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Angiotensin-Converting Enzyme 2 Ectodomain Shedding Cleavage-Site Identification: Determinants and Constraints

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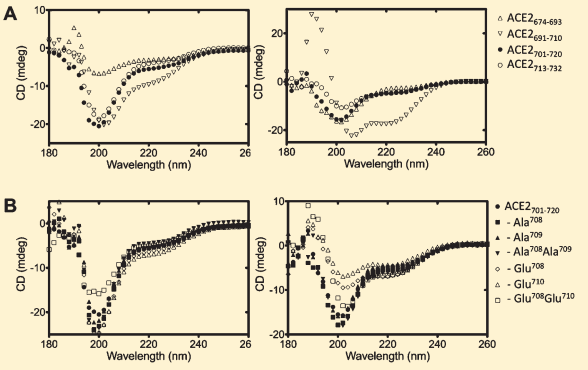
ABSTRACT: ADAM17, also known as tumor necrosis factor α-converting enzyme, is involved in the ectodomain shedding of many integral membrane proteins. We have previously reported that ADAM17 is able to mediate the cleavage secretion of the ectodomain of human angiotensin-converting enzyme 2 (ACE2), a functional receptor for the severe acute respiratory syndrome coronavirus. In this study, we demonstrate that purified recombinant human ADAM17 is able to cleave a 20-amino acid peptide mimetic corresponding to the extracellular juxtamembrane region of human ACE2 between Arg^{708} and Ser^{709}. A series of peptide analogues were also synthesized, showing that glutamate subtitution at Arg^{708} and/or Arg^{710} attenuated the cleavage process, while alanine substitution at Arg^{708} and/or Ser^{709} did not inhibit peptide cleavage by recombinant ADAM17. Analysis of CD spectra showed a minimal difference in the secondary structure of the peptide analogues in the buffer system used for the ADAM17 cleavage assay. The observation of the shedding profiles of ACE2 mutants expressing CHO-K1 and CHO-P cells indicates that the $Arg^{708} \rightarrow Glu^{708}$ mutation and the $Arg^{708}Arg^{710} \rightarrow Glu^{708}Glu^{710}$ double mutation produced increases in the amount of ACE2 shed when stimulated by phorbol ester PMA. In summary, we have demonstrated that ADAM17 is able to cleave ACE2 peptide sequence analogues between Arg^{708} and Ser^{709}. These findings also indicate that Arg^{708} and Arg^{710} play a role in site recognition in the regulation of ACE2 ectodomain shedding mediated by ADAM17.



Angiotensin-converting enzyme 2 (ACE2) is a type I membrane integral protein, with a large extracellular catalytic domain, a single membrane-spanning helix, and a short carboxy-terminal cytoplasmic domain.[1,2] ACE2, like its mammalian homologue, angiotensin-converting enzyme (ACE), can undergo ectodomain shedding to release a catalytically active ectodomain from the cell surface into the extracellular milieu.[3] To investigate this post-translational proteolysis of ACE2, we examined the regulation of its mammalian homologue, ACE.

Like ACE2, ACE is also a type I integral membrane-bound protein. The cleavage site of somatic ACE by its sheddase has been identified as the Arg^{1203}—Ser^{1204} bond (human somatic ACE numbering), which lies 24 residues upstream on the extracellular side of the membrane-spanning domain.[4] Similarly, the cleavage site of testicular ACE by its sheddase was also identified to be at an Arg^{627}—Ser^{628} bond (human testis ACE numbering), again 24 residues proximal to the transmembrane domain.[5,6] Like those of most of the membrane-bound proteins that undergo ectodomain shedding, the precise requirements for sheddase recognition of the ACE ectodomain are still unknown. Studies utilizing various ACE mutant constructs, such as specific point mutations, domain substitutions, and mutants containing deleted domains, were still able to undergo ectodomain shedding.[7−12] In terms of how ACE interacts with its sheddase, two studies have proposed the existence of a "recognition motif" within the ectodomain of ACE.[13,14] Specifically, it is has been previously shown that the degree of ectodomain shedding of somatic ACE appears to be lower than that of testicular ACE.[4] Somatic ACE consists of two homologous N- and C-domains, whereas testis ACE consists of a single C-domain.[15] It has been proposed that the N- and C-domains are flexible, and both domains come into each other's proximity for the hydrolysis of ACE's substrates.[14] The molecular flexibility of the N- and C-domains, however, results in occlusion of a proposed shedding recognition motif, which resides within the C-domain.[14] Subsequent studies have shown that this recognition motif is localized to a region within the C-domain of testicular ACE, within amino acid residues 191−214 (human testis ACE numbering).[16]

Our current understanding of ACE2 ectodomain shedding, however, is very limited compared to our understanding of ACE. One of the preliminary reports from our laboratory demonstrated in vitro that ACE2 is proteolytically shed from the cell membrane by ADAM17 and that the rate of release is increased following treatment with the phorbol ester PMA, an activator of the protein kinase C (PKC) signaling pathway.[3] In this study, a series of protease inhibitors in conjunction with siRNA knockdowns were used to show that ADAM17 clearly plays a role in the

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Table 1. Summary of MALDI-ToF Mass Spectrometry Analysis of ADAM17-Mediated Cleavage of ACE2 Peptide Mimetics

peptide	sequence	MH^+	fragment	cleaved
$ACE2_{674-693}$	NLKPRISFNFFVTAPKNVSD	2294.1	observed	no
$ACE2_{691-710}$	VSDIIPRTEVEKAIRMSRSR	2343.3	observed	no
$ACE2_{701-720}$	EKAIRMSRSRINDAFRLNDN	2405.2	observed	yes
	EKAIRMSR	989.5	observed	
	SRINDAFRLNDN	1433,7	observed	
$ACE2_{713-732}$	DAFRLNDNSLEFLGIQPTLG	2220.0	observed	no
$ACE2_{701-720}Ala^{708}$	EKAIRMSASRINDAFRLNDN	2321.6	observed	yes
	EKAIRMSA	904.5	–	
	SRINDAFRLNDN	1433.7	observed	
$ACE2_{701-720}Ala^{709}$	EKAIRMSRARINDAFRLNDN	2389.2	observed	yes
	EKAIRMSR	989.5	–	
	ARINDAFRLNDN	1417.7	observed	
$ACE2_{701-720}Ala^{708}Ala^{708}$	EKAIRMSAARINDAFRLNDN	2304.2	observed	yes
	EKAIRMSA	904.5	–	
	ARINDAFRLNDN	1417.7	observed	
$ACE2_{701-720}Glu^{708}$	EKAIRMSESRINDAFRLNDN	2378.2	observed	no
	EKAIRMSE	962.5	–	
	SRINDAFRLNDN	1433.7	–	
$ACE2_{701-720}Glu^{710}$	EKAIRMSRSEINDAFRLNDN	2378.2	observed	no
	EKAIRMSR	989.5	–	
	SEINDAFRLNDN	1406.6	–	
$ACE2_{701-720}Glu^{708}Glu^{710}$	EKAIRMSESEINDAFRLNDN	2351.1	observed	no
	EKAIRMSE	962.5	–	
	SEINDAFRLNDN	1406.6	–	

phorbol ester-stimulated shedding.[3] Interestingly, we were unable to completely block the stimulated shedding.[3] Furthermore, although the ADAM17 specific inhibitor TAPI-1 significantly reduced the level of stimulated shedding, it had little effect on constitutive shedding; in contrast, the less specific inhibitor, GM6001, a broad spectrum hydroxymate-based MMP inhibitor, had a far greater impact on constitutive shedding, suggesting that one or more metalloproteases other than ADAM17 play a significant role in the constitutive release of ACE2.[3] Another key study related to this topic is a recent report suggesting that the cleavage site for the ADAM17-induced ectodomain shedding of this enzyme is localized between amino acids 716 and 741 (human ACE2 numbering), residing within the juxtamembrane region.[17] Furthermore, this study using several mutant and chimeric ACE2 proteins also showed that the juxtamembrane, transmembrane, and cytoplasmic domains of ACE2 are not required for constitutive shedding.[17] This observation is consistent with another recent study that shows that ACE2 deletion mutants lacking the juxtamembrane region abolished ADAM17-mediated shedding but not the constitutive cleavage secretion.[18] To date, there has only been one report detailing a specific recognition motif for ACE2 ectodomain shedding, involving a critical leucine residue at position 584 in the ectodomain, 157 amino acids proximal to the transmembrane domain.[17] Site-directed mutagenesis of this residue, replacing the aliphatic residue with an alanine, demonstrated a significant attenuation of ACE2 shedding.[17] Taken together, these data suggest a similarity between ACE and ACE2 in the regulation of ectodomain shedding, particularly the roles of the juxtamembrane, transmembrane, and cytoplasmic domains, and also the presence of a recognition motif in a region distal from the membrane-spanning helix.

