THE CHARACTERISATION OF ECTODOMAIN SHEDDING OF ANGIOTENSIN-CONVERTING ENZYME 2 (ACE2)

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ERRATA

Pg x, line 10: “change” for “changes”

line 11: “decrement” for “decrease”

line 12: “has” for “have”

line 13: “evidences” for “evidence”

line 15: “proposed” for “propose”

line 20: “adds” for “add”

Pg 1, line 13: “diseases ” for “disease”

line 15: “; thus” for “. Thus”

Pg 2, line 8: “cleaves” for “cleave”

Pg 3, line 6: “four” for “five”

Pg 4, line 3: “amide” for “peptide”

Fig 1-2 legend: “SREBP” for “sterol regulatory element binding protein”

Pg 5, line 8 (and throughout the thesis): “in vitro” for “*in vitro*”

Pg 7, line 8: “most” for “best”

Pg 20, line 15: “lininopril” for “lisinopril”

Pg 46, line 10 (and throughout Chapter 2): “Rydralmere” for “Rydalmere”

Pg 62, line 7: “MiliQ” for Milli-Q”

ADDENDUM

Pg ix, line 9: add “TACE or” between “known as” and “ADAM17”

line 15: delete “from” and read “in”

line 16: delete “have”

line 18: add “to” between “responsive” and “protein”

line 19: delete “the one” and read “that”

line 23: add “an” between “of” and “ACE2”

Pg x, line 2: delete “by explore” and read “phorbol ester”

line 6: delete “under” and read “in”

line 7: delete “of” and read “post”

line 7: delete “there were”

line 8: add “were” between “increases” and “observed”

line 15: delete “the”

line 20: delete “does”

line 21: delete “actually”

Pg 7 line 17: Insert Table:

Table 1: The subgroup of human ADAMs (adapted and modified from (*1*)).

ADAM	Other aliases	Major sites of expression in human tissues	Inhibition by TIMPs	Substrates
ADAM1	Fertilin alpha, FTNAP, Ftna, PH-30a			
ADAM2	Fertilin beta, CRYN1, CRYN2, FTNB, PH-30b, PH30	Testis	No study	
ADAM3	CYRN1, tMDCI			
ADAM5	TMDCII			
ADAM6	t MDCIV; C14orf96			
ADAM7	EAPI, GP-83	Testis, erythrocytes		
ADAM8	CD156, MGC134985, MS2	Bone marrow lymphoid/ myeloid cells, lymphatic system, haematopoietic stem cells, peripheral blood lymphoid/myeloid cells	None	ADAM 8 (pro-domain cleavage), CD23, CD30-Ligand (CD153), CHL-1, L-selectin
ADAM9	KIAA0021, MCMP, MDC9,	Mesenchymal stem cells, placenta,	None	Pro-HB-EGF, APP, Kit-ligand, p75 neurotrophin

	Mltng, meltrin gamma	pancreas, adult stem cells, adipose tissue		receptor, Insulin B chain, Delta-like ligand-1 (DII1), IGFBP-5, ADAM10, Collagen XVII, Laminin, Pro-EGF, FGF Receptor 2 iib
ADAM10	CD156c, HsT18717, MADM, kuz	Mesenchymal stem cells, placenta, blood myeloid cells, bladder, bone marrow myeloid cells	TIMP-1 TIMP-3	Notch, APP, N-cadherin, E-cadherin, Proteo-cadherin-γ C3 and B4, VE-cahderin, CD23, DII1, Pro-EGF, Pro-betacellulin, Ephrin A5, c-Met, Fas-ligand, HER2/new (ERBB2), CD30, CD44, Collagen XVII, TRANCE/RANKL, L1-CAM, CX3CL1/Fractalkine, CXCL16, LAG-3, Desmoglein-2, Klotho, PrP^C, Thyrotopin Receptor (TSHR), Axl
ADAM11	MDC	Erythrocytes, central and peripheral nervous systems, liver and biliary system, salivary gland		
ADAM12	RP11-295J3.5, MCMP, MCMPMltna, MLTN, MLTNA, Meltrin alpha	Placenta, mesenchymal stem cells, adult stem cells	TIMP-2 TIMP-3	DII1, Gelatin, Type-IV collagen, Fibronectin, IGF-BP-3, IGF-BP-5, HB-EGF, S-Carboxymethylated transferring
ADAM15	MDC15, Metargidin	Widespread (highest in mesenchymal stem cells and urogenital system)	No Study	CD23, Pro-amphiregulin, Pro-HB-EGF, E-cadherin
ADAM17	CD156b, MGC71942, TACE, cSVP	Widespread (highest in lymphatic)	TIMP-3	Pro-TNF-α, Pro-TGF-α, Pro-Amphiregulin, Neuregulin, Epigen, P75 TNF receptor, P55 TNF receptor, IL-1 Receptor-II, APP, DII1, Notch1, TRANCE/RANKL, Kit ligand-1 and -2, L-selectin, Neurotrophin receptor, ErbB4/HER4, CD44, CD40, CD30, Growth hormone receptor, L1-CAM, VCAM-1, IL6 Receptor, PrP^C, CX3CL1/Fractalkine, LAG-3, Colony stimulating factor-1, Nectin-4, ALCAM, Desmoglein-2, Klotho, Vacuolar protein sorting Vps10-p, N-CAM, PTP-LAR, Collagen XVII, Pre-adipocyte factor-1 (Pref-1), ACE2, Semaphorin 4D, NPR (neuronal pentraxin receptor), MICA/MICB (MHC-class I-related chain A/B)
ADAM18	ADAM27, MGC41836, MGC88272, tMDCIII	Testis, erythrocytes, bone marrow, pancreas)		
ADAM19	FKSG34, MADDAM, MLTNB	Widespread (highest in placenta, mesenchymal stem cells, lymphatic system, heart)	None	Neuregulin, TRANCE/RANKL, Pro-TNF-α, ADAM19
ADAM20		Testis, erythrocytes, bone marrow	No Study	
ADAM21	ADAM31, MGC125389	Testis, erythrocytes, central and peripheral nervous systems	No Study	
ADAM22	MDC2, MGC149832	Peripheral and central nervous systems		
ADAM23	MDC3	Peripheral and central nervous systems, heart		
ADAM28	ADAM23, MDC-Lm, MDC-Ls, MDCL, eMDC	Haematopoietic stem cells, pancreas, gastrointestinal system, bone marrow myeloid cells, lymphatic system, respiratory system, bladder	TIMP-3 TIMP-4	CD23, IGFBP-3
ADAM29	svph1	Testis		
ADAM30	svph4	Testis	No Study	
ADAM32	FLJ26299, FLJ29004	Blood lymphoid cells		
ADAM33	RP5-964F7.2, DJ964F7.1, DKFZp434K0521, FLJ35308, FLJ36751, MGC149823, MGC71889	Uterus, other urogenital system, respiratory system, gastrointestinal system, tongue, endocrine system	TIMP-3 TIMP-4	Kit-ligand-1, CD23

Pg 8, line 5: Comment: 129-derived stem cells correspond to a mouse strain containing a large degree of genetic diversity among 129 substrains.

Pg 22, last sentence: delete "ACE2 is predominantly found in the heart, kidneys and testis and" and read "Although extensive studies have characterised ACE2 with respect its involvement in the RAS as well as the binding of SARS coronavirus,"

Pg 26, line 11: Comment: B^0AT1 is an amino acid transporter that is essential for neutral amino acid absorption in the intestine and kidney (*2, 3*). Gene mutations in B^0AT1 (SLC6A19) have been identified as a cause of Hartnup disorder, characterised by a pellagra-like light-sensitive rash, cerebellar ataxia, emotional instability and amino aciduria (*4, 5*).

Pg 31, para 1: Comment: CHO-P, CHO-K1, HEK 293, HEK 293T and HEK-NHA-AT1 cell lines have not been tested for Mycoplasma infection. Huh-7 cells were tested positive for Mycoplasma infection in Prof. Stephen Locarini group (VIDRL, Melbourne, VIC, Australia).

Pg 40, line 16: delete "Huh-7, stable ACE2 and ACE2 mutant cells, and transfected with the soluble ectodomain form of ACE2," and read "transfected cells".

Pg 42, line 10: Comment: Quenched fluorescent substrate used to measure is specific to ACE2 in the high nanomolar range, along with the incorporation of a highly specific ACE2 inhibitor (MLN 4760) (IC_{50} = 0.44 nM). It is not known if this custom synthesised peptide substrate is used for other enzymes such as prolyl endopeptidase. However, studies in vitro have shown that ACE2 to be 10- to 600-fold more potent in hydrolysing AngII compared to prolyl endopeptidase (*6*). Hence specific inhibitor for prolyl endopeptidase is also used when looking at ACE2 activity assay.

Pg 42, line 10: Add reference (*7*)

Pg 51, line 16: Add at the end of "autoMSn mode":
" [AutoMS(*8*); No. Precursor Ions: 4; Threshold Abs: 50,000; Threshold Rel: 5.0%; Acquisition Parameter: MS/MS Frag Ampl 1.00V]"

Pg 52, chap 2.22: Add at the end of para:
"Duplicate samples were performed for each CD experiment, using Milli-Q grade water or 50% (v/v) MeOH as sample buffers. The data was corrected against the baseline of blank solution (no peptide control). The analysis used for structure comparison is based on the database provided with the CD instrument."

Pg 51, chap 2.22: Comment: At 180 nm and below, the acquired spectra from the peptides contain high level of background noise, which affected the smoothing of spectra after correction. Hence the sample collection wavelengths were limited to above 190 nm.

