The Identification of a Calmodulin-Binding Domain within the Cytoplasmic Tail of Angiotensin-Converting Enzyme-2

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Angiotensin-converting enzyme (ACE)-2 is a homolog of the well-characterized plasma membrane-bound angiotensin-converting enzyme. ACE2 is thought to play a critical role in regulating heart function, and in 2003, ACE2 was identified as a functional receptor for severe acute respiratory syndrome coronavirus. We have recently shown that like ACE, ACE2 undergoes ectodomain shedding and that this shedding event is up-regulated by phorbol esters. In the present study, we used gel shift assays to demonstrate that calmodulin, an intracellular calcium-binding protein implicated in the regulation of other ectodomain shedding events, binds a 16-amino acid synthetic peptide corresponding to residues 762–777 within the cytoplasmic domain of human ACE2, forming a calcium-dependent calmodulin-peptide complex. Furthermore, we have demonstrated that ACE2 expressed in Chinese hamster ovary cells specifically binds to glutathione-S-transferase-calmodulin, but not glutathione-S-transferase alone, in pull-down assays using cell lysates. Finally, to investigate whether calmodulin has any effect on ACE2 ectodomain shedding in cells that endogenously express the enzyme, cells from a human liver cell line (Huh-7) expressing ACE2 were incubated with calmodulin-specific inhibitors, trifluoperazine and calmidazolium. Both trifluoperazine (25 μmol/liter) and calmidazolium, (25 μmol/liter) significantly increased the release of ACE2 into the medium (44.1 $\pm$ 10.8%, $P < 0.05$, Student's t test; unpaired, two-tailed, and 51.1 $\pm$ 7.4% $P < 0.05$, one-way ANOVA, respectively;), as analyzed by an ACE2-specific quenched fluorescence substrate assay. We also show that the calmodulin-specific inhibitor-stimulated shedding of ACE2 is independent from phorbol ester-induced shedding. In summary, we have demonstrated that calmodulin is able to bind ACE2 and suggest that the ACE2 ectodomain shedding and/or sheddase(s) activation regulated by calmodulin is independent from the phorbol ester-induced shedding. (*Endocrinology* 150: 2376–2381, 2009)

Since the discovery of angiotensin-converting enzyme (ACE)-2 (1, 2), there have been numerous studies aimed at both characterizing the enzyme and defining its precise physiological roles. These studies have shown that ACE2 is a carboxypeptidase able to efficiently hydrolyze the vasoconstrictor peptide angiotensin II (Ang II) into Ang 1–7, both *in vitro* (1, 2) and *in vivo* (3), suggesting its involvement in the renin-angio-

tensin system and cardiovascular regulation. More significantly, ACE2, which is normally expressed at low levels in tissues such as the heart, is up-regulated in the failing human heart as well as in rat models of myocardial infarction (4) along with an increase in the level of Ang 1–7 formation (5). This suggests that ACE2 likely counteracts the functions of its homolog ACE by inactivating Ang II with the resultant production of the putative va-

Abbreviations: ACE, Angiotensin-converting enzyme; ADAM, a disintegrin and metalloproteinase domain; Ang II, angiotensin II; CaM, calmodulin; CaMi, CaM inhibitor; CHO, Chinese hamster ovary; CHOP, CHO-K1 cell line stably transfected with the Polyomavirus (Py) large T (LT) antigen gene; CMZ, calmidazolium chloride; eNOS, endothelial isoform of nitric oxide synthase; GST, glutathione-S-transferase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SARS-CoV, severe acute respiratory syndrome coronavirus; TFP, trifluoperazine dimaleate; TrkA, tyrosine kinase.

sodilatory peptide Ang 1–7. Apart from its implicated role in the renin-angiotensin system, ACE2 also appears to play a protective role in mice suffering from severe acute lung injury (6).