Despite the high degree of sequence similarity between the ectodomains of ACE and ACE2, the cytoplasmic, transmembrane, and juxtamembrane domains, particularly the immediate extracellular amino acid sequences juxtaposed to the cell membrane, share no recognizable homology. Indeed, although the consensus sequence Ser-Ala-Arg-Ser-Glu-Gly has been identified as the ACE cleavage site,[19] no analogous sequence is present in the membrane proximal region of ACE2. Thus, direct comparisons are not useful for the prediction of either the likely cleavage sites in ACE2 or the proteases that mediate the shedding (as yet unidentified for ACE; ADAM17 is seen as the prime mediator for ACE2 shedding). Hence, our immediate aim was to investigate and understand the cleavage-site constraints in the ADAM17-mediated shedding of ACE2.

EXPERIMENTAL PROCEDURES

Construction of Plasmids. The expression construct for full-length human recombinant ACE2 containing the C-terminal FLAG sequence has been described previously.[20] All primers used to generate ACE2 mutants (single- and double-point mutations) were produced using site-directed mutagenesis to substitute targeted residues with alanine and glutamate by polymerase chain reaction amplification using the wild-type or mutant ACE2 cDNA as a template. All mutants were verified by automated DNA sequencing (Monash University DNA gene sequencing-Micromon Facility).

Cell Culture and Transfection. Chinese hamster ovary (CHO) cells stably expressing polyoma LT antigen (CHO-P) and CHO-K1 cells were obtained from ATCC. All cells were maintained at 37 °C in 5% CO_2. Culture media were as follows:

10% (v/v) FBS and 1% (v/v) penicillin/streptomycin in RPMI 1640 for CHO-P and CHO-K1 cells and 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, and 0.5 mg/mL neomycin selective antibiotic in RPMI 1640 for CHO-K1 cells stably expressing wild-type and mutant ACE2. All ACE2 mutant proteins were expressed in CHO-P and CHO-K1 cells. Transient expression of wild-type and mutant ACE2 in CHO-P cells was achieved using the DEAE-dextran method.[21] Stable expression of wild-type and mutant ACE2 in CHO-K1 cells was achieved using Lipofectamine 2000 (Invitrogen, Mulgrave, Australia) reagent according to the manufacturer's instructions.

Cell Treatment and Protein Extraction. Cells were grown to 80% confluence in 100 mm tissue culture dishes and rinsed twice with serum-reduced medium before experiments were conducted. All pharmacological agents, 1 μmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Sydney, Australia), 2 μmol/L protein kinase C inhibitor bisindolylmaleimide I (BIM) (Merck, Kilsyth, Australia), and ADAM17 specific inhibitor TAPI-1 (Sigma-Aldrich), were diluted using serum-reduced medium OptiMEM (Invitrogen). All incubations were conducted at 37 °C with 5% CO_2. After the cells had been treated, the medium was harvested and concentrated 50-fold by centrifugation in 30K MWCO Amicon concentrators (Millipore, Kilsyth, Australia) to a final volume of 200 μL. Cells were scraped into ice-cold PBS, harvested by centrifugation, and solubilized in 500 μL of PBS containing 1% (v/v) Triton X-100. Protein concentrations were determined using bicinchoninic acid with BSA as a standard.

ADAM17 Cleavage Assay. Reaction mixtures (20 μL) containing 3 μg of synthetic peptides corresponding to wild-type and mutated residues within the juxtamembrane region of the ACE2 ectodomain (see Table 1) were incubated with 0.1 μg of recombinant human protein ADAM17 in 25 mmol/L Tris (pH 9.0), 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 at 37 °C for 8 h. Specific ADAM17 cleavage was assessed in the presence of 50 μmol/L TAPI-1. After incubation, the reaction was terminated by the addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were cospotted onto the MALDI target plate with matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid (Laser Biolabs, Sopha-Antipolis, France) in 50% (v/v) TFA. The samples were analyzed on an Applied Biosystems 4700 Analyzer MALDI-ToF/ToF (Applied Biosystems, Foster City, CA) in reflectron mode with a mass range of 800−3500 Da and a focus mass of 1400 Da, at 1500 shots per spectra. The 4700 Series Explorer software automatically selects the 15 most intense peptides as precursor masses for tandem MS (MS/MS) analysis, acquiring them in the order of decreasing intensity. MS/MS analysis was conducted in reflector mode with a relative precursor mass window of 50 resolutions with metastable ion suppression on and spectra summed 2500 shots per spectrum.

ACE2 Activity Assay. The catalytic activity of recombinant wild-type and mutant ACE2 was measured using a highly specific fluorogenic substrate [(7-methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH; custom synthesis from Auspep (Tullamarine, Australia)] for ACE2, as previously described.[22] Briefly, protein (10 μg) was incubated with 50 μmol/L quenched fluorescent substrate at room temperature, and the fluorescence resulting from substrate hydrolysis was monitored over 4 h using a FLUOstar Optima plate reader (BMG Labtech, Offenburg, Germany). The specific activity was determined using 100 nmol/L ACE2 specific inhibitor, MLN-4760 ((*S*,*S*)-2-{1-carboxy-2-[3-(3,5-dichlorobenzyl)[^{3}H]amidazol-4-yl]ethylamino}-4-methylpentaoic acid), a gift from N. Dales (Millenium Pharmaceuticals),[23] in parallel with the use of quenched fluorescent substrate. As the fluorogenic substrate can also be hydrolyzed by prolyl endopeptidase, 1 μmol/L Z-Pro-prolinal (Bachem A. G., Bubendorf, Switzerland), a specific inhibitor of this enzyme, was incorporated in assay wells. Results were corrected and analyzed versus the appropriate controls using a Student's *t* test.

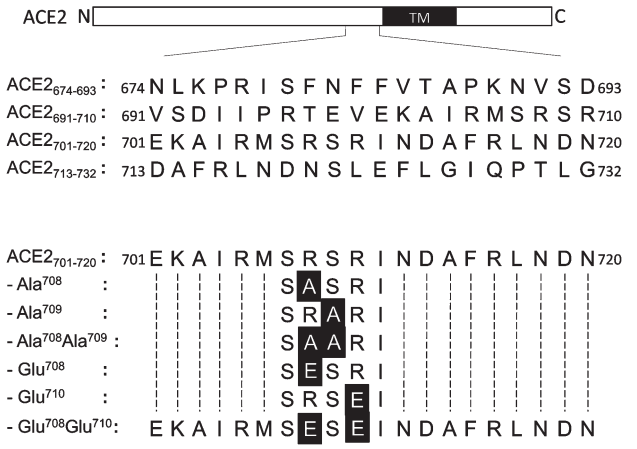

Figure 1. Schematic (top) of 20-amino acid peptide mimetics corresponding to ACE2 ectodomain residues (human ACE2 numbering) 674−693 ($ACE2_{674-693}$), 691−710 ($ACE2_{691-710}$), 701−720 ($ACE2_{701-720}$), and 713−732 ($ACE2_{713-732}$). Also shown are alignments (bottom) of peptide $ACE2_{701-720}$ and its mutant analogues. Alanine- and glutamate-subtituted residues are highlighted.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. Samples of medium and cell lysates were separated by SDS−PAGE and proteins electroblotted on polyvinylidene difluoride membranes (Millipore). After nonspecific protein binding sites had been blocked using 5% (w/v) skim milk in Tris-buffered saline containing 0.5% (v/v) Tween 20, the membranes were incubated with antibodies directed against the human ACE2 ectodomain (1:500) [BAF933 goat polyclonal (R&D Systems)]. A donkey anti-goat horseradish peroxidase-conjugated secondary antibody (1:4000) was diluted in 5% (w/v) skim milk in Tris-buffered saline containing 0.5% (v/v) Tween 20. Immunoreactive bands were visualized using enhanced chemiluminescence (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Circular Dichroism. CD measurements of the synthetic peptides were performed on a Jasco J-815 circular dichroism spectropolarizer (Jasco Inc., Tokyo, Japan) using quartz cuvettes with a 1 mm path length. Scans between 180 and 260 nm were performed at a scan speed of 50 nm/min, a bandwidth of 1.0 nm, a resolution of 0.1 nm, with a 1 s response time and five scan accumulations. The quartz cuvette temperature was controlled at 37 °C with a Peltier temperature controller, and the CD instrument was calibrated with (+)-10-camphorsulfonic acid. CD spectra of peptide mimetics were measured in 25 mmol/L Tris (pH 9.0), 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35, and spectra were smoothed using the Jasco Fast Fourier transform algorithm and then baseline corrected against the same buffer as the blank. The peptide concentration used was approximately 100 μmol/L for each analogue.