Pg 62, para 3: Add at the end of 4th sentence:
"Not all the CaM shift was observed at high peptide to CaM molar ratio possibly due to the poor solubility of this peptide at high concentrations. There is no evidence that the preparation contains inactive CaM. The observed shifts in CaM bands in all the assays require the presence of Ca^{2+}, suggesting this is a Ca^{2+}-sensitive interaction."

Pg 65, end of 4th sentence: delete "." and read ", with the exception of mutant 1. Mutant 1 displayed significant changes in conformational profile, indicating a possible helical structure in semi-organic environment. Given that the synthetic mutant peptides are investigated for their ability to bind CaM in an aqueous condition, we would assume that Mutant 1 will adopt the random confirmation (Fig 3-7*A*)."

Pg 65, 2nd last sentence: Comment: Although the experiment was repeated at least three times, there were some minor variations observed between Fig 3-4 and Fig 3-8 LHS. The multiple bands observed in Fig 3-8 LHS could be due to the peptides binding to various structural confirmations of CaM. Nonetheless the pivotal observation lies in the capability of wild-type peptide mimetic in shifting the CaM band, but not in mutant peptides.

Pg 66, Fig 3-6 legend: Add reference (*9, 10*)

Pg 87, para 1: Comment: It should be noted that the attempt in replicating the data from the recently published report using 2D-PAGE with proteomics approaches is only a small representation of the 'trial and error' experimentations at large. None of the lower MOWSE score "hits" from the fragment data were matched to ACE2. Although many other options were considered, including using MRM/SISCAPA approach, due to the time

constrain, we have proceeded with the peptidomimetic approaches, which has proven to be a successful and powerful approach.

Pg 91, line 5: delete “701-720” and read “711-730”

line 5: delete “711-730” and read “701-720”

Pg 91, para 2: Comment: The peptidomimetic approaches utilised in this study specifically looked at ADAM17’s ability to cleave ACE2 peptide mimetics. This was verified by the use of highly specific inhibitor of ADAM17, TAPI-1, thus ruled out any possibility that the synthetic peptides were cleaved by other proteases/ADAMs.

Pg 131, end of chap 5.3: Insert Table:

Table 2: Summary of angiotensin peptides mediated shedding of ACE2 from various cell lines.

Cell Line	AngI Stimulated Shedding	AngII Stimulated Shedding
Huh-7 constitutively expressing ACE2	YES	YES
HEK-NHA-AT1 cells transiently expressing ACE2	YES	YES
HEK-NHA-AT1 cells stably expressing ACE2 (pcDNA3.1 vector)	NO	YES
HEK-NHA-AT1 cells stably expressing ACE2 (pIRESpuro vector)	YES	YES

Pg 135, end of chap 5: Comment: It is possible that Ang1-7 may play a role in regulating ACE2 ecotodomain shedding via *mas receptor*. However, the main objective of this chapter aimed at investigating the whether the stimulation of AT1R, by AngI and/or AngII peptides, could lead to ACE2 shedding. Thus, the potential role of Ang1-7 on the regulation of ACE2 ecotodomain shedding has not been explored. Future experimental work will hopefully elucidate the role of Ang1-7 in regulating ACE2 shedding.

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FIGURES

TABLES

ABBREVIATIONS

2D-PAGE	2-dimensional Gel Electrophoresis
Aβ	Beta Amyloid
ACE	Angiotensin-converting Enzyme
ACE2	Angiotensin-converting Enzyme-2
ADAM	A Disintegrin and Metalloprotease
AngI	Angiotensin I
AngII	Angiotensin II
AngIII	des-Aspartyl^{1}-AngII
AngIV	Angiotensin IV / Angiotensin (3-8)
Ang(1-7)	Angiotensin (1-7)
Ang(1-9)	Angiotensin (1-9)
APP	Amyloid β Precursor Protein
AT1R	Angiotensin Type-1 Receptor
AT2R	Angiotensin Type-2 Receptor
AT4R	Angiotensin Type-4 Receptor
AXL Receptor	Tyrosine-protein Kinase Receptor UFO
B^{0}AT1	Sodium-dependent Amino Acid Transporter SLC6A19
BIM	Bisindolylmaleimide I
BSA	Bovine Serum Albumin
CaM	Calmodulin
CaMI	Calmodulin Inhibitors
CD	Circular Dichroism
CD18	Beta-2 integrin

CD40	Costimulatory Protein on surface of B lymphocytes
CD44	Hyaluronic Acid Receptor
CHO	Chinese Hamster Ovary
CHO-K1	CHO Cells Subclone K1
CHO-P	CHO Cells Stably Expressing Polyoma Large T-Antigen
CMZ	Calmidazolium Chloride
CoV	Coronavirus
DNP	Dinitropheyl
E-cadherin	Epithelial Cadherin
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol-bis-(2-aminoethyl)-N,N,N',N'-tetra-acetic Acid
eNOS	Endothelial Isoform of NO Synthase
FBS	Foetal Bovine Serum
GnRH	Gonadotropin-releasing Hormone
GPCR	G-protein Coupled Receptor
GPI	Glycosyl-phosphatidyliositol
GPIb-IX-V	von Willebrand factor
GPVI	Glycoprotein VI
GST	Glutathione S-transferase
HA	Hemagglutinin Antigen
HB-EGF	Heparin-binding Epidermal Growth Factor
HEK 293	Human Embyonic Kidney 293 Cells
HEK 293T	HEK 293 Cells stably Expressing Large T-Antigen of SV40
NEK-NHA-AT1	HEK 293 Cells stable Expressing N-terminally HA-tagged AT1R
HRP	Horse Radish Peroxidase
IGFR	Insulin-like Growth Factor-1 Receptor
I-CLiPs	Intramembrane Cleaving Proteases

IEF	Isoelectric Focussing
IPG	Immobilised pH Gradient
KKS	Kallikrein-kinin System
LC	Liquid Chromatography
LDL	Low Density Lipoprotein
MALDI	Matrix Assisted Laser Desorption Ionisation
MCA	7-methoxycoumarin-4-yl)acetyl
MMP	Matrix Metalloprotease
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MT-MMP	Membrane-type Matrix Metalloprotease
MWCO	Molecular Weight Cut-off
NAD(P)H	Nicotiamide Adenine Dinucleotide (Phosphate)
NO	Nitric Oxide
N-cadherin	Neural Cadherin
NF-κB	Nuclear Factor kappa-light-chain-enchancer of Activated B cells
P-cadherin	Placental Cadherin
PBS	Phosphate Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PDGFP	Platelet-derived Growth Factor Receptor
PKC	Protein Kinase C
PMA	Phorbol-12-myristate 13-acetate
PVDF	Polyvinylidene Difluoride
QFS	Quenched Fluorescent Substrate
RAS	Renin Angiotensin System
RFU	Relative Fluorescent Unit
SARS	Severe Acute Respiratory Syndrome

S1P	Site-1 Protease
S2P	Site-2 Protease
SPP	Signal Peptide Peptidase
SREBP	Sterol Regulatory Element Binding Protein (SREBP)
STAT	Signal Transducers and Activators of Transcription
TACE	Tumour Necrosis Factor Alpha-converting Enzyme
TBS	Tris Buffered Saline
TFP	Trifluoperazine Dimaleate
TGF-α	Transforming Growth Factor Alpha
TIMP	Tissue Inhibitor of Metalloprotease
TNF-α	Tumour Necrosis Factor Alpha
TOF	Time of Flight
Trk A	TRK1-transforming tyrosine kinase protein
VE-cadherin	Vascular Endothelial Cadherin

ABSTRACT

Angiotensin-converting enzyme 2 (ACE2) is the only mammalian homologue of the well-characterised angiotensin-I converting enzyme (ACE). ACE2 shares 40% identity and 60% similarity in overall protein sequence with ACE. Like ACE, ACE2 is a type I integral membrane protein and a zinc dependent metalloprotease. ACE2 has recently been shown to undergo a proteolytic cleavage event, releasing an active soluble ectodomain. This cleavage-event is also commonly known as ectodomain shedding.The studies outlined in this thesis were designed to characterise the regulation of ACE2 ectodomain shedding, specifically looking at the interaction of this carboxypeptidase with calmodulin, potential site of cleavage mediated by tumour necrosis factor-α converting enzyme (also known as ADAM17), as well as the mechanism(s) responsible for the activation of this cleavage event. Calmodulingel shift assays have showed that this calcium regulatory protein is able to bind to both peptides that mimic the cytoplasmic tail of ACE2. Furthermore,we showed that GST-calmodulin fusion proteins were successfully co-immunoprecipitatedalong with full-length ACE2, *in vitro*. By using increasing concentrations of the calmodulin specific inhibitor, trifluoroperazine and calmidazolium, we have observed increase in the shedding activity of ACE2 from endogenously expressed in Huh-7 cells ($n \geq 3$, one-way ANOVA, $P < 0.05$). We have also provided evidence that calmodulin-induced shedding of ACE2 in the Huh-7 cells were non-responsive protein kinase C inhibitor, suggesting that the activation pathway for this shedding mechanism differs from the one mediated by phorbol esters. To characterise the cleavage-secretion site for ACE2 ectodomain release, peptide mimetics of ACE2 juxtamembrane region were incubated with purified recombinant TACE for analysis by MALDI-TOF/TOF. Peptide fragment analysis showed that ADAM17 cleaves peptide mimetics between Arg^{708} – Ser^{709} bond. Shedding profiles of ACE2 mutant in CHO-K1 and Huh-7 cells

were carried out, indicating that $Arg^{708}\rightarrow Glu^{708}$ and double mutant $Arg^{708}/Arg^{710}\rightarrow Glu^{708}/Glu^{710}$ displayed increased shedding activity when stimulated by explore. Lastly, to explore the possibility that the phorbol ester (PMA) stimulated shedding could be a consequence of a G-protein coupled receptor activation, Huh-7 cells and HEK 293 cells expressing the AT1R receptor were transfected with ACE2 and treated with angiotensin peptides (angiotensin I and angiotensin II) under the presence of different angiotensin receptor antagonists. At various time points of angiotensin treatment, there were significant increases observed in ACE2 shedding in cells treated with either angiotensin I or angiotensin II peptides. However when Huh-7 cells were incubated with the metabolically stable AT1 receptor agonist, L-162,313, we observed significant change in cell morphology, suggesting the likeliness of cell death, and were also reflected in the gradual decrement in ACE2 activity detected in the medium. In summary these studies has providedstrongevidences suggesting the involvement of calmodulin in regulating the ectodomain shedding of ACE2, an event which is independent of the protein kinase C signaling pathway.We also proposed that ADAM17 is able to cleave ACE2 between Arg^{708} – Ser^{709}, and that AT1R receptor activation may lead to stimulation of ectodomain shedding in ACE2. Given that ACE2 has recently been shown to be the functional receptor for the severe-acute respiratory syndrome (SARS) coronavirus (SARS-CoV) and also its implications in cardiovascular, renal and respiratory regulations, these studies adds to our knowledge of how does the ectodomain shedding of this important membrane protein may be actually regulated.

