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Calmodulin interacts with angiotensin-converting enzyme-2 (ACE2) and inhibits shedding of its ectodomain

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Abstract **Angiotensin-converting enzyme-2 (ACE2) is a regulatory protein of the renin–angiotensin system (RAS) and a receptor for the causative agent of severe-acute respiratory syndrome (SARS), the SARS-coronavirus. We have previously shown that ACE2 can be shed from the cell surface in response to phorbol esters by a process involving TNF-α converting enzyme (TACE; ADAM17). In this study, we demonstrate that inhibitors of calmodulin also stimulate shedding of the ACE2 ectodomain, a process at least partially mediated by a metalloproteinase. We also show that calmodulin associates with ACE2 and that this interaction is decreased by calmodulin inhibitors.**

Keywords: ACE; ACE2; Collectrin; Calmodulin; Shedding

1. Introduction

Angiotensin-converting enzyme-2 (ACE2) is rapidly emerging from the shadow of its better-known homologue angiotensin-converting enzyme (ACE) as an important co-regulator of the renin–angiotensin system (RAS). Whilst the primary physiological role of ACE in the RAS is to hydrolyse angiotensin I (Ang I) to the potent vasoconstrictor angiotensin II (Ang II) [1], ACE2 is able to cleave Ang II to produce Ang (1–7), a peptide which has opposing effects [2,3]. The physiological significance of ACE2 in the RAS has been demonstrated in a variety of tissues including the heart, liver, kidney and lung [4–7]. In addition, ACE2 is the cellular receptor for the SARS coronavirus, the causative agent of severe-acute respiratory syndrome (SARS) [8]. ACE2, like ACE, is a type I transmembrane metallopeptidase with an extracellular ectodomain containing its zinc-coordinating catalytic site [9,10]. Here, it is positioned to hydrolyse circulating substrates and serve as a viral receptor. Regulation of its expression at the cell surface is therefore of prime importance to its physiological and pathophysiological functions.

We have previously shown that the ACE2 ectodomain can be cleaved from the cell membrane and be released into the extracellular milieu [11,12]. This 'shedding' event is stimulated by phorbol esters and involves a member of the ADAM (a disintegrin and metalloproteinase) family, ADAM17 (also known as TNF-α-converting enzyme, TACE) [12]. The function of diverse cell surface proteins is regulated by such shedding events, including enzymes (ACE, beta-site amyloid precursor protein cleaving enzyme), cytokines and growth factors (TNF-α, heparin binding epithelial growth factor) and neurodegenerative proteins (amyloid precursor protein, cellular prion protein) [13–18]. Whilst a great deal is known about the proteases which mediate these shedding events, the factors regulating this process still remain unclear. Ectodomain shedding is a complex event responding to a variety of stimuli (phorbol esters, calcium ionophores, growth factors) and involving a variety of interacting cellular proteins (protein kinase C, Eve), depending on the substrate [19–21].

In this study, we have identified the involvement of calmodulin in the regulation of ACE2 ectodomain shedding. Calmodulin is an ubiquitous calcium binding protein which is known to bind other transmembrane proteins, including L-selectin and ACE, and regulate their cell surface expression [22,23]. Here, we show that calmodulin interacts with ACE2, both in cells expressing ACE2 heterologously and endogenously, and inhibitors of calmodulin increase the release of the ACE2 ectodomain in a dose- and time-dependent manner. This stimulation of shedding is only partially abrogated by metalloproteinase inhibitors, suggesting the involvement of disparate sheddases. Furthermore, treatment with calmodulin inhibitors decreases the association between the two proteins, suggesting the interaction of ACE2 with calmodulin serves to retain catalytically active enzyme in the plasma membrane.

2. Materials and methods

2.1. Materials

All standard laboratory reagents were purchased from Sigma (UK) unless indicated otherwise. Anti-ACE2 polyclonal antibody was purchased from R&D Systems (UK), anti-calmodulin antibody and donkey anti-goat horseradish-peroxidase conjugated secondary antibodies were purchased from Sigma (UK). GM6001 was purchased from Chemicon (UK). The ACE2-specific fluorescent substrate Mca-APK(Dnp) was synthesized by Dr. G. Knight (Cambridge University, UK).