Despite the differences in their physiological roles and catalytic specificity, ACE and ACE2 are highly homologous, sharing an overall sequence identity of 42% (3). ACE2 is an 805-amino acid, glycosylated, type I integral membrane protein with an apparent molecular mass of 120 kDa (2, 7). The type I topology consists of an extracellular domain containing the active catalytic site, a juxtamembrane region, a single-transmembrane domain, and a short cytoplasmic tail (2, 7). We have recently shown that the ectodomain of ACE2, like ACE, undergoes proteolytic cleavage to release a catalytically active soluble form (8).

ACE2 has also been identified as the functional cellular receptor for the severe acute respiratory syndrome coronavirus (SARS-CoV) and recombinant soluble ACE2 can effectively block the association of SARS-CoV S1 protein with its cellular receptor; thus, the importance of ACE2 ectodomain shedding is clearly significant, not only in terms of the local metabolism of Ang II but also in mediating viral entry (9). Ectodomain shedding may thus represent a fundamental process allowing this enzyme to act not only locally but also systemically in the circulation. Alternatively, shedding may be a way of rapidly down-regulating enzyme activity in the vicinity of its site of expression as an alternative to internalizing the enzyme. Nonetheless, the precise mechanism of ACE2 ectodomain shedding is poorly understood and may involve one or more regulatory endodomains and/or the juxtamembrane region to initiate the recruitment of sheddase(s) to the membrane for the cleavage-secretion event to occur.

In other studies of ectodomain shedding, it has been shown that the intracellular regulatory protein calmodulin (CaM) can bind to several different transmembrane proteins, including the leukocyte cell surface adhesion protein, L-selectin, and the platelet collagen receptor glycoprotein (GPVI), and that CaM inhibitors (CaMi) increase ectodomain shedding of these proteins (10–12). Recently Lambert *et al.* (13) reported the interaction between CaM and ACE2 and that CaM is able to inhibit ACE2 ectodomain shedding. In the present study, we sought to determine how CaM modulates ACE2 shedding, and we show the formation of complexes between CaM and peptides mimicking a region in the cytoplasmic tail of ACE2 and provide evidence that CaM inhibition that increases ACE2 ectodomain shedding is independent from stimulated shedding by phorbol ester.

Materials and Methods

Cell culture and transfection

The human hepatic cell line, Huh-7 (kindly provided by Professor Stephen Locarini, Victorian Infectious Disease Reference Laboratory, Australia), was maintained in MEM-α (Invitrogen, Mount Waverley, Australia) containing 10% (vol/vol) fetal bovine serum and 1× penicillin-streptomycin-glutamine (pH 7.2). Chinese hamster ovary (CHO) cells stably expressing polyoma LT antigen [CHO-K1 cell line stably transfected with the Polymavirus (Py) large T (LT) antigen gene (CHOP)] were maintained in RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum and 1× penicillin-streptomycin-glutamine (Invitrogen). Transient expression of recombinant full-length human ACE2 in CHO cells was achieved by transfecting pcDNA3.1/V5-His-

TOPO expression plasmids (Invitrogen) encoding full-length ACE2 with a C-terminal FLAG tag (see below for construction details) using the DEAE-dextran method (14).

Plasmid construction

An expression construct for full-length human recombinant ACE2 containing a C-terminal FLAG sequence was made by PCR amplification of the cDNA from a human testis quick-clone cDNA library (catalog no. 7117-1; CLONTECH, Palo Alto, CA) using forward (5′-GGTACCAT-GTCAAGCTCTTCCTGGCTCC-3′) and reverse (5′-CGCTCGAG-TCACTTGTCATCGTCGTCCTTGTAGTCAAAGGAGGTCTGAAC-ATCATC-3′) primers. The PCR product was digested with *Kpn*I and *Xho*I and ligated into these sites of the pcDNA3.1/V5-His-TOPO according to the manufacturer's instructions (Invitrogen). The expression construct for secreted soluble ACE2 was generated by fusing the IL-3 signal sequence and a FLAG tag to the N terminus of ACE2 (amino acids 2–738) and a hexa-histidine tag on the C terminus, as previously described (15). The cDNA was ligated into pcDNA3.1/V5-His-TOPO vector (Invitrogen) for transient transfection into HEK 293-T cells by the calcium phosphate method (16). Secreted ACE2 was purified from media by sequential anion exchange and anti-Flag (Sigma-Aldrich, Castle Hill, Australia) chromatography, according to the manufacturer's instructions. The cDNA sequences were verified by DNA sequence analysis on a 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Cell treatment and protein extraction

Cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-free medium before experimentation. All pharmacological agents, phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), protein kinase C inhibitor bisindolylmaleimide I (Calbiochem, La Jolla, CA), and calmodulin-specific inhibitors; trifluoperazine dimaleate (TFP) (Sigma-Aldrich), and calmidazolium chloride (CMZ; Sigma-Aldrich), were diluted using serum-reduced media OptiMEM (Invitrogen). All incubations were carried out at 37 C with 5% CO_2. After treatment of cells, the medium was harvested and concentrated 50-fold by centrifugation in 30-kDa NanoSep concentrators (Pall Science, Cheltenham, Australia) to a final volume of 200 μl. Cells were scraped into ice-cold PBS, harvested by centrifugation, and solubilized in 500 μl of lysis buffer [PBS with 1% (vol/vol) Triton X-100]. Protein concentration was determined using bicinchoninic acid with BSA as a standard.

ACE2 activity assay

The catalytic activity of recombinant ACE2 was measured using a highly specific fluorogenic substrate [(7-methoxycomarin-4-yl)acetyl-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH; custom synthesis from Auspep, Parkville, Australia] for ACE2, as previously described (17). Protein (25 μg) was incubated with 50 μmol/liter of quenched fluorescence 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). Specific activity was determined using the ACE2-specific inhibitor, MLN-4760 [(S,S)-2-(1-carboxy-2(3-[3,5-dicloro-benzyl]-^{3}H-imidazol4-yl)-ethylamino)-4-methylpentaoic acid], a gift from Dr. Natalie Dales (Millennium Pharmaceuticals, Cambridge, MA) (18), at 100 nmol/liter in parallel to the use of quenched fluorescence substrate. The reaction product was quantified using a standard curve generated from known concentrations of 7-methoxycoumarin-4-acetic acid (Sigma-Aldrich). Results were analyzed against the appropriate controls and the significance of data were performed on normalized data using Student's *t* test.

Gel shift assay

Complexes formed between CaM and a 16-residue synthetic peptide corresponding to residues 762–777 within the cytoplasmic domain of ACE2 (ACE2T2 = CFTGIRDRKKKNKARSG-amide; Auspep) were analyzed by gel shift assay as described by Erickson-Viitanen and Degrado (19) and previously used to identify CaM-binding peptides (11, 20–22).

Reactions (30 μl) containing 300 pmol of bovine CaM (Sigma-Aldrich) and increasing amounts (0–15 nmol) of the peptides in 100 mM Tris-HCl (pH 7.5), 4 mol/liter urea, and either 0.1 mmol/liter CaCl$_2$ or 1 mmol/liter EGTA were incubated at 25 C for 30 min. After incubation, 15 μl of a 50% glycerol/0.1% bromophenol blue solution was added to each reaction and the complexes were resolved in 12.5% acrylamide gels containing 4 mol/liter of urea in either 1 mmol/liter CaCl$_2$ or 1 mmol/liter EGTA. The gels were fixed and stained with Coomassie Blue.

In vitro binding assay

Cell lysates (2 mg) of transiently transfected CHO cells were incubated with 20 μg of glutathione-S-transferase (GST) (Sigma-Aldrich) or GST-CaM fusion protein [prepared and purified as previously described (12)] and 50% (wt/vol) glutathione Sepharose resin (GE Healthcare Biosciences) in the presence of 0.5 μl EDTA-free protease inhibitor cocktail (Sigma-Aldrich) for 16 h at 4 C. Bound resins were rinsed three times with Tris-buffered saline [50 mmol/liter Tris-HCl, 150 mmol/liter NaCl (pH 7.4)].