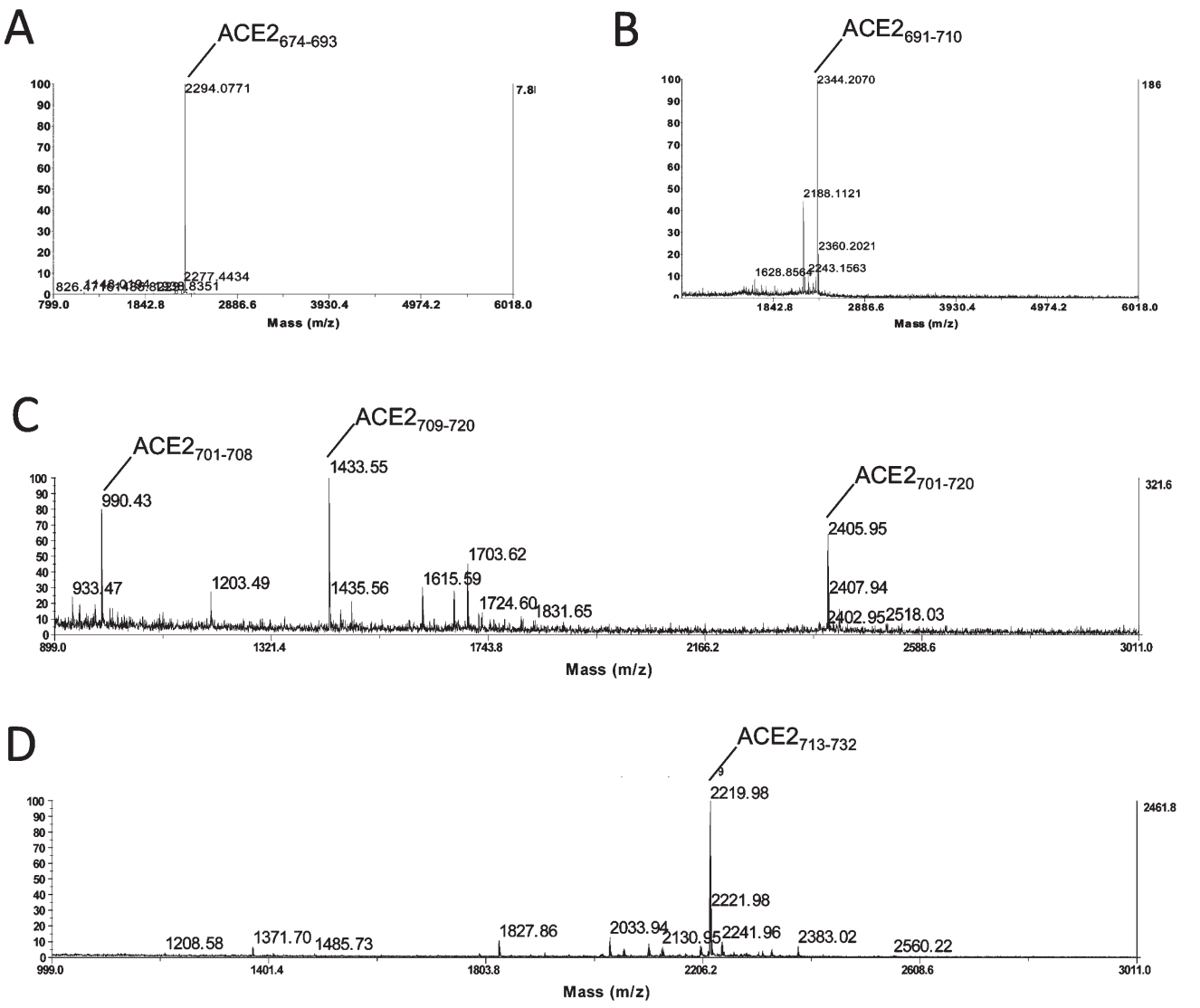

Figure 2. Mass spectrometry analysis of ACE2 peptide mimetics (A) $ACE2_{674-693}$, (B) $ACE2_{691-710}$, (C) $ACE2_{701-720}$, and (D) $ACE2_{713-732}$. Synthetic peptides (3 μg) were incubated with recombinant human protein ADAM17 (0.1 μg) in 25 mmol/L Tris (pH 9.0), 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 at 37 °C for 8 h. Cleavage reactions were terminated via the addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analyzed using MALDI-ToF/ToF mass spectrometry. Peptide substrates and cleaved fragments are labeled.

RESULTS

Cleavage of ACE2 Peptide Mimetics by Human Recombinant ADAM17. To identify the cleavage site for ACE2 ectodomain shedding mediated by ADAM17, we tested four overlapping peptide mimetics, corresponding to human ACE2 residues 674−693 ($ACE2_{674-693}$), 691−710 ($ACE2_{691-710}$), 701−720 ($ACE2_{701-720}$), and 713−732 ($ACE2_{713-732}$) (Figure 1, top panel), for their susceptibility to cleavage by human recombinant ADAM17. MALDI-ToF mass spectrometry analyses performed after incubation with human recombinant ADAM17 for 8 h at 37 °C revealed that $ACE2_{674-693}$ (2295 Da), $ACE2_{691-710}$ (2344 Da), and $ACE2_{713-732}$ (2219 Da) were resistant to cleavage by ADAM17 (panels A, B, and D of Figure 2, respectively). MALDI-ToF mass spectrometry analysis of $ACE2_{701-720}$ (2405.9 Da), however, indicated that this peptide was cleaved after incubation for 4 h. The resulting two shorter peptide fragment cleavage products were subsequently identified as $ACE2_{701-708}$ and $ACE2_{709-720}$ with masses of 990.4 and 1433.5 Da, respectively (Figure 2C). Tandem mass spectrometry analysis using the MALDI-ToF/ToF analyzer provided both mass and sequence data to confirm the cleavage site as being between Arg^{708} and Ser^{709}. To confirm this finding, the experiments were repeated in the presence of the ADAM17 specific inhibitor TAPI-1 at 50 μmol/L (Figure 3). As predicted, in the presence of TAPI-1, these shorter fragments (990.4 and 1433.5 Da) were poorly detected compared to those from which the ADAM17 inhibitor was absent (Figure 3B). The detection ratio of cleaved peptide $ACE2_{709-720}$ to full-length peptide $ACE2_{701-720}$ is comparatively lower in the presence of TAPI-1, as a result of the inhibition of ADAM17 proteolytic activity (Figure 3B). Additionally, cleaved peptide $ACE2_{701-708}$ was not detected in the presence of TAPI-1, possibly because of its poor ionization state compared to that of $ACE2_{709-720}$ (Figure 3A,B).

Effect of Amino Acid Substitutions of the $ACE2_{701-720}$ Peptide. Having established that human recombinant ADAM17 is able to cleave a specific peptide sequence within the human

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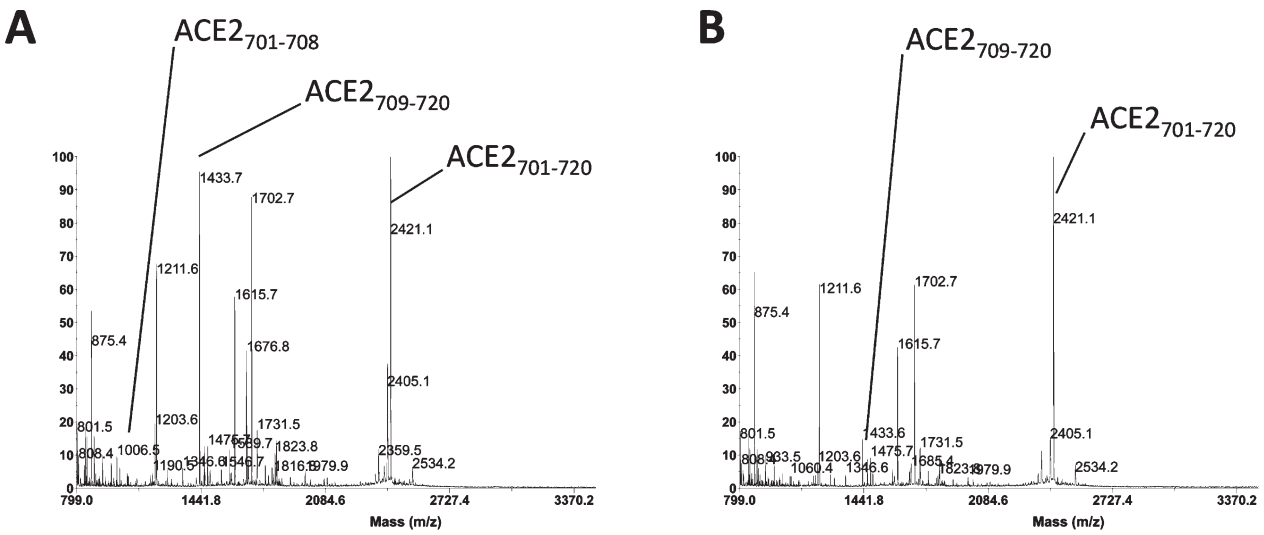

Figure 3. Mass spectrometry analysis of peptide fragment analogues mimicking human ACE2 residues 701−720 ($ACE2_{701-720}$) in the presence of ADAM17 specific inhibitor, TAPI-1. Recombinant human ADAM17 (0.1 μg) was incubated with synthetic peptides (3 μg) for 8 h in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0) (left) or in the presence of TAPI-1 (50 μmol/L) (right). Cleavage reactions were terminated by the addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analyzed using MALDI-ToF/ToF mass spectrometry. Peptide substrates and cleaved fragments are labeled.