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Abbreviations: ACE, angiotensin-converting enzyme; ADAM, a disintegrin and metalloproteinase; Ang, angiotensin; CaMi, calmodulin inhibitor; Dnp, dinitrophenyl; HEK, human embryonic kidney; Mca, 7-amido-4-methylcoumarin; RAS, renin–angiotensin system; RIPA, radio-immunoprecipitation assay; SARS, severe-acute respiratory syndrome; TNF-α, tumour necrosis factor-alpha

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2.2. Cell culture

HEK-ACE2 cells and Huh7 cells, a human hepatocellular carcinoma-derived cell line known to express and shed ACE2 [12], were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 2 mM essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Treatment of cells and protein extraction

Cells were grown to confluence in 80 cm^3 flasks and rinsed twice with OptiMem prior to experimentation. All pharmacological treatments were diluted in OptiMem (5 ml) and all incubations carried out at 37 °C. Medium was harvested and concentrated by centrifugation in 10 kDa cut-off Centricon tubes (VivaScience, UK) to a final volume of ≈200 μl. Cells were scraped into ice-cold phosphate-buffered saline, pelleted by centrifugation and solubilised in 500 μl radio-immunoprecipitation assay (RIPA) 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 [21].

2.4. Immunoprecipitation of cell lysates

Cell lysates (500 μg, prepared as described) were incubated with rotation for 3 h at 4 °C with 50 μl protein-A Sepharose beads (Sigma). Following centrifugation, the supernatant was incubated overnight at 4 °C with rotation in the presence of 10 μl anti-calmodulin monoclonal antibody (Sigma). Subsequently, 50 μl protein-A sepharose beads (1 g resuspended in 3 ml phosphate buffered saline) were added and incubation continued for a further 2 h. The conjugated beads were pelleted by centrifugation and rinsed three times in ice-cold RIPA buffer. The beads were then heated in SDS–PAGE sample buffer containing denaturing reagent (Invitrogen) for 10 min at 95 °C.

2.5. SDS–PAGE and immunoblotting

Immunoprecipitated proteins were separated by SDS–PAGE and proteins electrotransferred to nitrocellulose membranes (Invitrogen). Non-specific protein binding sites were blocked using 5% (w/v) dried milk, 3% (w/v) bovine serum albumin in Tris-buffered saline containing 0.5% (v/v) Tween-20 (TBS-T), and the membranes subsequently incubated in anti-ACE2 antibody (1:1000 for HEK-ACE2, 1:100 for Huh7) in the same solution for 3 h. Donkey anti-goat horseradish peroxidase-conjugated secondary antibody was used at a dilution of 1:5000 for 1 h in TBS-T. Immunoreactive bands were visualised using enhanced chemiluminescence (ECL; Pierce, UK) according to the manufacturer's instructions.

2.6. Fluorogenic assay of ACE2

The catalytic activity of ACE2 in concentrated media proteins (20 μg, HEK-ACE2; 50 μg, Huh7) was determined using a specific fluorogenic substrate (Mca-APK(Dnp)), as described previously [12].

2.7. Statistical analyses

Statistical significance of data were tested using Mann–Whitney *U*-test.

3. Results

3.1. The cytoplasmic domain of ACE2 contains a conserved predicted calmodulin binding motif

Analysis of the cytoplasmic domain of ACE2 using the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca; [24]) revealed the presence of a region strongly indicative of a potential calmodulin binding domain (Fig. 1A). This 10 amino acid region, encompassing residues 763–772, is evolutionarily conserved in both rat and mouse (Fig. 1A), suggesting this domain may be functionally significant.

3.2. Calmodulin associates with ACE2

In order to ascertain whether the predicted calmodulin binding domain of ACE2 was indeed functional, we next performed immunoprecipitation using an anti-calmodulin monoclonal antibody in cellular lysates collected from