SDS-PAGE and immunoblotting

Media samples, cell lysates, and bound resins were separated by SDS-PAGE and proteins electroblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking of nonspecific protein binding sites using 5% (wt/vol) dried milk in Tris-buffered saline containing 0.5% (vol/vol) Tween 20, the membranes were incubated with antibodies directed to the human ACE2 ectodomain (1:500) (BAF933-goat polyclonal; R&D Systems, Minneapolis, MN), GST (1:10,000) (27-4577-01-goat polyclonal; GE Healthcare Biosciences), or FLAG peptide (1:4,000) (F3165-mouse monoclonal; Sigma-Aldrich). Donkey antigoat and goat antimouse (Millipore) horseradish peroxidase-conjugated secondary antibodies were diluted 1:4000. All antibodies were diluted in 5% milk in Tris-buffered saline containing 0.5% (vol/vol) Tween 20. Immunoreactive bands were visualized using enhanced chemiluminescence (PerkinElmer, Rowville, Australia) according to the manufacturer's instructions. Bands were compared against soluble recombinant human ACE2, which served as a positive control.

Results

Calmodulin binds to the cytoplasmic tail of ACE2

To determine whether CaM is able to bind to the cytoplasmic tail of ACE2, we made a synthetic peptide mimicking the 16-amino acid sequence of the ACE2 cytoplasmic region initially proposed by Lambert *et al.* (13) as well as four other peptide variants (mutations 1–4) containing mutations at different charged residues (Fig. 1). The synthetic peptides were incubated with CaM in the presence of Ca^{2+} or EGTA. We observed formation of a CaM-peptide complex as a shift in the migration of the CaM band of the wild-type sequence (dubbed ACE2T2). At a synthetic peptide to CaM molar ratio of 2.5:1, a single faint band was observed above the CaM band, corresponding to the Ca^{2+}-dependent CaM-peptide complex, which gradually increased in intensity as the molar ratio increased (Fig. 2). This observation strongly suggests that CaM is able to bind to the synthetic peptide. As predicted, no shift in the migration of CaM was observed in samples containing EGTA (Fig. 2). None of the peptide variants (mutations 1–4) showed a shift in CaM band (data not shown).

Calmodulin binds to full-length ACE2

After showing that CaM was able to bind synthetic peptide corresponding to a stretch of amino acids in the cytoplasmic tail
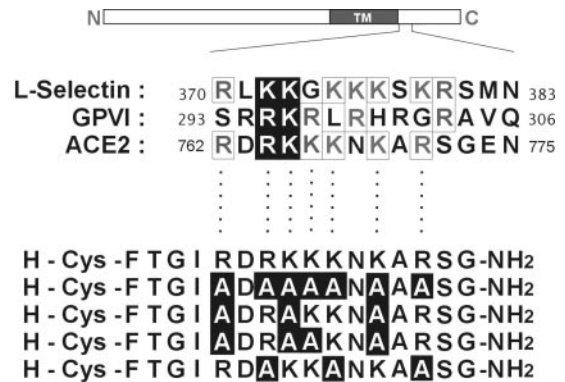

FIG. 1. Sequence comparison of ACE2 to GPVI and L-Selectin. *Top panel*, Cytoplasmic juxtamembrane sequence of human ACE2 compared with functional CaM-binding juxtamembrane sequences that regulate metalloproteinase-mediated ectodomain shedding of platelet GPVI and leukocyte L-selectin (40, 41). Shown are alignments of residues in the cytoplasmic tails of ACE2, GPVI, and L-selectin. Conserved residues are *highlighted* (*bottom panel*). Synthetic peptides corresponding to residues 762–777 of human ACE2 and peptide variants (mut 1–4) containing mutated amino acid residues.