STATEMENT

This thesis contains no material that has been submitted or accepted for the award of any other degree or diploma in any other tertiary institution.

To the best of my knowledge, this thesis contains no material previously published or written by another person, with the exception where due reference has been made in the text of the thesis.

Zon Weng Lai

October 2010.

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1 INTRODUCTION

The cell membrane is home to a wide variety of proteins. The proteins expressed on the outer surface of the membrane provide an important means of communication between the host cell and its environment, allowing interactions with surrounding cells and foreign pathogens/compounds, as well as mediating the effects of autocrine and endocrine messengers. For example, surface proteins may serve as functional receptors or may facilitate the trafficking of various molecules across the plasma membrane. Moreover, the surface proteins also may have a role in the regulation of the immune system (*1*). Considering this range of functions, membrane proteins appear to be promising drug targets, as well as potential biomarkers for molecular diagnostics and therapeutics. In bacterial cells, surface proteins are used as both recognition and adhesion targets to initiate the invasion of the host cell (*2*). In mammalian cells, membrane protein expression may be significantly different in diseased cells compared to healthy cells, as observed, for example, in many types of cancer and cardiovascular diseases (*3, 4*). The extent of the changes in expression, followed by a proteolytic cleavage event called ectodomain shedding, a post-translationalproteolysis of specific proteins at the cell surface, may reflect the severity of disease; thus, the identification and quantification of these cleaved or shed proteins in blood or urine may provide useful insights into disease severity and/or may represent early markers of disease (*3*).

1.1 Ectodomain Shedding

1.1.1 Protein shedding from the membrane surface

Membrane-associated proteins may be released into the extracellular space by either constitutive or regulated proteolytic cleavage from the cell surface, a process known as protein or ectodomain shedding. These proteins are generally cleaved in the extracellular domain close to membrane proximal region (juxtamembrane), or within the transmembrane domain, resulting in the liberation of the 'ectodomain', also known as ectoproteins, which are often physiologically and functionally active. Figure 1-1 (below) illustrates how a catalytically active protein expressed on the cell surface can undergo cleavage at the juxtamembrane region by a membrane-bound protease. These membrane-bound proteases that cleaves other membrane-bound proteins are often known as 'sheddases'.

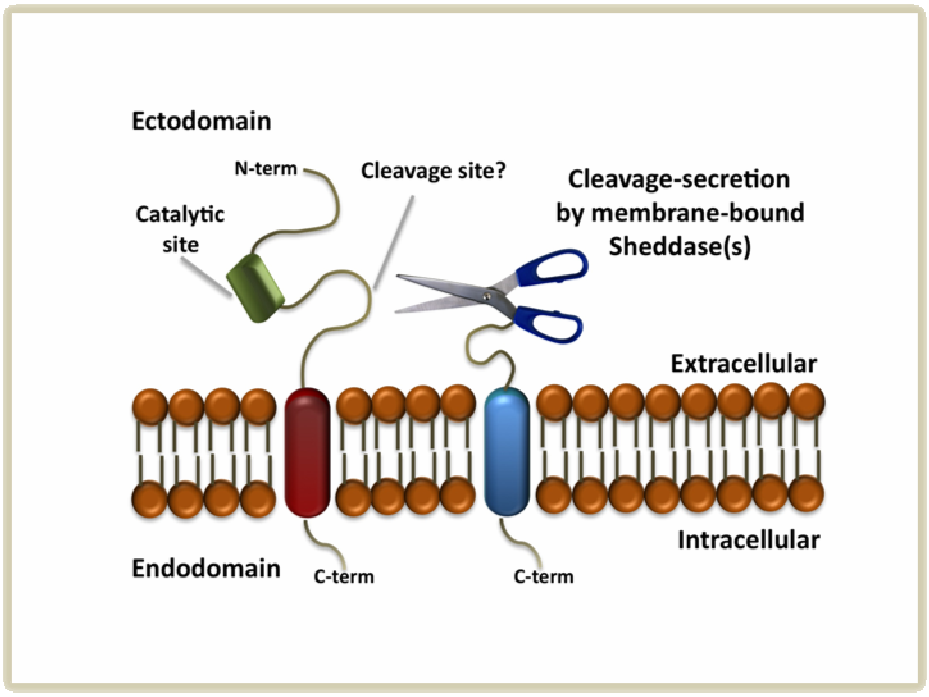


Figure 1-1:Ectodomain shedding. A schematic showing how proteins expressed on the cell surface undergo ectodomain shedding. Surface proteins are cleaved at the membrane-proximal region by proteases or 'sheddases' to release a soluble form of ectoprotein, which can be detected by ligand binding assays, immunodetection or, if sufficiently abundant, by mass spectrometry-based proteomic approaches. Quantitative analysis of catalytically active ectoproteins can be measured using activity-based assays.

Ectodomain shedding is crucial for regulating the activity of many surface proteins, including growth factors, adhesion molecules and cytokine receptors (*5-7*). The release of a soluble and sometimes active form (in terms of its ability to bind to other proteins such as receptors or to catalyse reactions) of a protein may reflect changes in the biological roles and actions of the protein, further allowing it to function systemically. Alternatively, protein shedding may also provide a means of preventing pathogens from binding to the host cells. For example, the ectodomain shedding of transforming growth factor-beta receptor (betaglycan) is able to partially suppress the invasion of tumour cells in pancreatic cancer specimens (*8*). The true physiological consequences of protein shedding from cell surface are largely unknown; however, there is potential value in examining these proteins as possible biomarkers of disease.

1.1.2 Intramembrane cleavage as a mechanism for protein shedding

Intramembrane cleavage of membrane-bound proteins is quickly becoming a well-established process to actively liberate ectoproteins. A family of proteases, collectively known as intramembrane cleaving proteases (I-CLiPs), has been reported to be able to cleave amino acid residues within the hydrophobic phospholipid bilayer. I-CLiPs are a family of proteases that are involved in the feedback regulation of lipid biosynthesis in mammalian cells (*9*). This family consists of four classes of proteases: presenilin aspartyl proteases (γ-secretase complex), rhomboid serine proteases, signal peptide peptidase (SPP), aspartyl proteases, and site-2 protease (S2P). Unlike other sheddases, which were identified as water-soluble enzymes, the I-CLiP family of proteases is localised uniquely within the phospholipid bilayer. Despite the highly hydrophobic environment, I-CLiPs are able to hydrolyse the peptide bonds within membrane-spanning domains of type II integral proteins (*10*). In type II integral proteins, the conformation of the transmembrane regions is usually alpha helical,

where all backbone amide bonds are buried within the helical core. For this reason, it is thought to be difficult or even impossible for any other proteases to gain access to these amide bonds for the nucleophilic attack process for hydrolysis of its substrates, reflecting the steric hindrance by the amino acid side chains. To overcome this, I-CLiPs are able to create a microenvironment for water and hydrophilic residues within the phospholipid bilayer (*11*).

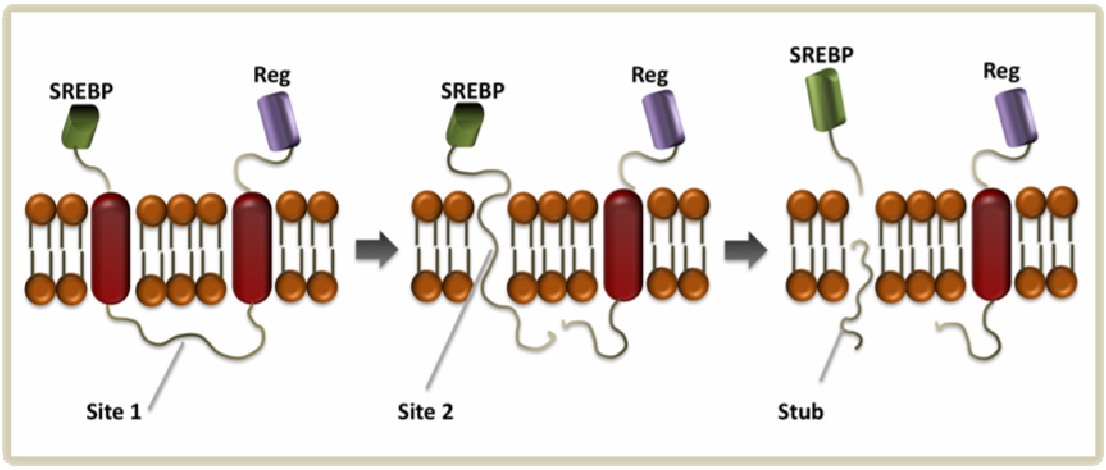


Figure 1-2:A schematic of SREBP shedding by S1P protease (cleaving at site 1) and S2P protease (cleaving at site 2).

