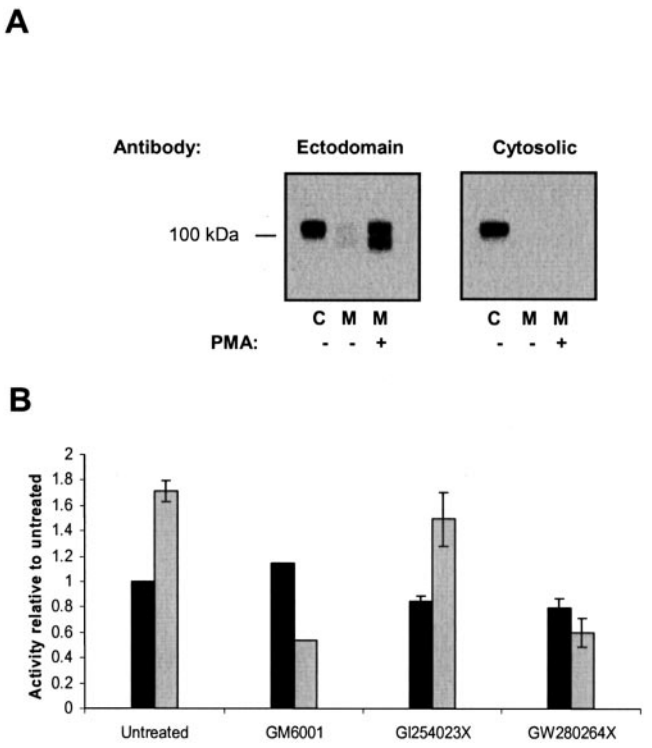

FIG. 8. **PMA-stimulated endogenous ACE2 shedding is sensitive to ADAM17 inhibition.** *A*, OptiMEM containing 0.1 μM PMA or an equal volume of Me_2SO carrier was added to exponentially growing Huh7 cells and collected after 6 h. Following sedimentation of cells, the media were concentrated, and 100 μg of media proteins (*M*) was separated by SDS-PAGE (4–12% v/v) alongside 50 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." *B*, Huh7 cells were incubated for 15 min in the presence of 50 μM GM6001 or 1 μM the ADAM10 inhibitor GI254023X or the mixed ADAM10/ADAM17 inhibitor GW280264X or an equal volume of Me_2SO. Subsequently, incubation was continued for 4 h in the presence or absence of PMA (0.1 μM). Media were harvested and concentrated as described under "Materials and Methods." Concentrated media samples (20 μg) were assayed for their ability to cleave an ACE2-specific fluorogenic substrate, as described under "Materials and Methods." *Black shading*, −PMA; *gray shading*, +PMA.

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-

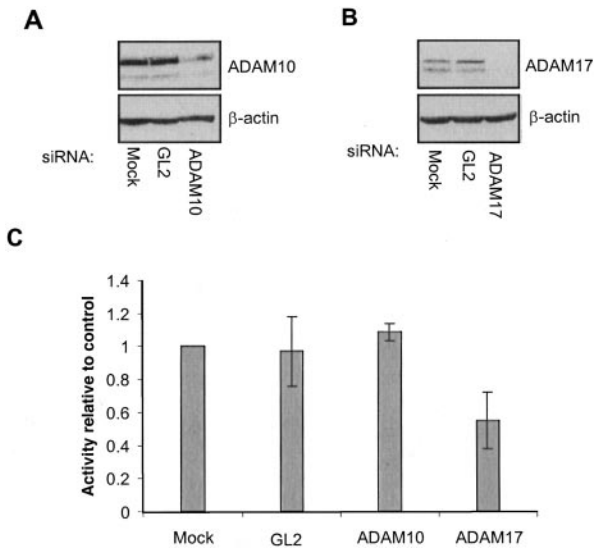

FIG. 9. **Ablation of ADAM17 expression by siRNA reduces regulated endogenous ACE2 shedding.** Huh7 cells (50% confluent) were transfected with double-stranded RNA oligomers (as described under "Materials and Methods") to ADAM10, ADAM17, or a negative control sequence. After 40 h, the media were aspirated and replaced with OptiMEM containing 100 nM PMA or an equal volume of Me_2SO. After a further 5-h incubation, the media were concentrated, and cell lysates were prepared as described under "Materials and Methods." Cell lysates (50 μg) were separated by SDS-PAGE and immunoblotted for ADAM10 (*A*) and ADAM17 (*B*). Concentrated media samples (20 μg) were assayed for their ability to cleave an ACE2-specific fluorogenic substrate as described under "Materials and Methods."

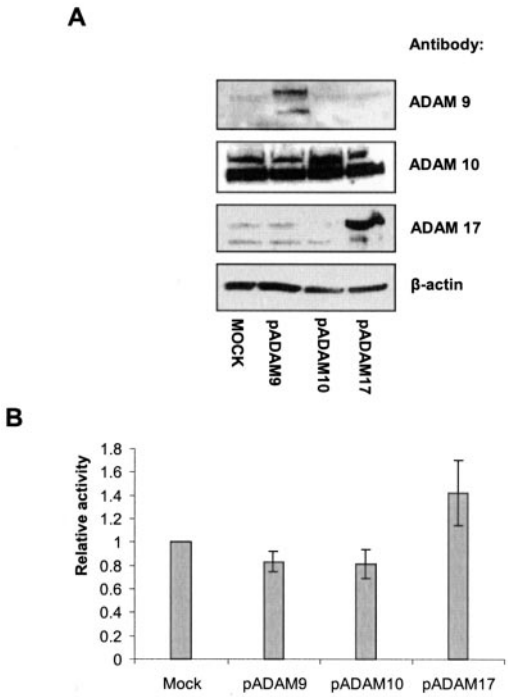

FIG. 10. **Overexpression of ADAM17 increases PMA-stimulated endogenous ACE2 shedding.** HEK-ACE2 cells were transiently transfected with an expression vector encoding ADAM9, ADAM10, or ADAM17, as described under "Materials and Methods." Thirty-six hours after transfection, cells were incubated in OptiMEM containing 0.1 μM PMA for 4 h. Media were concentrated as described, and detergent cell extracts were harvested. *A*, detergent cell extracts (50 μg) were separated by SDS-PAGE and immunoblotted for ADAM9, ADAM10, or ADAM17. Membranes were stripped and reprobed for β-actin as a loading control. *B*, concentrated media samples (20 μg) were assayed for their ability to cleave an ACE2-specific fluorogenic substrate as described under "Materials and Methods."

clude that this is due to the divergence of sequence in the juxtamembrane 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 secretase 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 juxtamembrane 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),[2] where it may be responsible for interstitial formation of the natriuretic peptide . In addition to the possible importance of the shedding of ACE2 to its roles in the heart and the renin-angiotensin system, up-regulation of ACE2 shedding could modulate SARS infectivity. Li *et al.* (7) recently demonstrated that recombinant soluble ACE2 could effectively block the association of the SARS-CoV S1 protein with its cellular receptor. Thus, high levels of shed ACE2 could inhibit the infectivity of the SARS virus.

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Protein Synthesis, Post-Translation Modification, and Degradation:

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)

Daniel W. Lambert, Mike Yarski, Fiona J. Warner, Paul Thornhill, Edward T. Parkin, A. Ian Smith, Nigel M. Hooper and Anthony J. Turner

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