ACE2 juxtamembrane region, we aimed to characterize the contribution of the amino acid(s) both within and adjacent to the proposed cleavage site to the recognition and regulation of ACE2 ectodomain shedding in the next series of experiments. A series of analogues of $ACE2_{701-720}$ were synthesized in which residues 708−710 (human ACE2 numbering) were systemically substituted with alanine or glutamate (Figure 1, bottom). These peptides were investigated for their susceptibility to cleavage by human recombinant ADAM17. Mass spectrometry analysis revealed that the alanine substitutions had no effect on ADAM17-mediated cleavage (Table 1). All alanine-substituted analogues were succesffuly cleaved by ADAM17 after incubation for 8 h, as illustrated in Table 1. However, only one of the expected cleavage fragments, along with the peptide substrates, was detected as an intact peptide following mass spectrometry analysis (Figure 4). However, the glutamate-substituted analogues showed no detectable changes in their respective mass spectrometry profiles compared to controls, suggesting these peptides are not cleaved by human recombinant ADAM17 after incubation for 8 h (Figure 5). A summary of peptide cleavages analyzed by MALDI-ToF/ToF mass spectrometry is given in Table 1.

Solution Structure of the ACE2 Peptide Mimetics. CD spectra of ACE2 of peptide mimetics $ACE2_{674-693}$, $ACE2_{691-710}$, $ACE2_{701-720}$, and $ACE2_{713-732}$ in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 at pH 9.0 and 37 °C were recorded and are shown in Figure 6A (left panel). The data suggest that the peptide analogues exist as a number of interconverting species and/or comprise a mixture of different secondary structure forms. The CD spectrum for all peptide mimetics shows a minimum centered at approximately 200−203 nm and a broad region from 210 to 235 nm. The maximum present at 190 nm of the $ACE2_{674-693}$ CD spectrum and the broad though weak negative signal at wavelengths greater than 205 nm indicate that elements of helical or β-structure could be present. CD spectra for peptide analogues $ACE2_{691-710}$, $ACE2_{701-720}$, and $ACE2_{713-732}$, however, show no defined maxima. No significant changes were observed when the peptide analogues were dissolved in a solution containing 50% (v/v) methanol at 37 °C (Figure 6A, right panel). However, the maximum present at 190 nm and the broad region from 210 to 235 nm of the $ACE2_{674-693}$ CD spectrum have increased in intensity, which could indicate a higher proportion of β-structure in this peptide. In addition, CD spectra for $ACE2_{691-710}$ reveal the adoption of a significant degree of helical structure with the double minima observed at 205 and 222 nm.

To understand the potential importance of secondary structure in the ADAM17-mediated cleavage of peptide analogue $ACE2_{701-720}$, we further synthesized a series of $ACE2_{701-720}$ analogues in which arginine at position 708 and/or 710 and/or serine at position 709 was systematically substituted with alanine or glutamate. The CD spectra for each analogue were recorded in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0) or in 50% (v/v) methanol, at 37 °C. The CD spectra shown in Figure 6B (left panel) suggest that the majority of the subsitutions exerted little or no effect on secondary structure compared to that of the parent peptide $ACE2_{701-720}$. The CD spectrum for all peptide analogues shows a minimum centered at approximately 200−203 nm and a broad region from 210 to 235 nm, suggesting extended coil structure. The overall structures of the peptide analogues in 50% (v/v) methanol remained unchanged, although significant increases in the intensity of the maxima at 188−190 nm were observed in $ACE2_{701-720}Glu^{708}$, $ACE2_{701-720}Glu^{710}$, and $ACE2_{701-720}Glu^{708}Glu^{710}$, while significant decreases in the intensity of the minima at 200−202 nm were observed in all peptide analogues except $ACE2_{701-720}Glu^{708}Glu^{710}$ (Figure 6B, right panel), indicating that perhaps the solubility of the peptides differs in both solutions.

ACE2 Mutants Undergo Ectodomain Shedding in CHO-P and CHO-K1 Cells. The next series of experiments examined the effect the amino acid substitutions in the ACE2 juxtamembrane (as detailed above) have on the ectodomain shedding of full-length human recombinant ACE2 expressed in mammalian cell lines. Mutant ACE2 full-length proteins containing point

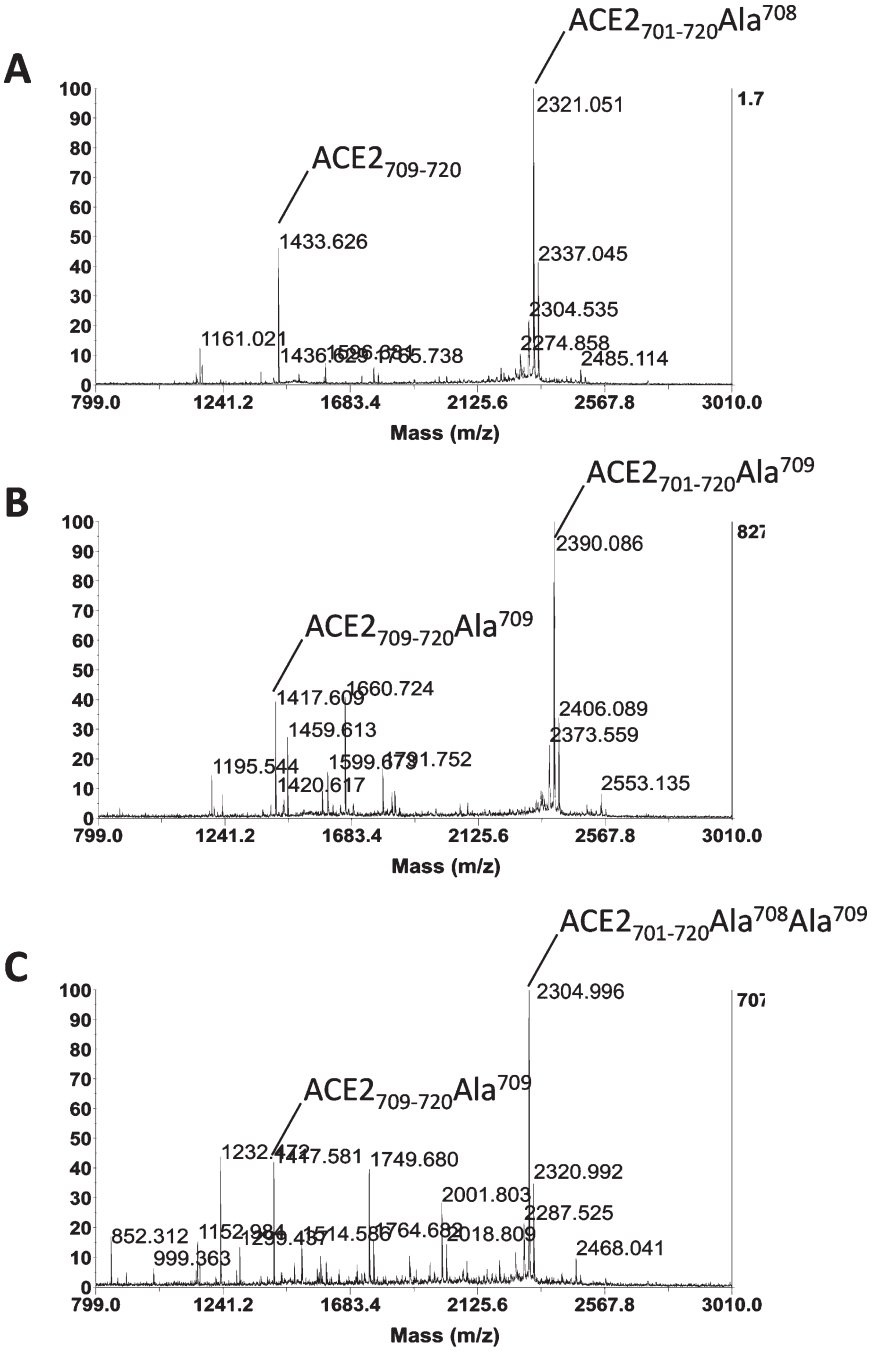

Figure 4. Mass spectrometry analysis of peptide fragment analogues containing alanine-substituted mutations of human ACE2 residues 708 and 709. Recombinant human ADAM17 (0.1 μg) was incubated with synthetic peptides (3 μg), (A) $Arg^{708} \rightarrow Ala^{708}$ ($ACE2_{701-720}Ala^{708}$), (B) $Ser^{709} \rightarrow Ala^{709}$ ($ACE2_{701-720}Ala^{709}$), and (C) $Arg^{708}Ser^{709} \rightarrow Ala^{708}Ala^{709}$ ($ACE2_{701-720}Ala^{708}Ala^{709}$), for 8 h in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0). Cleavage reactions were terminated by the addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analyzed using MALDI-ToF/ToF mass spectrometry. Peptide substrates and cleaved fragments are labeled.