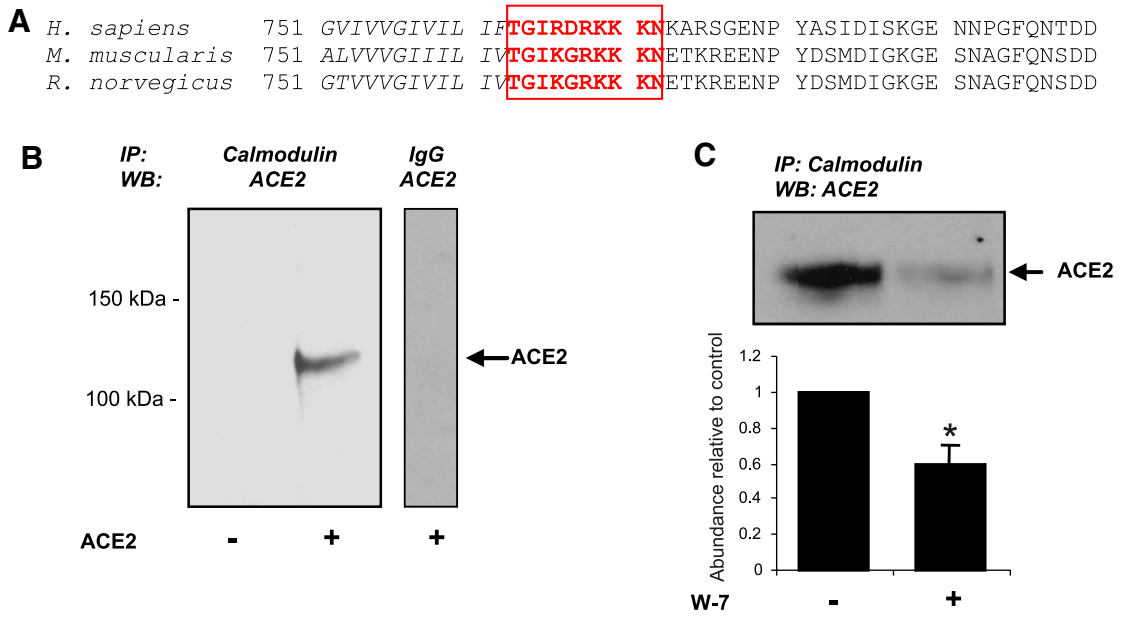

Fig. 1. ACE2 interacts with calmodulin. (A) Software analysis of the cytoplasmic domain of ACE2 (http://calcium.uhnres.utoronto.ca) reveals the presence of a calmodulin binding motif (bold, highlighted in red) proximal to the transmembrane domain (*italics*). The sequence is highly conserved in humans, mice and rats. (B) Cell lysates collected from untransfected HEK293 cells or cells stably transfected with ACE2 (HEK-ACE2) were immunoprecipitated with an anti-calmodulin antibody or IgG (referred to as IP) and immunoblotted for ACE2 (referred to as WB). (C) HEK-ACE2 cells were treated with the calmodulin inhibitor W-7 (25 μM) for 30 min, lysates collected and immunoprecipitated with an anti-calmodulin antibody before being immunoblotted for ACE2. A blot representative of the results of three experiments is shown together with the results of densitometrical analysis performed on the blot, ±S.E.M. $^*P < 0.05$.