S2P protease is a major contributor to the cleavage-secretion process of membrane-bound proteins by I-CLiPs, as demonstrated in the study of sterol regulatory element binding protein (SREBP) synthesis. SREBPs are transcriptional factors that promote gene expression of cholesterol and fatty acid synthesis enzymes in the feedback regulation pathway (*12*). When cholesterol levels become low, SREBP is synthesised via ectodomain shedding from the membrane-bound precursor formby S2P proteases in the Golgi apparatus (*12*). The ectodomain shedding of the membrane-bound SREBP precursor consists of a two-step mechanism in which a co-operative association with a separate protease, site-1 protease (S1P), is required (Figure 1-2). In the first step, S1P protease cleaves within the luminal domain at a specific site and causes the alpha helix to unwind (*9*). The unwinding mechanism of the alpha helix lies in the conserved asparagine-proline (Asn-Pro) sequence within the

SREBP precursor protein (*13*). These key residues have the lowest propensity to form the helical structure, suggesting that this region of the transmembrane is metastable (*13*). Consequently, when S1P cleaves within the luminal site, the Asn-Pro sequence may facilitate the helical unwinding, thus exposing the buried amide bonds. In step two of the reaction, the active site residues in the conserved HEXXH motif of S2P proteases will shed the precursor proteins, leaving behind a membrane-embedded 'stub' protein, where its post-shedding regulatory processing is still unknown (*14*).

Presently, there are no purification or in vitro assay techniques that have been described to investigate the presence or activity of S2P proteases. All the information available to date has been based on genetic studies of mutant cells containing the deletion of S2P gene. Furthermore, there are no antibodies or inhibitors currently available to specifically isolate and biochemically charaterise this class of proteases (*14*).

1.1.3 Protein shedding in disease states: Biomarker potential

Accurate disease diagnosis is particularly important for many potentially chronic conditions, such as cardiovascular diseases, neurological diseases and cancers. Many of these conditions do not have obvious symptoms in the early stages of disease. Early diagnosis of disease can allow for the initiation of effective treatments before the development of more advanced stage of the diseases, which are often beyond effective treatment.

In diseased cells, cell-surface proteins often undergo a change in their expression, which can lead to a change in protein shedding profile. As such, shed proteins may have a role as potential markers of disease status or progression. Information is accumulating regarding the physiological mechanisms and consequences of protein shedding in diseases. One of the classic examples in human neurological disease is the accumulation of β-amyloid (Aβ) in

plaques as a consequence of aberrant secretase activities (for reviews, see references (*15, 16*)). This accumulation of Aβ is believed to contribute to the pathology of Alzheimer's disease (*17-20*). Furthermore, the measurement of Aβ in plasma and cerebral spinal fluid may have predictive potential for the diagnosis and assessment of severity of Alzheimer's disease (*21, 22*). One of the surface proteins to be considered as a potential biomarker is platelet glycoprotein VI (GPVI), a member of the immunoglobulin receptor family that binds collagen to regulate homeostasis and thrombosis (*23, 24*). Patients with acute coronary diseases usually exhibit a significant increase in the expression level of platelet GPVI (*24*), which subsequently leads to increased shedding of the receptor (*25*). The regulation of ectodomain shedding is tightly controlled, both in healthy and diseased cells, as observed in a study of ectodomain shedding of membrane anchored heparin-binding epidermal growth factor (HB-EGF). Mice that expressed non-cleavable HB-EGF mutants exhibited heart failure, whereas mice that expressed only the soluble form of HB-EGF had hyperplasia in the skin and heart (*26*).

Finally, many cancers exhibit a change in membrane protein profiles as a consequence of malignancy, and many of these proteins appear to be selectively shed from the cell surface. An increasing number of reports in the literature suggest that shed proteins could be specific biomarkers for many cancers, including ovarian cancer (*27*) and thyroid cancer (*28*); alternatively, some proteins may be suitable as more general markers of tumours (*29*). In summary, proteins shed from the cell surface maynot only contribute to and/or are a consequence of disease pathology, but may also have a role as potential biological markers of disease and its progression.

1.2 Sheddases in Ectodomain Shedding

The regulation and functional complexity of protein degradation often involves proteolytic processes. Cellular enzymes called proteases or peptidases are responsible for catalysing this process. In ectodomain shedding, proteolysis is catalysed by enzymes collectively referred to as secretases or 'sheddases'. This review will highlight one of few major classes of proteases, known as the metalloproteases, for both its function as a sheddase and also as a substrate for ectodomain shedding. The general properties and types of metalloproteases will also be outlined in this section.

1.2.1 ADAM metalloproteases as sheddases

One of the most characterised families of sheddases is the ADAM (a disintegrin and metalloprotease) family of metalloproteases. This class of protease contains a disintegrin-like (may encode integrin ligands that regulate intercellular and cell-matrix interactions) domain and a metalloprotease-like domain, and is currently thought to consist of more than forty different members (*30*). The ADAM metalloproteases are a family of multidomain transmembrane glycoproteins that have critical roles in physiology and disease, with some members being validated drug targets for cancer and inflammation therapies. This section will provide a brief overview of some of the ADAMs involved in ectodomain shedding of membrane-bound proteins.

The first of the ADAMs shown to be involved in the ectodomain shedding was ADAM17, also known as the tumour necrosis factor alpha-converting enzyme (TACE). This enzyme is able to cleave its substrate, a 26-kDa membrane-bound form of tumour necrosis factor alpha (TNF-α) into a 17-kDa soluble and physiologically active form of TNF-α(*31*). During oxygen and glucose deprivation, the expression of ADAM17 is up-regulated (*32*), leading to increased shedding of the membrane-bound TNF-α. The release of a soluble form of TNF-α results in the activation of a transcription factor, NF-κB, which leads to inhibition of

cell apoptosis (*32*). Apart from hydrolysing TNF-α, ADAM17 was subsequently demonstrated to mediate the shedding of a number of other membrane-bound proteins, such as transforming growth factor-alpha (TGF-α) (*33*), angiotensin-converting enzyme (ACE) (*34*), p75 TNF receptor (*35-37*), LDL receptor (*38*), SorLA (*39*), L-selectin (*40-44*), and growth hormone receptor (*45*). Studies of 129-derived stem cells from mice expressing an inactive form of ADAM17, in which the catalytic zinc-binding domain was deleted, showed that membrane-bound TNF-α failed to be released (*31*). Another recent study highlighted the involvement of ADAM17 in the shedding of angiotensin-converting enzyme-2, the only mammalian homolog to ACE (*46*). The involvement of ADAM17 in shedding of this angiotensin enzyme is the focus of my PhD research study and will be discussed in the latter part of this review*(Chapter 1.5.1)*.

The second most studied ADAM member involved in protein shedding is ADAM10. ADAM10-mediated shedding events have biological implications in the central nervous system, and in inflammatory and cancer pathways. Together with ADAM17, ADAM10 is responsible for proteolytic processing and shedding of amyloid β precursor protein (APP) (*47-49*), prion protein (*50*), Notch receptor (*51, 52*), interleukin-6 receptor (*53-57*), CXC-chemokine ligand 16 (*58-60*), fractalkine (*61-63*), epithelial growth factor receptor (*64*), and CD44 (*65-68*). There is also some evidence for the increased expression of ADAM10 and ADAM17 in various types of cancer (reviewed in (*69*)). Studies have indicated the involvement of these ADAMs in early stages of tumour development, including stimulation of proliferation by released growth factors, and evading immune surveillance (*70, 71*). ADAM10 has other unique membrane-bound substrates such as N-cadherin (*72*), ephrins (*73, 74*), VE-cadherin (*75, 76*), AXL receptor (*77*), and betacellulin precursor protein (*64, 78*). ADAM10 itself is a membrane-bound metalloprotease and undergoes regulated intramembrane proteolysis to release its ectodomain. Interestingly, the sheddases responsible for this cleavage-secretion process are ADAM9 and ADAM15 (*79*). While ADAM10 functions as a

sheddase in its membrane-bound form, it is likely that the shed form of ADAM10 plays a potential role as a signaling protein.

Another characterised member in the ADAM metalloprotease family that has been shown to be involved in ectodomain shedding is ADAM9, also known as meltrin γ (MDC9) (*80*). ADAM9 contains a signaling motif in its cytoplasmic domain and a catalytic site zinc-binding motif bearing the consensus sequence HEXXH in its metalloprotease domain (*81*). ADAM9 is able to cleave naturally occurring substrates, including insulin B chain (*82*), collagen XVII (*83*), and amyloid β precursor protein (*84*). Its involvement in ectodomain shedding was demonstrated in the study of transmembrane cleavage of HB-EGF (*85*). The over-expression of ADAM9 shows a positive correlation to HB-EGF shedding, suggesting that ADAM9 may well be one of the sheddases responsible for cleaving membrane-bound HB-EGF and converting it into a mitogenically active protein. The exact regulation of ADAM9, however, is not fully understood. Although ADAM9 is activated from its pro-domain by a pro-protein convertase, its entire regulation pathway is not known (*82*). Studies have also shown that this metalloprotease is significantly over-expressed in brain and bone metastasis (*86*). However, ADAM9 knockout mice show normal development and no phenotypic changes throughout development and in adult life (*87*).

Some of the other ADAMs that are also worth mentioning in this context include ADAM8, which is potentially responsible for shedding membrane-bound proteins that are associated with angiogenesis (*88*); ADAM12, which is known to shed EGF ligands (*89*); ADAM 15, which sheds membrane-bound growth factors (*90*); and ADAM19, which is known to be involved in neuregulin (*91, 92*) and TNF-α(*93*) shedding.