mutations, at P1 or P1′ and/or P2′ of the proposed ACE2 cleavage site ($ACE2_{701-720}Ala^{708}$, $ACE2_{701-720}Ala^{709}$, $ACE2_{701-720}Glu^{710}$, and $ACE2_{701-720}Glu^{708}Glu^{710}$), were transiently expressed in CHO-P cells. Analysis of ACE2 activity using the concentrated medium from the transfected CHO-P and CHO-K1 cells showed that each of these ACE2 mutants ($ACE2_{701-720}Ala^{708}$, $ACE2_{701-720}Ala^{709}$, $ACE2_{701-720}Glu^{708}$, and $ACE2_{701-720}Glu^{708}Glu^{710}$) was able to undergo constitutive ectodomain shedding and also showed increased shedding activity in the presence of phorbol ester PMA (Figure 7), consistent with the obsevation made for recombinant wild-type human ACE2 expressed in these cells.[18,20] Concentrated medium harvested from CHO-P cells transiently expressing the $ACE2_{701-720}Glu^{708}Glu^{710}$ mutant showed a marked increase in ACE2 activity (216.9 ± 16.5%) in the presence of PMA, while in the presence of ADAM17 inhibitor TAPI-1, the activity is significantly decreased (−30.0 ± 2.7%) (illustrated in Figure 8A). As previously observed in recombinant wild-type

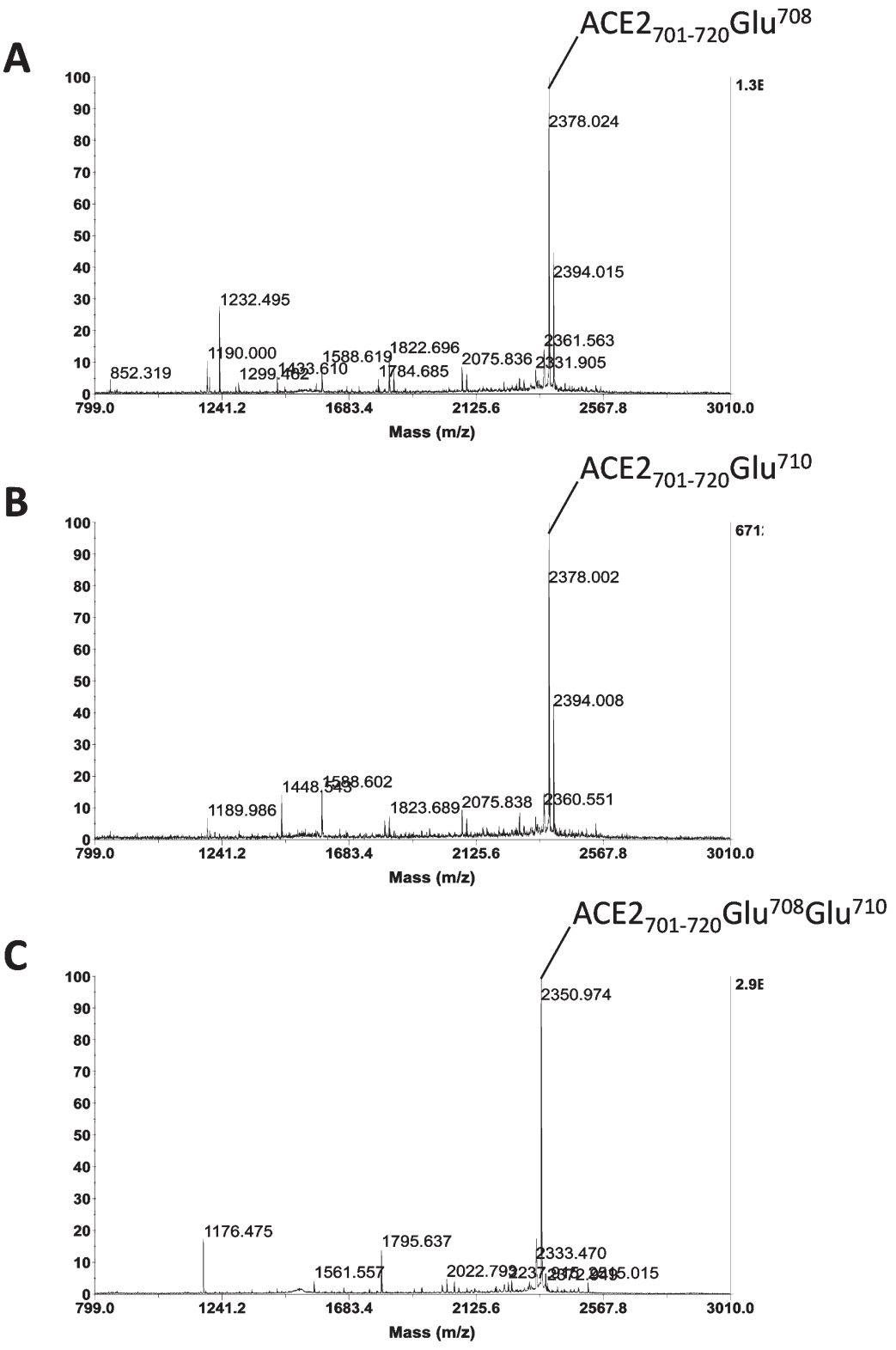

Figure 5. Mass spectrometry analysis of peptide fragment analogues containing alanine-substituted mutations of human ACE2 residues 708 and 710. Recombinant human ADAM17 (0.1 μg) was incubated with synthetic peptides (3 μg), (A) $Arg^{708} \rightarrow Glu^{708}$ ($ACE2_{701-720}Glu^{708}$), (B) $Arg^{710} \rightarrow Glu^{710}$ ($ACE2_{701-720}Glu^{710}$), and (C) $Arg^{708}Arg^{710} \rightarrow Glu^{708}Glu^{710}$ ($ACE2_{701-720}Glu^{708}Glu^{710}$), for 8 h in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0). Cleavage reactions were terminated by the addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analyzed using MALDI-ToF/ToF mass spectrometry. Peptide substrates and cleaved fragments are labeled.

human ACE2,[3] the shedding $ACE2_{701-720}Glu^{708}Glu^{710}$ mutant protein is not completely abolished in the presence of TAPI-1, suggesting there is another sheddase (or more than one) involved in the shedding of ACE2. Meanwhile, $ACE2_{701-720}Glu^{708}$-transfected

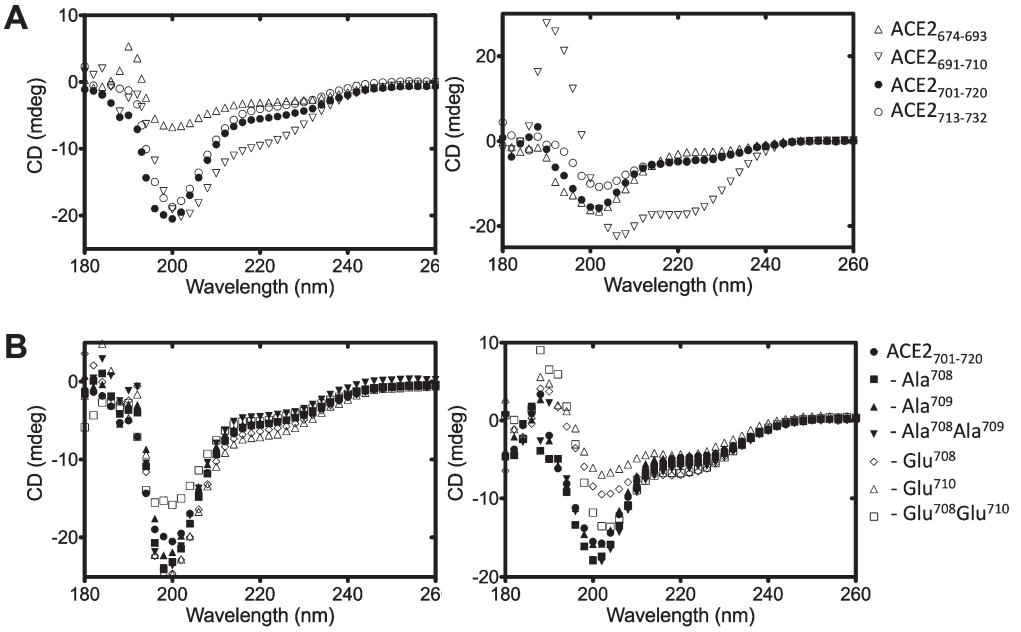

Figure 6. Circular dichroism spectra of ACE2 peptide analogues in solutions. (A) ACE2 peptide mimetics (100 μmol/L) in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0) (left) or in 50% (v/v) methanol (right). (B) $ACE2_{701-720}$ peptide and its mutant analogues (100 μmol/L) in 25 mmol/L Tris, 2.5 μmol/L $ZnCl_2$, and 0.005% (v/v) Brij 35 (pH 9.0) (left) and in 50% (v/v) methanol (right).

