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

Daniel W. Lambert[a,*], Nicola E. Clarke[a], Nigel M. Hooper[b], Anthony J. Turner[a]

[a] *Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK*
[b] *Leeds Institute of Genetics, Health and Therapeutics (LIGHT), University of Leeds, Leeds LS2 9JT, UK*

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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).

*Corresponding author.
E-mail address: d.w.lambert@leeds.ac.uk (D.W. Lambert).

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

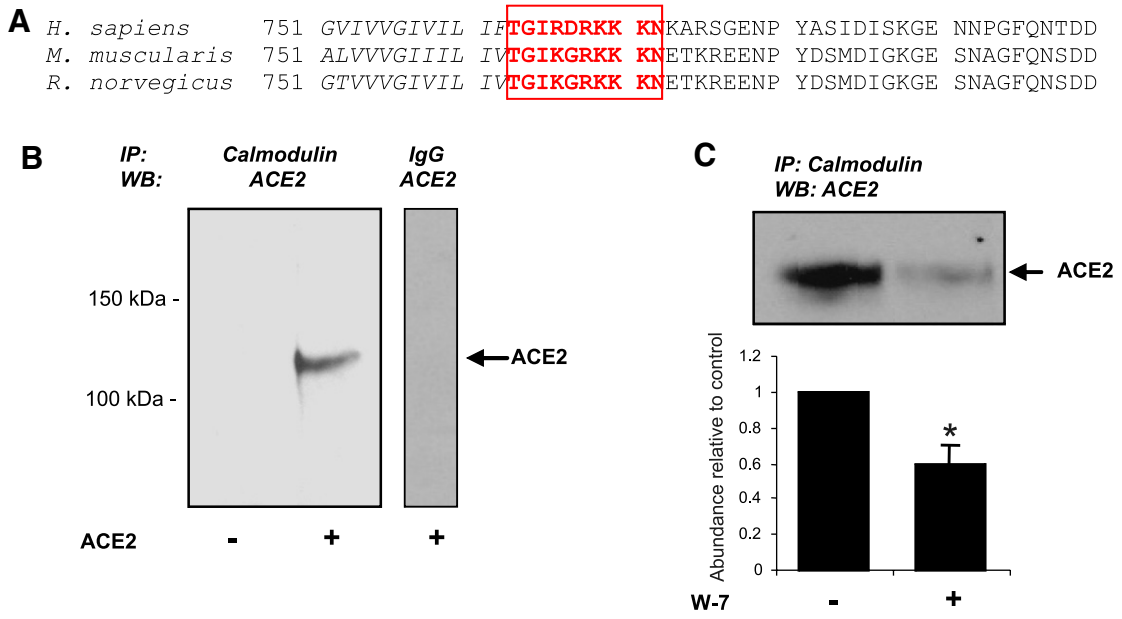

Fig. 1. ACE2 interacts with calmodulin. (A) Software analysis of the cytoplasmic domain of ACE2 (http://calcium.uhnres.utoronto.ca) reveals the presence of a calmodulin binding motif (bold, highlighted in red) proximal to the transmembrane domain (*italics*). The sequence is highly conserved in humans, 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. $^*P < 0.05$.