of ACE2, we next examined whether CaM was able to bind to full-length ACE2. A purified recombinant GST-CaM fusion protein or GST alone was incubated with cell lysates prepared from CHOP cells transfected with the FLAG-tagged full-length ACE2 plasmid. After washing, material that bound to glutathione Sepharose beads was separated using SDS-PAGE and analyzed by Western blotting. ACE2 was present in the cell lysate, migrating as the 120-kDa band and the soluble form of ACE2 used as a positive control (Fig. 3A). In the lane containing the GST-CaM fusion protein, a band corresponding to ACE2 is present, suggesting CaM is able to bind full-length ACE2. As a specificity control, there were no bands corresponding to ACE2 when cell lysates were incubated with GST alone. The polyvinylidene difluoride membranes were also reprobed with anti-GST antibody to validate that equivalent bait proteins were present in the appropriate lanes and running at the appropriate molecular mass (Fig. 3B). In separate experiments (data not shown), we have shown that the binding of CaM to ACE2 can be disrupted by the peptide corresponding to ACE2 762–777, whereas a scrambled sequence of this peptide was unable to displace the recombinant ACE2.
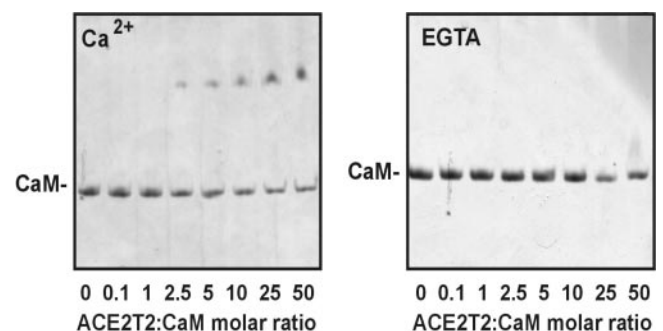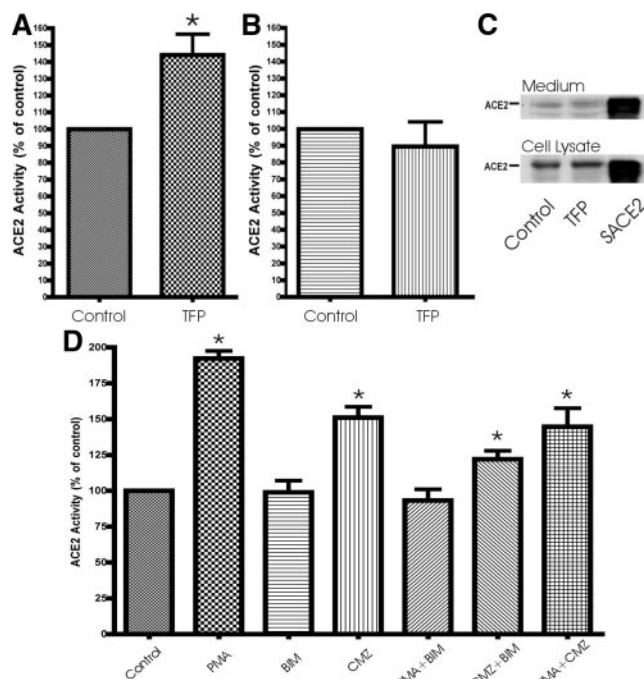