CHO-P cells did not have any detectable levels of ACE2 activity (Figure 8A, right panel), suggesting that perhaps the tranfection approach used in an attempt to establish the transient expression of this particular strain of ACE2 mutant is not suitable in this mammalian cell line. Hence, the experiments were repeated in CHO-K1 cells that are capable of the stable expression of $ACE2_{701-720}Glu^{708}$ or $ACE2_{701-720}Glu^{708}Glu^{710}$. The results from these experiments showed that the mutant proteins displayed a shedding profile similar to that of the transient system. PMA stimulation of CHO-K1 cells expressing $ACE2_{701-720}Glu^{708}$ and $ACE2_{701-720}Glu^{708}Glu^{710}$ showed significant increases in ACE2 acitivity in the concentrated medium (207.0 ± 15.6%) compared to controls (185.8 ± 3.8%) (Figure 8B). In the presence of TAPI-1, both mutant proteins showed significant decreases in ACE2 activity in the concentrated medium (40.8 ± 3.5%) compared to controls (74.3 ± 2.7%) (Figure 8B). As demonstrated previously,[3] the treatment of both cell lines with both PMA and TAPI-1 has little effect on the stimulated release of ACE2 into the medium compared to TAPI-1 treatment alone, suggesting that ADAM17 is responsible for the cleavage secretion of ACE2 ectodomain (Figure 8A,B).

To assess the ectodomain shedding profile of each mutant compared to that of recombinant wild-type human ACE2, each of the ACE2 mutants was stably expressed in CHO-K1 cells. Statistical analysis showed that at a transfection ratio of 3:1 (v/w) (Lipofectamine 2000:plasmid DNA), FLAG-tagged full-length human recombinant ACE2 and all of the ACE2 mutants showed a consistent level of ACE2 activity in the cell lysate (Figure 9A) [FLAG-tagged full-length human recombinant ACE2, 485.1 ± 53.9; $ACE2_{701-720}Ala^{708}$, 607.2 ± 111.5; $ACE2_{701-720}Ala^{709}$, 461.1 ± 37.2; $ACE2_{701-720}Glu^{708}$, 539.2 ± 60.4; and $ACE2_{701-720}Glu^{708}Glu^{710}$, 541.1 ± 64.1 (in relative fluorescent unites per minute per milligram of protein)]. For specificity control, the presence of ACE2 in the cell lysate was confirmed using Western immunoblotting (Figure 9A).

Analysis of the concentrated media from cells treated with the phorbol ester PMA showed a significant increase in secreted/shed ACE2 activity compared to control media (in Figure 9B, ACE2 activities are shown as the percent activity compared to control media) (FLAG-tagged full-length recombinant ACE2, 167.4 ± 17.7%; $ACE2_{701-720}Ala^{708}$, 137.9 ± 7.4%; $ACE2_{701-720}Ala^{709}$, 196.0 ± 24.6%; $ACE2_{701-720}Glu^{708}$, 237.5 ± 18.3%; $ACE2_{701-720}Glu^{708}Glu^{710}$, 248.5 ± 18.4%). In cells that were treated with bisindolylmaleimide I or a combination of both bisindolylmaleimide I and PMA, there were no significant changes in ACE2 activity in the concentrated media compared to the control after treatment for 4 h (Figure 9B). Statistical analysis of the concentrated media from $ACE2_{701-720}Glu^{708}Glu^{710}$-expressing cells indicated a significant increase in ACE2 activity when compared to that of recombinant wild-type human ACE2- and $ACE2_{701-720}Ala^{708}$-expressing cells treated with PMA (via one-way analysis of variance, $P < 0.05$; Tukey's multiple-comparison test). In addition, $ACE2_{701-720}Glu^{708}$-expressing cells also showed an increase in ACE2 activity when compared to that of $ACE2_{701-720}Ala^{708}$-expressing cells when treated with PMA (via one-way analysis of variance, $P < 0.05$; Tukey's multiple-comparison test). As an additional specificity control, the presence of ACE2 in the cell lysate was confirmed using Western immunoblotting (Figure 9B).

DISCUSSION

As ACE2 was discovered only relatively recently (compared to ACE), the majority of studies thus far have been largely focused on deducing its physiological functions, as well its relevance in disease. However, a handful of reports have looked into the ectodomain shedding event of this enzyme, none of which have offered much, if any, insight into the precise cleavage site or the regulation and mechanisms involved in this shedding event. Via examination of other membrane-bound protein families that can