The metalloprotease activity of ADAMs can be studied with ease using four different classes of inhibitors. The first type of inhibitor consists of reducing agents, which denature

the ADAM metalloproteases. The second type of inhibitor is the cation chelators, which chelate the central zinc ion, thus preventing the activation of the metal-bound water molecules for hydrolysis. The third type of inhibitor comprises a family of hydroxamate-based inhibitors that bind competitively to the active site and have proved to be an important tool for the study of ADAMs (*94*). The fourth type of inhibitor is the endogenous proteinaceous inhibitor called tissue inhibitors of metalloproteases (TIMPs). TIMP inhibitors are represented by four members: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. The expression of TIMP-1 and TIMP-2 is generally widely distributed across mammalian tissues, whereas TIMP-3 and TIMP-4 expression is found to be restricted to specific tissues (*95*). The regulation of TIMP-1, TIMP-3 and TIMP-4 is by transcription at specific tissues, while TIMP-2 is synthesised at a constant rate regardless of the physiological demand (*95*). TIMPs, however, were found not to have any inhibition effect on ADAM8 and ADAM9 (*96*). Like hydroxamate-based inhibitors, TIMPs are also able to inhibit matrix metalloproteases (briefly reviewed in the next section).

1.2.2 Matrix metalloproteases as sheddases

Matrix metalloproteases (MMPs), or matrixins, are another large family of zinc-dependent metalloproteases involved in the regulation of cell matrix composition (*97, 98*) (Table 1-1). This group of metalloproteases has a HEXXHXXGXXH sequence motif in its metalloprotease domain, reflecting the zinc ion binding ligands (*98*). A glycine residue is usually found between the second and third histidines, which serve as zinc ligands. The presence of this glycine enables the formation of a beta turn that brings the zinc ligands closer together. To make up for the lack of disulphide bonds in the catalytic domain, an additional zinc ion, plus one or two calcium ions, are required to achieve structural stability in the MMPs (*98*).

Table 1-1: The subgroups of secreted and membrane-bound matrix metalloproteases

MMP Type	Subgroup	MMP Family
Secreted	Minimal-domain MMPs	7, 26
	Simple hemopexin domain-containing MMPs	1, 3, 8, 10, 12, 13, 18, 19, 20, 22, 27,
	Furin-activated secreted MMPs	11, 28
	Gelatin-binding MMPs	2, 9
	Vitronectin-like MMPs	21
Membrane-bound	Glycosyl phosphatidylinositol-attached MMPs	17, 25
	Type-I transmembrane MMPs	14, 15, 16, 24
	Type-II transmembrane MMPs	23

MMPs can be subdivided into two different classes: secreted and membrane-bound MMPs. Secreted MMPs can be further divided into minimal-domain MMPs, simple hemopexin domain-containing MMPs, furin-activated secreted MMPs, gelatin-binding MMPs, and vitronectin-like MMPs. Similarly, the membrane-bound MMPs can be further divided into glycosyl-phosphatidyliositol (GPI)–anchored MMPs, type-I transmembrane MMPs and type-II transmembrane MMPs (Table 1.1) The structural differences of these subgroups are as illustrated in Figure 1-3.

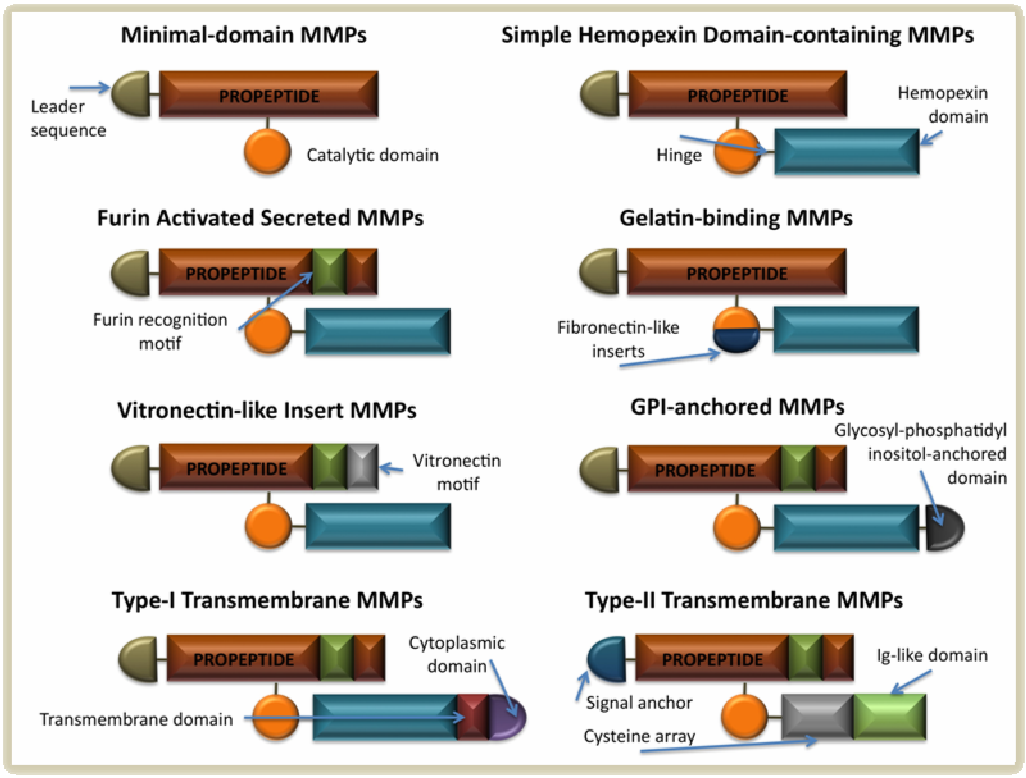


Figure 1-3:Classification of secreted and membrane-bound MMPs and their domain structure.

All of the MMPs contain a leader sequence attached to a catalytic domain-bound pro-peptide. This leader sequence plays a role in targeting the protein for secretion. Apart from the catalytic zinc ion, the catalytic domain consists of additional zinc ion and calcium ions. In some MMPs, a hinge region is present connecting the catalytic domain to other domains. The proline-rich region is very flexible and plays a role in influencing substrate specificity. The type-I transmembrane MMPs and GPI-anchored MMPs are also known as membrane-type matrix metalloproteases (MT-MMP) 1-6, each named according to the sequential discoveries of the different MT-MMPs. The members of type-I transmembrane MMPs are highly homologous, sharing 50% sequence homology amongst each member of the family. The GPI-anchored MMPs have slightly lesser sequence similarities compared to the type-I transmembrane MMPs (*98*). The other member of the membrane-bound family, the type-II transmembrane MMP, is not recognised as part of the membrane-type matrix metalloproteases. It distinguishes itself as being the first MMP to have a signal anchor in the

amino-terminal region instead of a leader sequence, as well as a novel cysteine array and an immunoglobulin-like domain in the carboxyl-terminal region of a hemopexin domain (*99*).

One of the better-characterised MMP members known to play a role in ectodomain shedding of membrane-bound proteins is MMP-14. MMP-14 was initially reported to activate pro-MMP2 (progelatinase A), mediated through the formation of a trimolecular complex with TIMP-2 inhibitor (*100*). Subsequent studies showed that MMP-14 is involved in the activation of pro-MMP-14 (procollagenase 3) (*101*), and it was suggested to play a role in leukocyte transmigration during the inflammatory response (*102*). Furthermore, MMP-14 is able to degrade a wide range of extracellular matrix components, such as collagen types I, II and III, gelatin, fibronectin, laminins 1 and 5, vitronectin, and the shedding of hyaluronic acid receptor (CD44) from the cell surface (*101, 103*). CD44 is a cell adhesion molecule responsible for cell-cell and cell-matrix interaction. Ectodomain shedding of CD44 has been shown to promote the migratory potential of the endothelial cells and the metastatic tumour cells. The over-expression of MMP-14 is frequently observed in various types of human cancer tumours, such as in carcinoma cells from the lung, cervix, intestine, colon and the brain (*104*). These observations suggest a possible role of MMP-14 in the ectodomain shedding of CD44 in the tumour invasion response pathway (*103*). Unlike ADAM10 and ADAM17, the mechanism of MMP-14-mediated CD44 shedding and its regulation leading to the migration of cells in the tissue is not fully understood (*103*). MMP-14 is also involved in facilitating other tumour invasions. For instance, MMP-14 mediates tumour cell surface major histocompatibility complex class I chain-related molecule-A, which contributes to tumour progression (*105*), and the shedding of stromal syndecan-1, which stimulates breast carcinoma cell proliferation (*106*). Inhibitors that are used to inhibit MMP-14 are TIMP-2, TIMP-3 and TIMP-4 (*107-109*).

Another MMPknown to contribute to ectodomain shedding of surface protein is MMP-7, also referred to as matrilysin. MMP-7 is a minimal-domain MMP that exhibits proteolytic activity against components of the extracellular matrix. Like MMP-14, MMP-7 plays a role in promoting cancer progression apoptosis, growth and angiogenesis. It is involved in the extracellular cleavage of E-cadherin to promote tumour invasion in human stomach cancer cells (*110*), shedding proHB-EGF to soluble HB-EGF to promote cellular proliferation (*111*), shedding of the membrane-bound Fas ligand to promote apoptosis of cells adjacent to tumour cells (*112*), and the shedding of TNF-α into its soluble form to promote apoptosis (*113*).