FIG. 2. Calmodulin-peptide complex formation. Purified CaM (300 pmol/tube) was incubated with increasing amounts (0; 30; 300; 750; 1,500; 3,000; 7,500; and 15,000 pmol) of peptide ACE2T2 in the presence of 4 mol/liter urea and either Ca^{2+} or EGTA. Complexes were run on gels containing 4 mol/liter urea and either Ca^{2+} or EGTA, reflecting the same condition as in the samples. The gels were stained with Coomassie blue and observed as a shift in the migration of CaM in the presence of calcium (Ca^{2+}) (*left panel*). No CaM shift was observed in the presence of EGTA at any concentration of the added peptide (*right panel*).
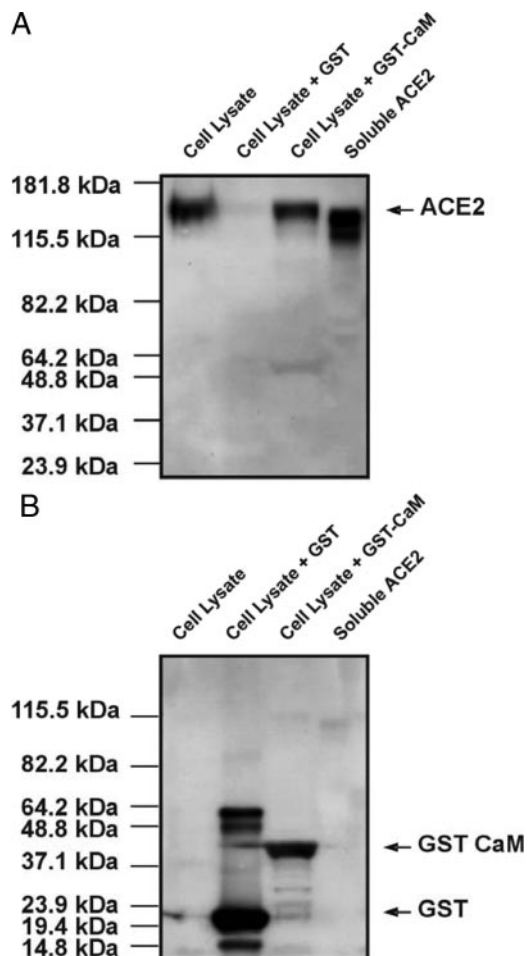
FIG. 3. Binding of ACE2 from cell lysates by GST-CaM. Cell lysates (~2 mg protein) from ACE2-transfected CHOP cells were incubated with glutathione Sepharose beads and GST (20 μg), or GST-CaM (20 μg) bait for 16 h. After incubation, samples were separated by SDS-PAGE and immunoblotted for ACE2 (A) and GST (B) antibodies. Cell lysates (transfected with ACE2) and soluble ACE2 were used as controls for Western blotting.

FIG. 4. Shedding of ACE2 is increased by CaM inhibitors. A, Huh-7 cells that endogenously express ACE2 were incubated in OptiMEM containing 25 μmol/liter TFP or in an equal volume of carrier (Me$_2$SO) for 16 h. The medium was subsequently harvested and concentrated 50-fold, whereas the cells were pelleted and detergent solubilized, as described in *Materials and Methods*. Media (25 μg protein) (A) and cell lysates (25 μg protein) (B) were assayed for their ability to cleave an ACE2 fluorogenic substrate. The individual mean control ACE2 activity with TFP treatment in media and cell lysate were calculated. The data are normalized against the controls from at least four independent experiments (n $\geq$ 4, Student's *t* test; unpaired, two tailed). *Asterisk* denotes significant difference (*P* < 0.05) compared against control. C, One hundred micrograms total protein from the media and cell lysates were separated by SDS-PAGE and Western blotted with monoclonal ACE2 antibody. Soluble ACE2 (SACE2) was used as positive control for immunoblotting. D, Huh-7 cells were incubated in OptiMEM containing combinations of 25 μmol/liter CMZ, BIM (2 μmol/liter), or PMA (1 μmol/liter) and an equal volume of carrier (Me$_2$SO) for 1 h. The medium was prepared and analyzed as described above. The mean control ACE2 activity after CMZ chloride treatment in the media was calculated. The data are normalized against the controls from at least four independent experiments (n $\geq$ 4, one-way ANOVA). *Asterisk* denotes significant difference (*P* < 0.05) compared against control.