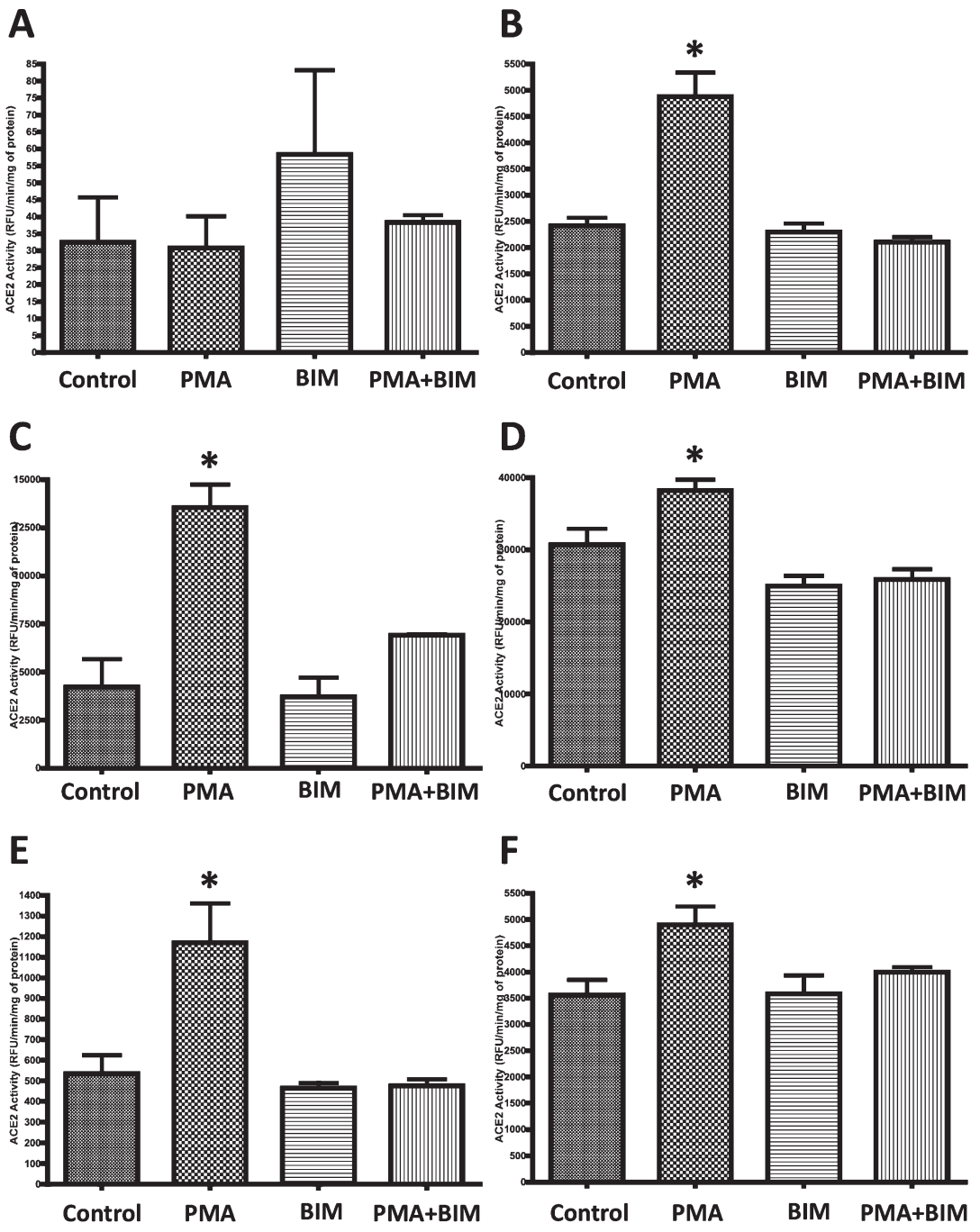

Figure 7. ACE2 activity from the concentrated medium of CHO-P cells transiently expressing ACE2 mutants. Ten micrograms of plasmid DNA [(A) mock, (B) wild-type recombinant human ACE2, (C) $ACE2_{701-720}Ala^{708}$, (D) $ACE2_{701-720}Ala^{709}$, (E) $ACE2_{701-720}Glu^{708}$, or (F) $ACE2_{701-720}Glu^{708}Glu^{710}$] were used to transfect 60% confluent CHO-P cells in 100 mm cell culture dishes using the DEAE-dextran method. Forty-eight hours post-transfection, cells were washed and replenished with serum-reduced medium prior to further incubation for an additional 16 h. Cells were stimulated with phorbol ester PMA (1 μmol/L) in the presence or absence of PKC inhibitor BIM (2 μmol/L) for 4 h. Secreted proteins (10 μg) were analyzed using a specific ACE2-quenched fluorescent substrate assay. Asterisks denote significant differences ($P < 0.05$) compared to the no-treatment control ($n = 3$; Student's t test).

undergo ectodomain shedding, it would appear that many of these proteolytic events, including the shedding of ACE2,[3] are mediated by zinc-dependent metalloproteases, including ADAMs (a disintegrin and metalloproteinases) and MMPs (matrix metalloproteinases) (for reviews, see refs 24−26).

The catalytic extracellular domain of ACE2 undergoes proteolytic release or ectodomain shedding when expressed in various eukaryotic cells in culture.[3,18,20,27,28] An identical process has been observed in ACE2-expressing tissues in situ,[17,29−32] and this mechanism is likely responsible for the generation of the soluble form of ACE2 that can be detected in bodily fluids such as human blood plasma and urine.[28,29] However, the precise mechanism(s) that confers both the specificity and regulation of the juxtamembrane domain cleavage and thus ectodomain

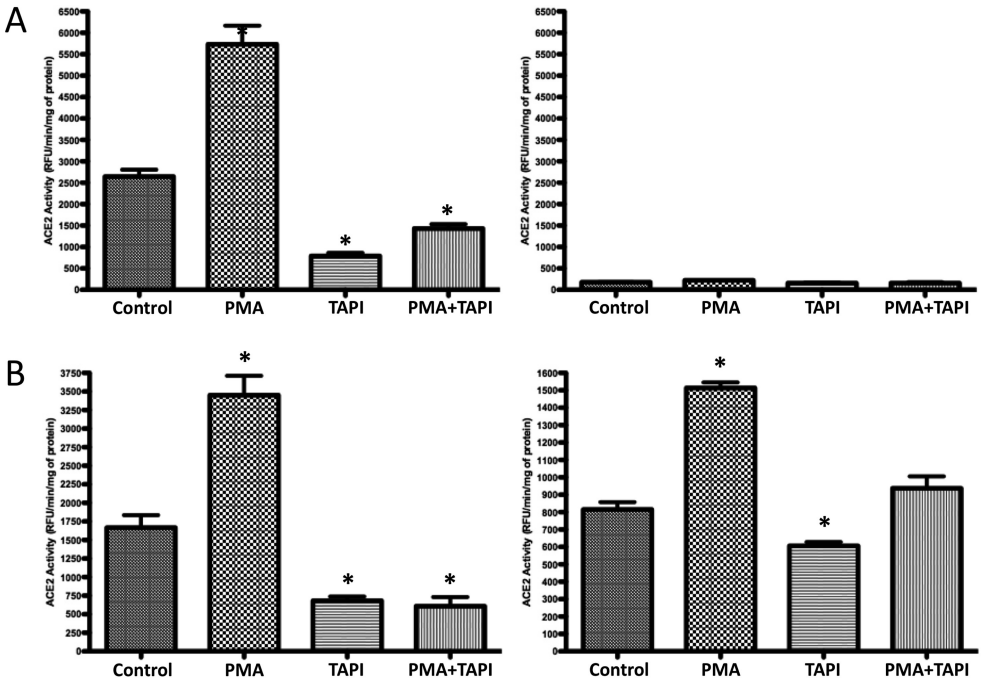

Figure 8. ACE2 activity from the concentrated medium of CHO-P and CHO-K1 cells expressing ACE2 mutant proteins. (A) CHO-P cells transiently expressing $ACE2_{701-720}Glu^{708}Glu^{710}$ (left) and CHO-K1 cells transiently expressing $ACE2_{701-720}Glu^{708}$ (right) were generated using 10 μg of plasmid DNA according to the DEAE-dextran transfection method.[21] (B) CHO-K1 cells stably expressing $ACE2_{701-720}Glu^{708}Glu^{710}$ (left) and $ACE2_{701-720}Glu^{708}$ (right) were generated using the Lipofectamine 2000 (Invitrogen) transfection method according to the manufacturer's instructions. All cells were grown to 80% confluence prior to incubation with serum-reduced medium for 16 h prior to stimulation with phorbol ester, PMA (1 μmol/L), ADAM17 specific inhibitor, TAPI-1 (50 μmol/L), or a combination of both, for 4 h. Secreted proteins (10 μg) were analyzed using an ACE2 specific quenched fluorescent assay. Data are analyzed from three independent experiements ($n \geq 3$; one-way analysis of variance) and expressed as relative fluorescent units per minute per milligram of protein used. Asterisks denote significant differences ($P < 0.05$) compared to other treatments.

release of ACE2 is unresolved. A recent report has proposed that the cleavage site in human ACE2 expressed in airway epithelia, Calu-3 cells, is localized to a region within the juxtamembrane domain, predicted to be between amino acid residues 716 and 741 (human ACE2 numbering).[17] Thus, to understand and in an attempt to elucidate the cleavage site(s) within ACE2 that mediate ectodomain shedding, we tested the ability of recombinant human ADAM17 to cleave a series of synthetic peptides whose design was based on the extracellular membrane-proximal sequences of ACE2. This approach has been previously employed to demonstrate the cleavage site for glycoprotein VI ectodomain shedding mediated by ADAM10.[33]

Overlapping peptide mimetics corresponding to amino acid residues 674−730 were incubated with recombinant ADAM17, and the resulting digestion was analyzed by mass spectrometry. ADAM17 cleaved the ACE2-based peptide (residues 701−720) at the $Arg^{708}-Ser^{709}$ bond. A second series of synthesized peptides in which residues at the P1, P1′, and P1 and P1′ positions were substituted with alanine were also analyzed for their susceptibility to cleavage by ADAM 17, with none of these substitutions having any impact. In contrast, however, glutamate substitution of residues in the P1, P2′, and P1 and P2′ positions blocked ADAM17-mediated cleavage. Although there is an apparent lack of a defined consensus cleavage motif by ADAM17, the proposed $Arg^{708}-Ser^{709}$ cleavage site does resemble the motif identified in its mammalian homologue, somatic ACE ($Arg^{1203}-Ser^{1204}$),[4] and is similar to those of other membrane-bound proteins such as the human L-selectin ($Arg^{321}-Ser^{322}$)[34] and human amphiregulin ($Lys^{177}-Ser^{178}$).[35] Although it is known that ADAM17 plays a role in the phorbol ester-stimulated shedding of ACE2, it does not appear to be involved in the constitutive shedding of ACE2.[3]

It is perhaps not surprising to see that the proposed cleavage site does not fall within the stretch of amino acid residues 716−741 as previously reported.[17] The potential cleavage site identified in this study is 32 amino acids proximal to the membrane-spanning domain and is thus still localized within the required juxtamembrane stalk region proposed for cleavage secretion by ADAM17.[36] However, it must be kept in mind that there may be substantial conformational differences in the cleavage efficiencies upon comparison of full-length proteins on the cell surface to their peptide analogues.[37] Morever, the similarity in the CD spectra of the peptide analogues of $ACE2_{701-720}$ suggests only minimal differences in the structure of these peptides in the same buffer system used for digestion by recombinant ADAM17. This indicates that the sensitivity of the cleavage event of these peptides is more likely due to the difference in amino acid sequence and/or conformation. In contrast, in a study by Black et al. of native membrane-bound full-length tumor necrosis factor α and its peptide analogues, both identical cleavage sites are readily hydrolyzed by ADAM17, whereas peptide mimetics of other substrates are cleaved far less efficiently.[37] Nonetheless, the differences in the efficiency of ADAM17-mediated cleavage of synthetic peptides and full-length proteins have been comprehensively discussed in previous reviews [24,25]