There are several other MMPs known to be responsible for the ectodomain shedding of other membrane-bound proteins. MMP-1 and MMP-2 are secreted during over-expression of P-cadherin, which in turn sheds P-cadherin itself (*114*). MMP-2 alone is responsible in shedding surface-expressed CD40 ligand (*115, 116*), fractalkine (*117*), and beta-2 integrin subunit (CD18) from macrophages (*118*). MMP-3 (also known as stromelysin-1) is able to cleave membrane-bound Fas ligand in synovial fluid of patients with rheumatoid arthritis (*102*). MMP-3 and MMP-9 shed intercellular adhesion molecule-5 to promote long-term potentiation by inhibiting dendritic spine enlargement (*119*). Both MMP-9 and MMP-12 are also involved in N-cadherin shedding to regulate vascular smooth muscle cell proliferation (*120*). Furthermore, studies show that MMP-9 knockout mice failed to shed kit-ligand, resulting in impairment in hematopoiesis recovery, measured from the count of white blood cells and platelets, following treatment with cytotoxic agent and increased mortality(*121*). Subsequent introduction of exogenous soluble form of kit-ligand was able successfully restores hematopoiesis and survival after bone marrow ablation in these mice (*121*).

1.3 Renin Angiotensin System

1.3.1 Angiotensin Peptides

The renin angiotensin system (RAS) (Figure 1-4) is a central regulator of cardiovascular and renal homeostasis and is responsible for maintaining body fluid and electrolyte balance, which in turn helps to regulate long-term blood pressure and blood volume, as well as many other associated cardiovascular functions in the human body (*26*).

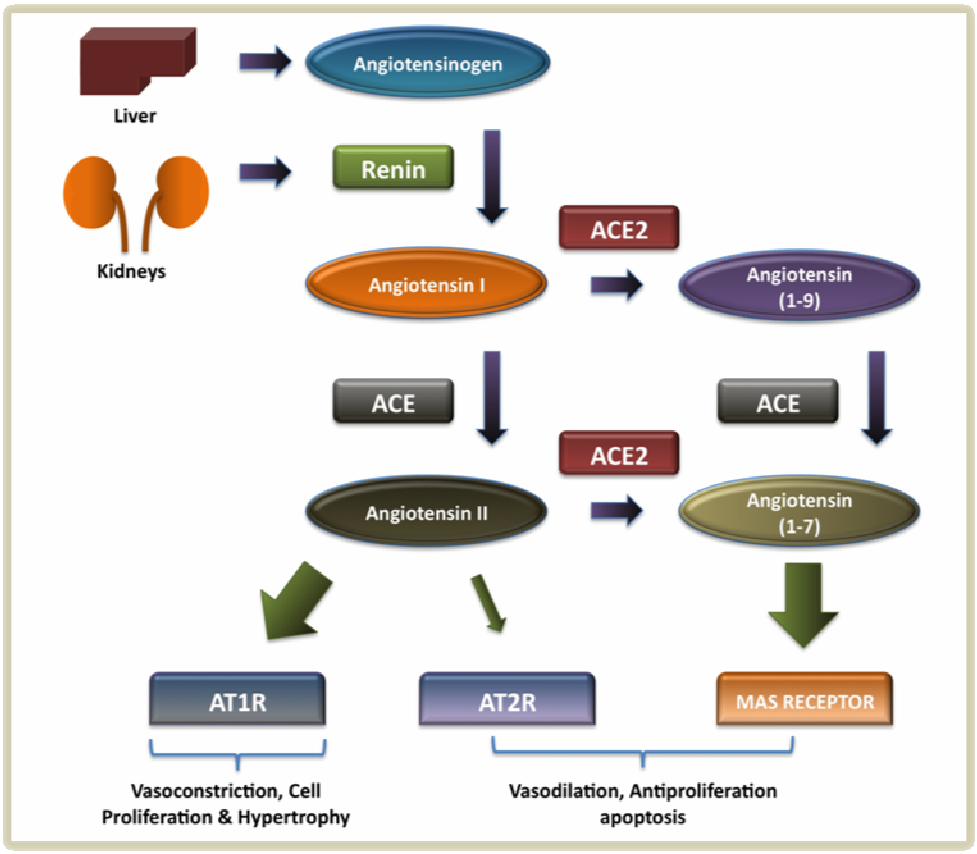


Figure 1-4:A scheme showing the renin angiotensin system, RAS. The left side shows the classical view, and the right side shows the likely role ACE2 plays.

Within the RAS, one of the key peptide products is angiotensin II (AngII). During RAS activation, cells from the juxtaglomerular apparatus of the kidney secrete a protease called

renin, which cleaves circulating angiotensinogen, to generate angiotensin I (AngI), which is essentially inactive and from which all other angiotensin molecules are derived. AngI is then transformed into active AngII following cleavage within the circulation by a peptidyl dipeptidase called ACE (Figures 1-4 and 1-5). Following its synthesis, AngII is transported into peripheral tissues by the systemic circulation and its action on target cells is mediated via two specific G-protein coupled receptors (GPCRs): the angiotensin type-1 receptor (AT1R) and type-2 receptor (AT2R). The activation of AT1R mediates most of the cardiovascular actions attributed to AngII, such as vasoconstriction, mitogenic and hypertrophic effects, inflammatory responses, and salt and water retention (*122*). These effects are mediated via complex interacting signaling pathways involving phospholipids (*123-126*), stimulation of nicotiamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase and reactive oxygen species (*127, 128*), induction of gene transcription, such as the proto-oncogenes c-fos, c-jun, and c-myc(*129*),and activation of tyrosine kinases (*130, 131*). Some of these actions may be mediated directly or indirectly via trans-activation of tyrosine kinase receptors including PDGFP, epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGFR)(*132*). Given the dramatic effects of AngII on blood pressure, first-line drugs used in the treatment for patients suffering from hypertension inhibit the generation of AngII via ACE inhibition, while second-line drugs inhibit its binding to the AT1R. To a much lesser extent, AngII can also act through the less abundant AT2R; its actions are essentially opposite to that of the AT1R receptor, in that it is proposed to induce apoptosis, natriuresis, and vasodilation (*122*).

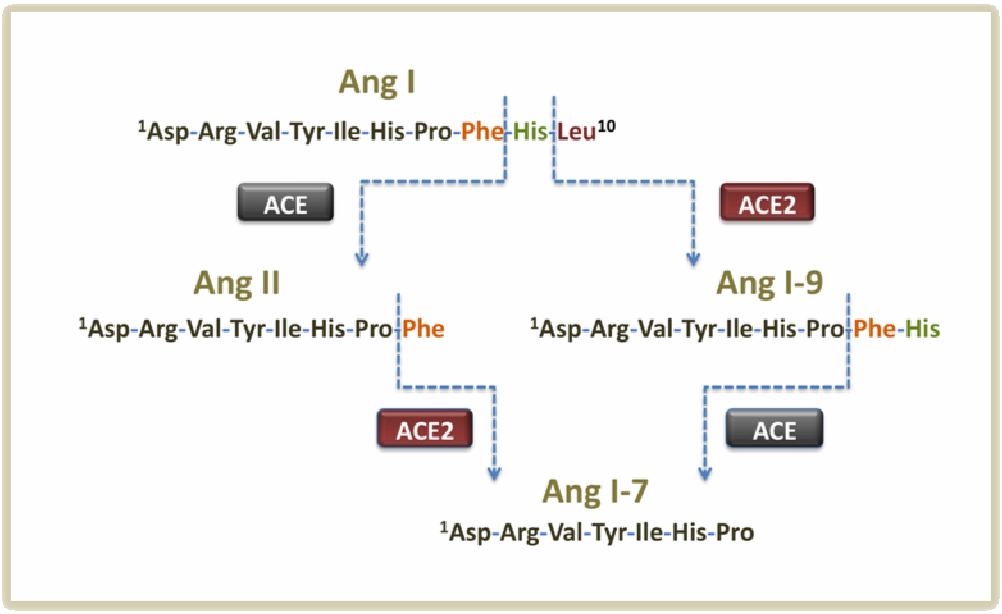


Figure 1-5:A scheme illustrating where ACE and ACE2 cleave angiotensin I, as well as showing how the cleavage products are processed by the two metalloproteases.

After production, AngII is rapidly degraded by circulating and tissue peptidases to des-aspartyl[1]-AngII (AngIII), angiotensin (1-7) (Ang(1-7)), and angiotensin (3-8) (AngIV), extensively reviewed in (*133*). Although once assumed to be inactive, these angiotensin peptide fragments are now recognised as possessing unique biological activities distinct from AngII (*133-136*). In particular, Ang(1-7) is thought to induce vasodilation in part via the stimulation of bradykinin, which in turn elicits generation of the vasodilatory radical nitric oxide (NO) and prostaglandins (*137, 138*). Recently, a putative Ang(1-7) receptor, the GPCR *mas receptor*, has been identified, which is thought to mediate the beneficial actions of Ang(1-7) (*139*). Ang(1-7) can be generated via three separate but related pathways. Two pathways involve the stepwise hydrolysis of AngI by ACE and ACE2 consecutively, in either order. The third pathway involves the hydrolysis of AngI by neutral endopeptidase.

Angiotensin(1-9) (Ang(1-9)) is another inactive peptide associated with RAS, generated from AngI via the actions of ACE2 and other carboxypeptidases, which remove a

single amino acid from the carboxyl-terminal end. The actions of Ang(1-9) are unknown at this stage, but studies suggest it acts as an endogenous inhibitor of ACE, as it is also an alternate substrate for ACE, competing against AngI for hydrolysis (*140*).