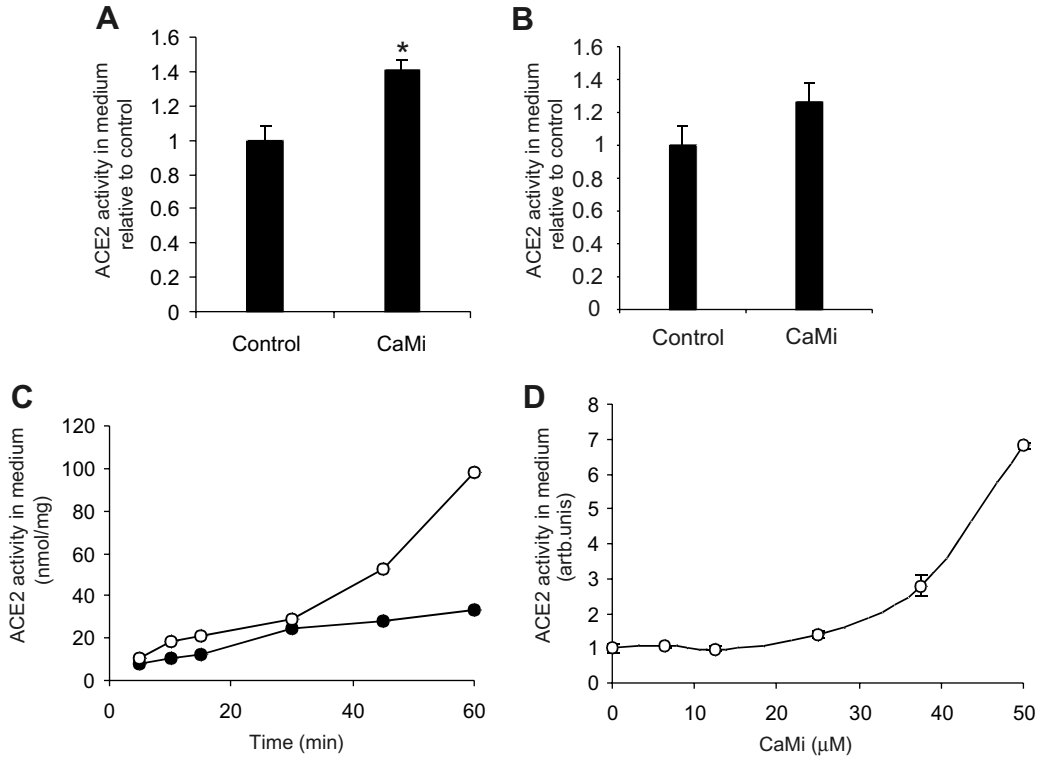

Fig. 2. Inhibition of calmodulin increases ACE2 ectodomain shedding. HEK-ACE2 cells (A) or Huh7 cells (B) were incubated in the presence or absence of calmidazolium (25 μM; CaMi) for 30 min in OptiMem. HEK-ACE2 cells were incubated in the presence (closed circles) or absence (open circles) of CaMi (25 μM) for the times indicated (C), or for 30 min at the concentrations indicated (D). Media was collected, concentrated and assayed for ACE2 activity as described in Section 2. The results represent data collected from at least three independent experiments, ±S.E.M. $^{*}P < 0.05$.

HEK293 cells stably transfected with full-length ACE2 [12]. Subsequent immunoblotting of immunoprecipitates for ACE2 revealed a band of the expected size (120 kDa) in HEK-ACE2 cells but not untransfected HEK cells (Fig. 1B) or HEK-ACE2 cell lysates subjected to immunoprecipitation with mouse IgG. Similar results were obtained for Huh7 cells, which endogenously express ACE2 ([12]; data not shown). Incubation of HEK-ACE2 cells with W-7, a specific calmodulin antagonist, reduced the ACE2:calmodulin association (Fig. 1C).

3.3. Calmodulin inhibitors stimulate ACE2 ectodomain shedding

Given that interaction of membrane proteins with calmodulin has been shown to influence their retention in the plasma membrane, we next analysed the effect of inhibiting the association of ACE2 with calmodulin on the shedding of its ectodomain. Incubation of HEK-ACE2 or Huh7 cells with the calmodulin antagonist (CaMi) calmidazolium resulted in increased ACE2 activity in the media (Fig. 2A and B, respectively). This CaMi-mediated stimulation of ACE2 shedding was time- and dose-dependent (Fig. 2C and D, respectively). Similar results were obtained with other calmodulin inhibitors (trifluoperazine, W-7; data not shown).

3.4. CaMi-stimulated shedding is reduced by the metalloproteinase inhibitor, GM6001

We have previously demonstrated that ADAM17 (also known as TACE, tumour necrosis factor-α converting enzyme) is involved in mediating stimulated ACE2 shedding in response to phorbol ester [12]. We therefore next analysed whether a metalloproteinase is also involved in CaMi-stimulated ACE2 shedding. Pre-incubation of HEK-ACE2 cells with the hydroxamate-derived metalloproteinase inhibitor GM6001 reduced the increased ACE2 shedding in response to CaMi (Fig. 3A). GM6001 treatment had little effect on basal shedding of ACE2 from HEK-ACE2 cells as shown previously [12]. Inhibitors of other classes of proteinases (serine, aspartic and cysteine) had no observable effect on shedding (Fig. 3B). GM6001 did not have any effect on the activity of ACE2 itself (Fig. 3C).