Calmodulin inhibitors stimulate ACE2 shedding (phorbol ester independent)

Having established that CaM is able to bind full-length ACE2 *in vitro*, we next investigated the regulation of ACE2 ectodomain shedding by CaM. For these studies, we used Huh-7 cells, a cell line previously used to demonstrate a significant level of ACE2 expression (8). Analysis of the concentrated medium from cells treated with CaM-specific inhibitors showed a significant increase in soluble ACE2 activity compared with control medium (Fig. 4). In cells that were treated with trifluoperazine (25 μmol/liter), a 44.1 $\pm$ 10.8% increase (*P* < 0.05 Student's *t* tests; unpaired, two tailed) in ACE2 activity after 16 h incubation was observed (Fig. 4A). When we switched to using a more potent CaM-specific inhibitor, calmidazolium (25 μmol/liter), in the presence of PMA and Bisindolylmaleimide I, we also observed significant ACE2 activity (51.1 $\pm$ 7.4%) in the concentrated medium compared with control after 1 h treatment (*P* < 0.05, one-way ANOVA) (Fig. 4D). As demonstrated previously (8), PMA is able to stimulate ACE2 shedding (92.3 $\pm$ 5.3%). The increase in ACE2 activity in the medium after CaM inhibitor treatments suggests that that CaM plays a role in the cleavage

secretion of the ACE2 ectodomain. Cell lysates from the trifluoperazine treatment did not show significant changes in ACE2 activity reflecting that only a small percentage (<5%) of ACE2 is actually shed from the cell surface. There was no significant change in the cell lysates of the calmidazolium treated cells (data not shown).

Discussion

Ectodomain shedding is known to be crucial for regulating the cellular responses and biological activities of many membrane-bound proteins such as growth factors, adhesion molecules, and cytokine receptors (23). We previously demonstrated that ACE2 similarly undergoes ectodomain shedding *in vitro* (8) and that ACE2 is present in both human urine and plasma (24, 25). The physiological significance for ACE2 ectodomain shedding is not clearly understood; however, like ACE, ACE2 may need

to be present locally at the cell surface and/or secreted for the appropriate maintenance of local and systemic Ang II levels in the body (26).

The recent discovery of CaM involvement in the phosphorylation of ACE (27) and the ectodomain shedding of both ACE and ACE2 (13, 27) led us to look into the mechanism of how CaM regulates ACE2 ectodomain shedding. Interestingly, the CaM-binding site identified in ACE2 is structurally very different from the CaM binding motif identified for ACE; thus, the mechanism by which calmodulin regulates ACE2 shedding also must be very different; this is not entirely surprising, given that the homology between ACE and ACE2 resides primarily in the extracellular domain (2). Apart from ACE, CaM is known to bind to a number of other membrane-bound proteins that are shed from the cell surface. In a previous study, we showed that CaM binds the membrane-proximal cytoplasmic sequences of the platelet membrane glycoprotein GPVI (12). Similarly, CaM was shown to bind to the cytoplasmic domain of L-selectin (10). The juxtamembrane cytoplasmic sequence of ACE2 is homologous to membrane-proximal sequences of GPVI and L-selectin (Fig. 1), which were both previously shown to bind CaM (10, 11). This sequence similarity suggests, and our results confirm, that CaM is able to bind to the proposed CaM-binding region of ACE2 (13). Study of the interaction between CaM and a synthetic peptide analog of the putative CaM-binding site in ACE2 demonstrated that these proteins associate at a 1:2.5 molar ratio, within the range of values reported for other CaM-binding peptides in gel shift assays (11, 20–22). One of the common characteristics for CaM binding motifs in its target proteins is a conserved region of positively charged amino acids, which often is predicted to form amphipathic α-helices (28, 29). The design of the peptide variants illustrated in Fig. 1 was based on the hypothesis that the charged residues in the wild-type sequence will form an amphipathic α-helical structure. However, none of the peptides, including the wild-type sequence, adopted any defined conformations when analyzed by circular dichroism (data not shown). This indicates that the binding of CaM to the peptides is based on the amino acid sequence rather than the structural characteristic of the region.