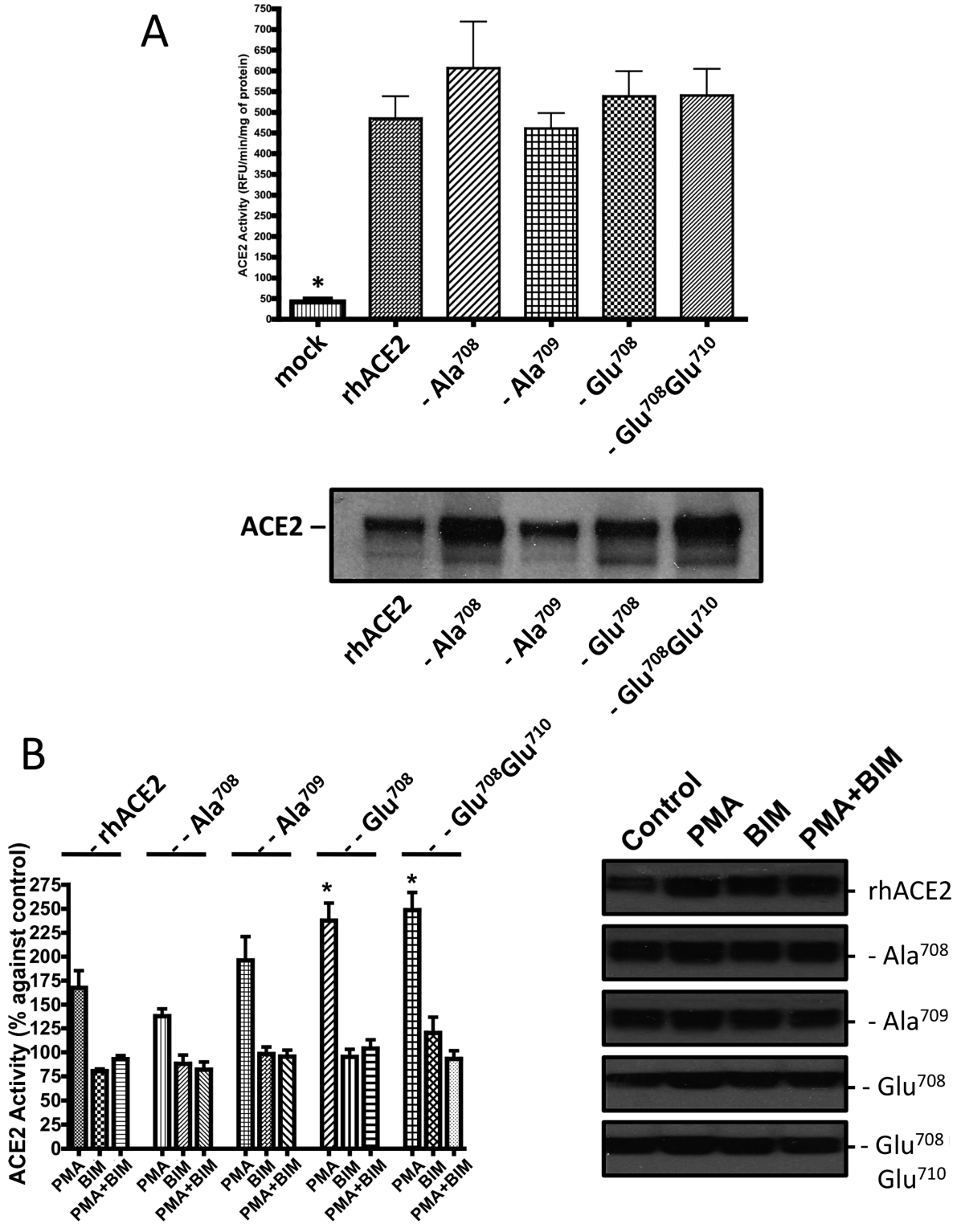

Figure 9. ACE2 activity from CHO-K1 cells stably expressing ACE2 mutant proteins. (A) The top panel shows the optimized selection of CHO-K1 cells stably expressing ACE2 mutants. A Lipofectamine 2000:plasmid DNA ratio of 3:1 (v/w) of each ACE2 mutant was selected for the level of ACE2 expression in the cell lysate (10 μg). The data were compared from six independent experiments (consistencies within passage 7−9) ($n = 6$; one-way analysis of variance). In the bottom panel, cell lysates (100 μg) of CHO-K1 cells stably expressing ACE2 mutant proteins were separated by SDS−PAGE and Western immunoblotted with an ACE2 monoclonal antibody. (B) The left panel shows PMA-stimulated shedding of ACE2 in CHO-K1 cells stably expressing ACE2 mutant proteins. Cells were incubated in serum-reduced medium containing PMA (1 μmol/L), BIM (2 μmol/L), a combination of both or in an equal volume of Me_2SO for 4 h as described in Experimental Procedures. The total protein (25 μg) from the concentrated medium was assayed for ACE2 activity. The data are normalized against the controls from at least four independent experiments ($n \geq 4$; one-way analysis of variance). Asterisks denote significant differences ($P < 0.05$). In the right panel, cell lysates (100 μg) were separated by SDS−PAGE and Western immunoblotted with an ACE2 monoclonal antibody.

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In CHO-P cells transiently expressing ACE2, mutating the residues at the P1, P1′, and P1 and P1′ positions to alanine and the P1, P2′, and P1 and P2′ positions to glutamate did not block PMA-induced shedding. The lack of an effect on shedding of these mutations is not unexpected given previously published mutagenesis studies of other ADAM17 substrates, such as L-selectin, glycoprotein VI, and ACE, which showed similar results.[9,33,38] This may indicate that perhaps the regulation of ACE2 ectodomain shedding in cells and tissues is less stringent, or perhaps that ADAM17 is able to cleave ACE2 at alternate site(s). However, it has been previously shown that the cleavage secretion process is not always constrained by a specific amino acid sequence, but rather by topological parameters.[6] Results from CHO-K1 cells stably expressing ACE2 indicate that by reversing the positive charge of the arginine residues in the P1 position and the P1 and P2′ positions to negatively charged glutamate showed a significant increase in the level of PMA-induced shedding (Figure 8B). A similar observation was made for the ACE2 homologue ACE, these studies also showing that mutation around the cleavage site led to an increase in the level of ectodomain shedding.[7] This study suggested that leucine substitution at the P4 position at the ACE cleavage site leads to more accessibility at the juxtamembrane stalk region for the ACE secretase and is thus responsible for the enhancement of shedding.[7] The molecular mechanism for ACE2 cleavage secretion and the spatial role of each of the amino acids within the proposed cleavage site still warrant further investigation. Furthermore, elucidation of the structural conformation of the ACE2 juxtamembrane region would perhaps be able to provide valuable insights into both the regulated and constitutive shedding of ACE2 and perhaps also contribute to our understanding of shedding of other membrane-bound proteins.

In summary, this study identifies a putative cleavage site within ACE2 for ADAM17-mediated shedding of the catalytically active ectodomain. Our studies demonstrate that ADAM17 is able to cleave peptide mimics at a position that corresponds to the Arg^{708}—Ser^{709} bond, and this cleavage is attenuated in the presence of ADAM17 specific inhibitor TAPI-1. Studies involving mammalian cells stably expressing ACE2 mutants highlighted that alanine substitution at the P1 or P1′ position did not influence this cleavage event. In contrast, glutamate substitution at P1 and/or P2′ positions significantly enhanced ADAM17-mediated shedding, though we cannot rule out the possibility that this could be occurring at a different cleavage site. Taken together, these findings provide valuable insights into ACE2 ectodomain shedding, as well as identifying the potential site of regulated cleavage mediated by ADAM17. Given the likely very important role of ACE2 ectodomain shedding in the regulation of cardiovascular and respiratory function, further studies such as purifying and sequencing shed ACE2 will allow us to definitively characterize the precise cleavage sites for both the constitutive and regulated shedding of ACE2.

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ABBREVIATIONS

ADAM17, also known as tumor necrosis factor α-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACE, angiotensin-converting enzyme; ADAM, a disintegrin and metalloproteinases; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SARS, severe acute respiratory syndrome; CHO, Chinese hamster ovary; BIM, bisindolylmaleimide I; MALDI, matrix-assisted laser desorption ionization; ToF, time-of-flight; CD, circular dichroism.

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