4. Discussion

ACE2 is increasingly recognised to have a pivotal role in regulating the local levels of the hypertensive and mitogenic peptide angiotensin II [25–27]. In addition, it is also the cellular receptor of the causative agent of SARS, SARS-CoV [8]. ACE2 is a type I transmembrane protein which is subject to a juxtamembrane cleavage event releasing a catalytically active ectodomain [12]. While the precise physiological role of this 'shedding' event is unknown it is clear, given the well documented pathophysiological roles of Ang II, that the mechanisms regulating the cell surface expression of ACE2 are of critical importance. We have previously demonstrated that the shedding of ACE2 can be stimulated by phorbol ester, a process mediated at least in part by the promiscuous metalloproteinase 'sheddase', ADAM 17 [12]. Here, we show that the

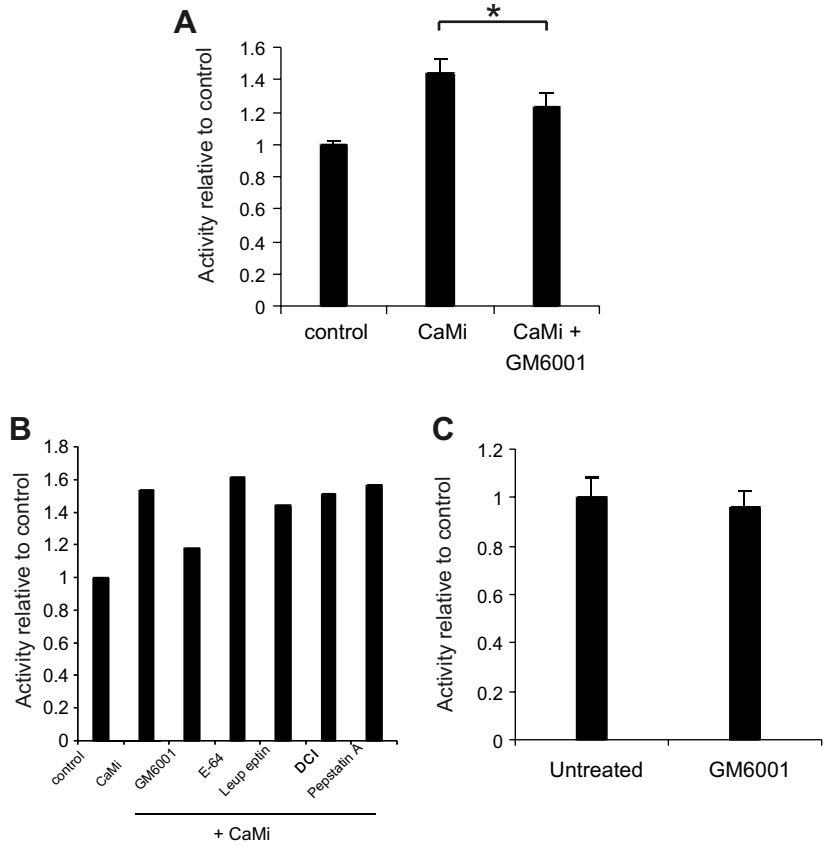

Fig. 3. CAMi-mediated ectodomain shedding is blocked by metalloproteinase inhibitor. HEK-ACE2 cells were pre-incubated with either the sheddase inhibitor GM6001 (25 μM); (A and C) or the cysteine proteinase inhibitors E-64 and leupeptin, the serine proteinase inhibitor 3,4-dichloroisocoumarin or the aspartic proteinase inhibitor pepstatin A (B) for 30 min before the addition of CaMi (25 μM) for 30 min. Media from these and from untreated control cells was collected, concentrated and assayed for ACE2 activity as described in Section 2. As an additional control, media were collected from untreated cells and incubated in the presence or absence of 50 μM GM6001 before being assayed for ACE2 activity as described. The results are presented relative to untreated control flasks and represent data collected from four (A), two (B) or three (C) independent experiments. $^{*}P < 0.1$.