1.3.2 RAS and ectodomain shedding

As in many other biological systems, ectodomain shedding plays a pivotal role in the catalytic regulation of the RAS. Both the key enzymes ACE and ACE2 are membrane-bound zinc metalloproteases. ACE, and possibly ACE2, are both shed from the cell surface by a post-translational proteolytic cleavage event leading to the release of the soluble ectodomain form into the extracellular space. Compared to ACE2, the shedding of ACE is very well characterised. Since its discovery, most of the established physiological functions of ACE have been ascribed to the membrane-associated form. However, the exact function(s) of this enzyme in the soluble or shed form are not clear. Nonetheless, ectodomain shedding may well be a fundamental process in allowing these important enzymes to act locally as well as systemically via the blood vessels in the body. Ectodomain shedding may also be an alternative mechanism for the rapid down-regulation of specific enzyme activity in the vicinity of its expression site, rather than classical protein internalisation strategy in these enzymes. The two peptidases, ACE and ACE2, will be reviewedseparately below.

1.4 Angiotensin-converting Enzyme (ACE)

ACE was initially discovered in 1956 and was then referred to as the hypertensin-converting enzyme (*141*). As briefly mentioned previously, ACE is able to convert AngI, transforming it into a highly potent vasoconstrictive peptide hormone called AngII. Apart from hydrolysing AngI, ACE is able to hydrolyse the vasodilatory peptide bradykinin, thus

further enhancing the deleterious effects of AngII on vascular cells, or more generally, blood pressure (*142*). Other naturally occurring substrates for ACE include substance P, cholecystokinin, and luliberin or gonadotropin-releasing hormone (GnRH)(*142*).

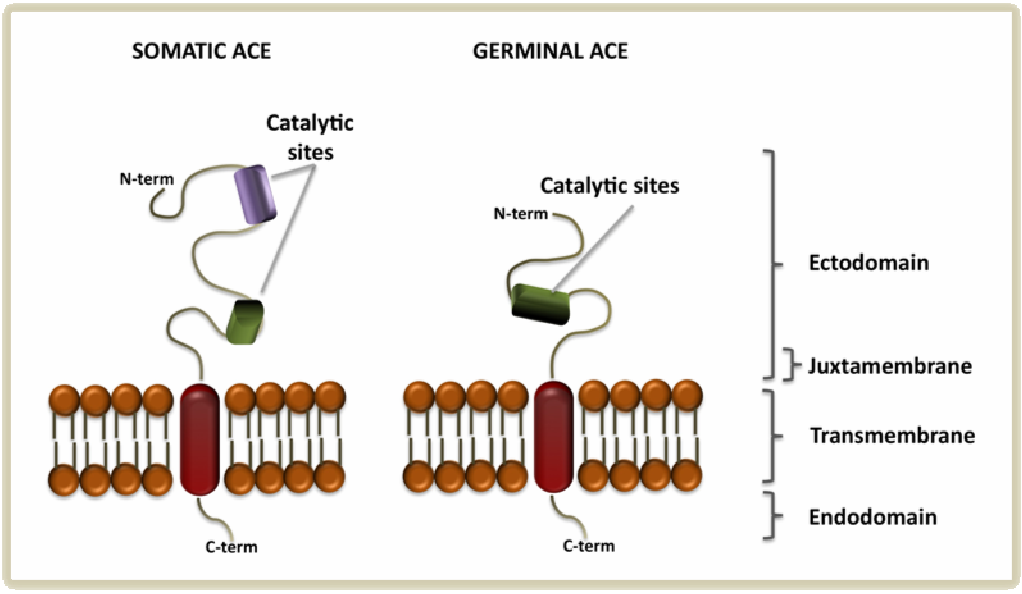


Figure 1-6:Structural representation of the various domains and catalytic sites of both somatic and germinal ACE.

Structurally, ACE is a type-I integral protein that is anchored onto the surface of endothelial cells. Its carboxyl-terminal end sits within the cytosol, while the amino-terminal end is situated in the extracellular space. The endodomain and ectodomain of ACE are separated by a transmembrane domain, an alpha helix built from 21 hydrophobic amino acids, spanning across the lipid bilayer of the cell membrane (*143*), as illustrated in Figure 1-6. The expression of ACE is most abundant in the lung, but it is also expressed in other tissues such as the heart, brain, kidney, striated muscles and testis. ACE is found in three isoforms: somatic, germinal and soluble forms. The somatic form of ACE consists of 1306 amino acids that encode two catalytic domains and is expressed at the tissue-fluid interfaces throughout the body (*144*). The germinal form of ACE, however, is a shorter enzyme, consisting of only 732 amino acids with only one catalytic domain (*145*). The expression of

germinal ACE is limited to the spermatozoa in the testis (*145*). The somatic and germinal forms of ACE are both transcribed from the same gene, but by different promoters (*146*). The soluble form of ACE, on the other hand, is formed as the result of protein shedding from either of the membrane-associated ACE isozymes and is present in blood plasma, cerebrospinal and seminal fluid, and other bodily fluids (*147*).

ACE is a member of the M2 family of the MA(E) clan of zinc metalloproteases(*148*). These clan members consist of a conserved HEXXH + E motif in their zinc-binding site (*149*). Depending on the isoform, ACE can have either one or two catalytic sites, each characterised by the HEMGH (zinc-binding) catalytic motif. Somatic ACE contains a tandem repeat structure of two homologous domains (amino- and carboxyl-end domains), each contain one zinc-binding motif (Figure 1-7). The carboxyl-domain of somatic ACE is identical to the single domain of germinal ACE. Moreover, both somatic and germinal ACE shares high sequence identity in the transmembrane and cytoplasmic domains. The first crystal structure of human ACE was solved in 2003 (*150*). This structure was deduced using the a truncated testiscularform of ACE, lacking the amino-terminal *O*-glycosylated and transmembrane regions,complexed together with one of the most widely used inhibitor, lininopril, to promote crystallisation (*151*). The structure of testis ACE, revealed that this enzyme is a largely α-helical globular enzyme, and divided down the middle by a deep cleft, which substrates could gain access to the active site(*150*). This crystallisation approach was similarly used laterto solve the structure of N-domain of human somatic ACE (*152*). To date, no full intact structure has yet been reported for human somatic ACE.

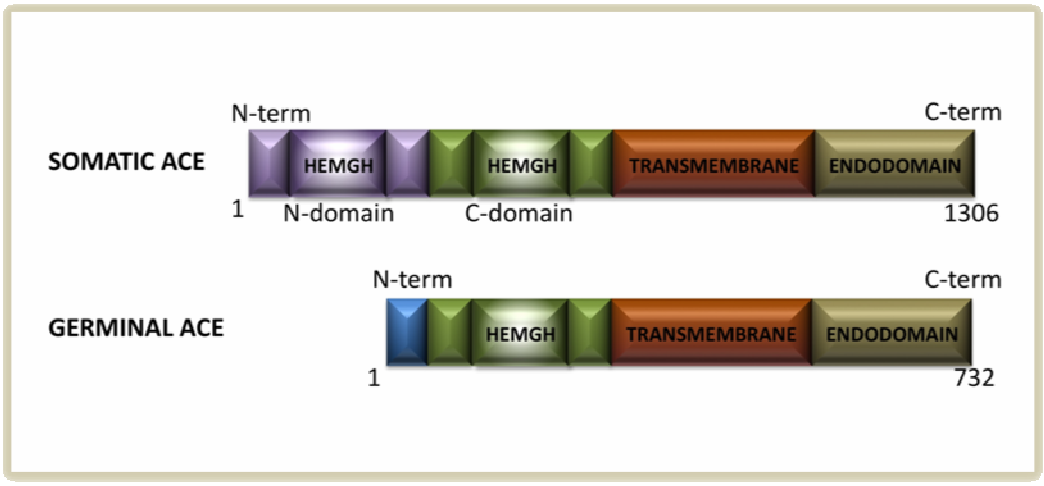


Figure 1-7:HEMGH zinc-binding motifs and the domain homology of the somatic and germinal forms of ACE.

1.4.1 Characterisation of ACE ectodomain shedding

The shedding site of soluble ACE from both membrane-associated isozymes is localised to the juxtamembrane stalk region. In human somatic ACE, the ectodomain cleavage-secretion site is between the Arg^{1203}-Ser^{1204} bond, 24 residues proximal to the transmembrane domain (*153*). Similarly, in human testis ACE, the cleavage site is between Arg^{627}-Ser^{628} bond, also 24 residues upstream to the transmembrane domain (*153*).In contrast, there was a conflicting report of the cleavage site of somatic ACE at an Arg-Leu bond, 100 residues proximal to the transmembrane domain (*154*). This earlier report suggested that the amino-terminal domain of somatic ACE could play a role in modulating the activity of the sheddase, redirecting it to an alternative cleavage site(*154*). Howeverthis cleavage site is not consistent with the data indicating that the size of the residual anchoring 'stubs' domain, and that another report showing that a deletion of 47 residues proximal to the identical transmembrane domain the testis ACE renders it catalytically inactive(*155, 156*).Nevertheless, the precise requirements for the sheddase recognition of the ACE ectodomain are not known. There have been multiple studies that also proposed that the

amino-terminal domain play a role in modulating ACE shedding (*157-159*). However, a recent study has showed that a stretch of unhindered amino acid residues in the juxtamembrane region, which may play a role in for ACE shedding (*160*). Subsequent studies have reported the existence of a 'recognition motif' for ACE ectodomain shedding within the juxtamembrane domain(*158*). A more detailed review about the regulation and mechanism of ACE ectodomain shedding will be discussed in *Chapter 4.1*.

Currently, the identity of the ACE sheddase(s) remains unknown. Some evidence suggests that there may be a primary sheddase involved, as well as the recruitment of alternative sheddases (*155*). It is certain, however, that the ACE sheddase(s) is a membrane-bound metalloprotease, as ACE shedding activity can be inhibited by hydroxamic acid-based inhibitors (*155*). ACE sheddase(s) was also suggested to be related to, but distinct from, ADAM17 (*161*). Recently, a different study has demonstrated an involvement of a different class of protease, a serine protease, in the shedding of germinal ACE (*162*).