CaM plays a pivotal role in the regulation of signal transduction for many biological processes, including regulating the function of integral membrane proteins (30, 31). The presence of a putative CaM binding site in ACE2 may thus indicate the involvement of CaM in the regulation of this membrane-bound carboxypeptidase. CaMis are often used to investigate the association of CaM and membrane-bound proteins. In this study, we observed a significant increase in ACE2 activity in the medium when Huh-7 cells, which endogenously express ACE2, were incubated with CaMi. This finding is consistent with studies of other cell surface molecules in which CaMi treatment stimulated ectodomain shedding. A previous study of the receptor tyrosine kinase (TrkA) showed that the ectodomain of this integral membrane protein can be shed by PMA and CaMi (32). PMA activates the protein kinase C (PKC) pathway, leading to increased cleavage-secretion of many membrane proteins, including ACE2 (33). In contrast, CaMi-induced ectodomain shedding of TrkA was reported to be independent of PKC activity, indicating that

CaMi and PMA mediate cleavage-secretion by two distinctive signaling pathways (32). CaMi-induced shedding was also observed for other cell surface molecules such the adhesion molecule CD44 and the membrane-anchored precursor for TGF-α (pro-TGF-α) (34, 35). Two different sheddases, a disintegrin and metalloproteinase domain (ADAM)-10 and ADAM17, have been reported to cause ectodomain shedding of CD44 by CaMi and PMA, respectively (34, 35), whereas CaMI and PMA were shown to mediate pro-TGF-α shedding through two distinctive signal transduction mechanisms (34, 35). We previously reported that the ectodomain shedding of ACE2 was stimulated by PMA, which could be inhibited using both ADAM17-specific inhibitors and small interfering RNA technology (8). These results suggest that ADAM17 is involved in the regulated, but not constitutive, shedding of ACE2 (8). Although the sheddase(s) involved in ACE2 shedding regulated by CaMi is not known, we propose that the mechanism of activation for ACE2 ectodomain shedding by CaMi differs from that affected by phorbol ester. Our findings demonstrated that CaMi-induced shedding were unaffected by the inhibition of PKC pathway.

Recent studies have shown that CaM binding to the endothelial isoform of nitric oxide synthase (eNOS), which is involved in regulating vascular resistance and blood circulation in the liver (36, 37), is reduced after liver injury (38). Wang Abdel-Rahman (38) demonstrated that, although the level of CaM remained unchanged in the diseased liver, the binding of CaM to eNOS significantly decreased. Similarly, in one of our recent studies (39), we reported that ACE2 expression is significantly up-regulated in human and rat liver after chronic liver injury. Although the direct association of CaM and ACE2 in the liver has yet to be investigated, it is possible that, like eNOS, CaM binding to the overexpressed ACE2 may be reduced in the liver, ultimately leading to an increase in ACE2 secretion. In diseased tissues such as the heart and liver, an increase in secreted ACE2 could counteract the effect of increased Ang II production caused by other overexpressed components in the renin-angiotensin system (40, 41).

There is increasing evidence to suggest that ACE2 is a critical regulator of cardiovascular function. The ectodomain shedding of ACE2 is potentially an important mechanism by which local ACE2 activity can be regulated. In addition, the shedding of ACE2 may represent a mechanism by which viral entry and infection may be controlled (*e.g.* SARS-CoV). Our studies demonstrate that CaM binds to a peptide mimetic of ACE2 cytoplasmic tail, and we show that CaMi- and PMA-induced shedding of ACE2 ectodomain occur via two independent mechanisms by which the ACE2 sheddase(s) may be activated. This finding provides valuable information about ACE2 shedding as well as identifying potential intracellular targets for the pharmacological and therapeutic regulation of ACE2.

Acknowledgments

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