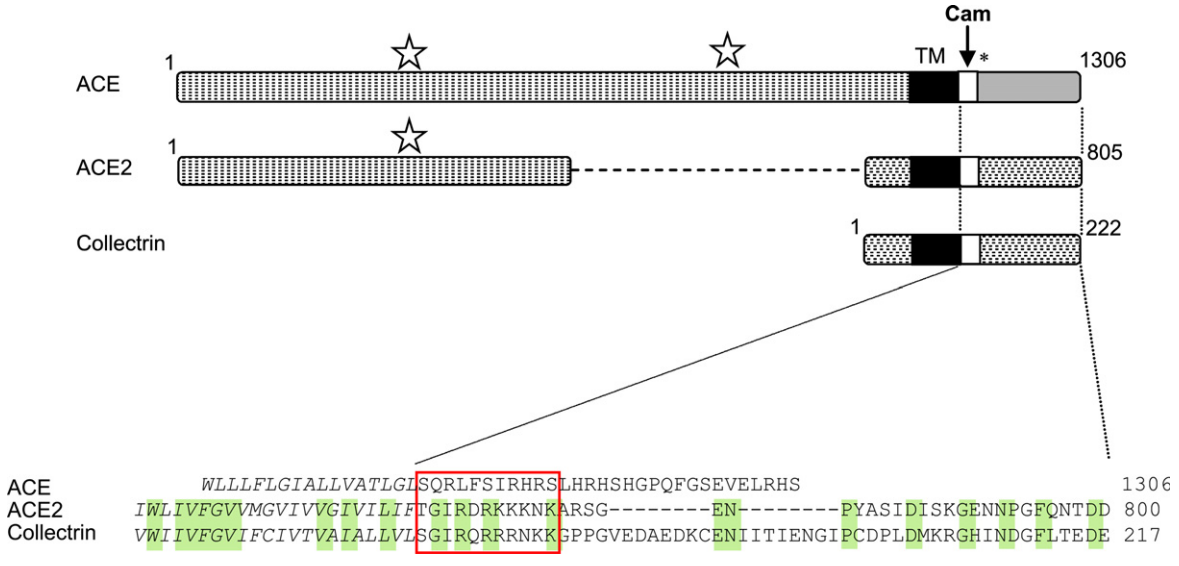

Fig. 4. The ACE2 homologue collectrin contains a putative calmodulin binding motif. Alignment of human somatic ACE, ACE2 and collectrin functional domains. Stars indicate catalytic sites, black rectangles the transmembrane domains and white rectangles denote observed (*) or predicted calmodulin binding domains. The ACE2 and collectrin peptide sequence alignment shows conserved residues (highlighted) and predicted calmodulin binding domain (outlined). There is no homology between ACE and ACE2 or collectrin in this region. The transmembrane hydrophobic regions of all three proteins are in italics.

shedding of ACE2 can also be upregulated by inhibitors of the ubiquitous calcium binding protein, calmodulin, both in cells endogenously expressing ACE2 (Huh7) and in those heterologously over-expressing it (HEK-ACE2). The CaMi-mediated increase in ACE2 shedding was reduced by the hydroxamate-derived metalloproteinase inhibitor GM6001, suggesting the involvement of an ADAM (a disintegrin and metalloproteinase). Inhibitors of other classes of proteinases (serine, aspartic, and cysteine) had no effect on cleavage. Further studies are required to determine whether the CaMi-stimulated shedding of ACE2 is also mediated by ADAM17, or whether phorbol esters and calmodulin inhibitors invoke distinct sheddases.

Computational analysis of the cytoplasmic domain of ACE2 revealed a conserved consensus calmodulin binding motif. Immunoprecipitation experiments revealed that calmodulin associates with ACE2 suggesting that this motif may be functional. While further studies are required to elucidate the precise binding site of calmodulin on ACE2, these results clearly indicate that association of calmodulin with ACE2 has a regulatory role in its cleavage-secretion from the plasma membrane. It is noteworthy that the cytoplasmic tail of ACE2 is quite distinct to that of ACE, the shedding of which is also modulated by association with calmodulin [23]; however, the ACE2 tail bears significant sequence identity to collectrin, a developmentally regulated protein recently shown to have a critical role in amino acid retrieval in the kidney [28,29] (Fig. 4). Analysis of the sequence of the cytoplasmic domain of collectrin also identifies a putative calmodulin-binding motif (Fig. 4). Recent studies have revealed that collectrin is also subject to ectodomain shedding [30], suggesting a possible role for calmodulin in regulating the cell surface expression and release of this protein.

In summary, we have demonstrated that ACE2 interacts with calmodulin and that this association down-regulates shedding of the ACE2 ectodomain. This is the first study to identify a regulatory binding protein for ACE2 in vitro, revealing a hitherto unknown mechanism for regulating its cell surface and circulating activity.

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