1.5 Angiotensin-converting Enzyme 2 (ACE2)

ACE2 was discovered relatively recently compared to ACE, and was initially described by two independent research groups using different molecular strategies. The first group discovered ACE2, previously known only as ACE homologue, by screening a cDNA library of human lymphoma (*163*). The other group discovered ACE2 from a cDNA library prepared from the ventricular tissue of a heart failure patient (*140*). To date, ACE2 is the only known mammalian homologue of ACE. ACE2 is predominantly found in the heart, kidneys and testis and its precise role is not known (*164*).

1.5.1 Biochemical properties and general structure of ACE2

ACE2 is a type I integral membrane protein that shares an overall protein sequence identity of ~40% with ACE. Unlike ACE, which functions as a peptidyl-dipeptidase, ACE2, on the other hand, is a carboxypeptidase. ACE2 cleaves a single amino acid from the carboxyl-terminal end of its substrate. ACE2 substrates include AngII, des-Arg^{9} bradykinin, and apelin 13 (*165*). Characterisation of ACE2 shows that this enzyme could also play a role in regulating RAS by the conversion of the vasoconstrictor AngII into the vasodilatory peptide, Ang (1-7), thereby modulating the levels of AngII produced by ACE. Like ACE, ACE2 is also a member of the M2 family of the MA(E) clan of zinc metalloproteases. ACE2 consists of 805 amino acid residues and has an apparent mass of approximately 120 kDa in the mature form (*163*). The overall sequence homology shared with ACE lies at the catalytic site of ACE2. This catalytic site is closely related to the catalytic site of the amino-terminal domain of somatic ACE, and thus contains only one zinc-binding site (*140*), as illustrated in Figure 1-8. Despite the close similarity at the catalytic site, studies have shown that ACE inhibitors, such as captopril, lisinopril and enalaprilat, fail to inhibit the enzymatic activity of ACE2 (*163*). The carboxyl-terminal domain of ACE2, however, shows a sequence homology of 48% with collectrin, a catalytically inactive glycoprotein found in the kidney (*166*).

The first crystal structure of ACE2, lacking the transmembrane and cytoplasmic domains, was solved in 2004 (*148*).Observations of these structures, in native and inhibitor-bound states (2.2 Å and 3.0 Å, respectively), have revealed that a large hinge-bending motion within the catalytic domain plays important roles in substrate catalysis, and also a target for inhibitor binding (*148*).Electron density analysis resulting from the X-ray diffraction of the crystal have previously suggested that there are six possible N-linked glycosylation sites in the ectodomain of ACE2; namely at Asn^{53}, Asn^{90},Asn^{103},Asn^{322},Asn^{432},and Asn^{546}(*148*).Additionally, mass spectrometry analysis showed that the ectodomain form of

ACE2 is ~6 kDa greater than the theoretical molecular mass, which believed to be due to the glycosylation at these sites (*148*). Similarly, observations in our laboratory also showed that the recombinant soluble ectodomain form of human ACE2 and full-length human ACE2, both showed significant decrease in molecular mass when separated using gel electrophoresis following deglycosylation using peptide*N*-glycosidase F enzyme(*167*).In human testicular ACE, it is known that two of the seven potential glycosylation sites play roles in facilitating intracellular transport and also cell surface localisation (*168*).However, no studies thus far have described the importance for glycosylation in ACE2.

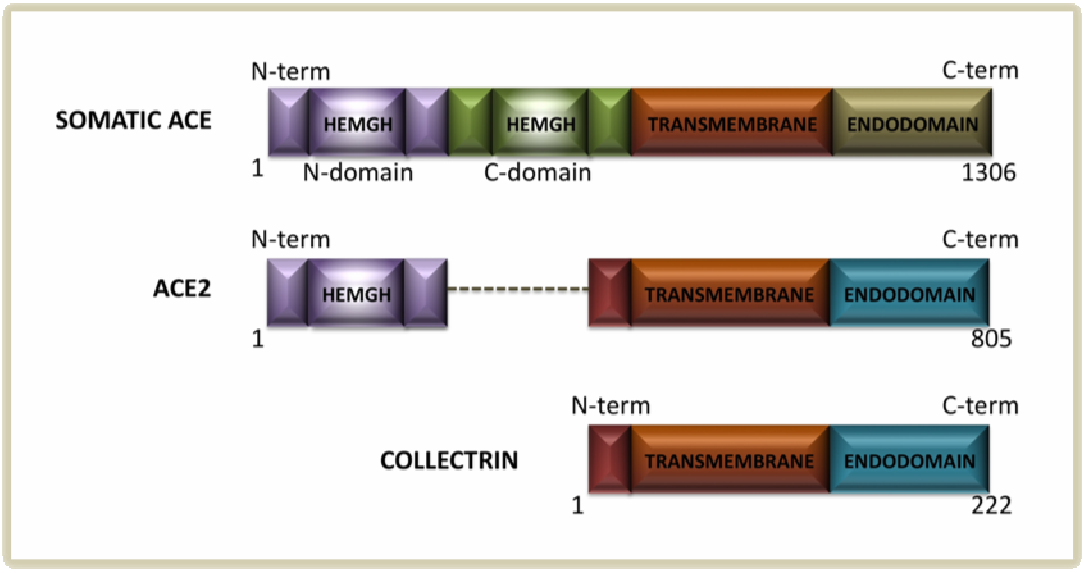


Figure 1-8:HEMGH zinc-binding motifs and the domain homology of somatic ACE, ACE2 and collectrin.

1.5.2 Physiological roles of ACE2

Based on the localisation of ACE2, one may suggest ACE2 may play a role in cardiovascular and renal regulation, as well as a reproductive role. The ability of ACE2 to hydrolyse important components of RAS, such as the angiotensin peptides, suggests an active role in the regulation of RAS. Studies have shown that transgenic mice that over-express

ACE2 die prematurely due to atrioventricular block and ventricular tachycardia (*169*). In contrast, ACE2-deficient mice showed a selective decrease in first-phase insulin in response to glucose, but no increase in insulin-sensitivity, energy intake or feeding pattern. Thus, ACE2 might play a role in regulating glucose metabolism (*170*). This perhaps ties in with one of the angiotensin receptors, angiotensin type-4 receptor (AT4R)(*171*), which has been identified as an insulin-regulated aminopeptidase, thus suggesting a more intimate link between insulin and energy regulation and the RAS than previously thought. Also, unlike ACE-deficient mice, ACE2-deficient mice are fertile (*172*). This is possibly because ACE2 is localised only in Leydig cells, and is not as directly linked to fertility compared to ACE in germ cells (*173*). Moreover, the expression of ACE2 activity in rat testis cells was not significantly altered by manipulation of the pituitary-testicular hormonal axis with testosterone implants (*173*). Nevertheless, this does not suggest that ACE2 does not play a functional role in human reproduction, as ACE2 is also found in human Sertoli cell cytoplasmic extensions surrounding the germ cells (*173*). The presence of ACE2 in these cells may suggest a regulatory role for the enzyme in steroidogenesis, perhaps to limit testosterone inhibition by AngII (*173*). Another later study into knockout mice has demonstrated other phenotypic effects in ACE2-deficient mice. The loss of ACE2 appeared to cause heart defects in the mice, such as thinning of the anterior left ventricular wall and an increase in chamber dimensions (*172*). Subsequent assessment showed a reduction in cardiac contractility, specifically in males, with a progression in phenotype with age (*172*). Hence, it is unclear if the reduction in blood pressure in ACE2-deficient mice is attributed to the reduction in cardiac contractility or the loss of ACE2 activity indirectly (*172*). No difference in ACE mRNA levels was found in the hearts and the kidneys of these ACE2 mutant mice compared to control (*172*). Interestingly, double-mutant mice lacking both ACE and ACE2 failed to show any defects in cardiac functions (*172*). Perhaps this suggests that heart defects are not so much caused by ACE2 directly, but by the effect that over-expression or deficiency of ACE2 has on ACE expression.

Apart from cardiovascular regulation, studies have also showed that ACE2 plays a protective role in the respiratory system. Compared to wild-type mice, ACE2 knockout mice displayed severe diseased state of acute respiratory distress syndrome when insulted by endotoxin, acid aspiration or sepsis (*174*). In addition, there have been recent evidences that show ACE2 as a therapeutic target for the prevention of pulmonary fibrosis and pulmonary hypertension (*175-177*). In a recent review, ACE2 has been described to play an associated role with an amino acid transporter, based on the high homology of ACE2 shares with collectrin, a crucial binding partner for neutral amino acid transporters (*178*). There are some evidences that support this hypothesis, such as immunofluorescence studies, co-immunoprecipitation detection, and functional experiments, all indicating that ACE2 is necessary for the expression of a sodium-dependent amino acid transporter, B^0AT1 in the rat intestine (*178*). Genetic mutations of B^0AT1 gene is an autosomal recessive disease, characterised by light sensitive rash, cerebral ataxia, emotional instability and amino aciduria (*179*). However, ACE2 knockout mice displayed no phenotypic changes compared to wild-type, showing no sign of aminoaciduria in the kidney (*180*). Nevertheless, as this hypothesis was initially deduced based on the recent research on collectrin as amino acid transporter, further studies will further warrant the functional similarity of these homologues.

Lastly, one of the other most significant findings for this metalloprotease is its role as the functional receptor for the coronavirus (*181*) of severe acute respiratory syndrome (SARS) (*182*), which will be discussed in detail in *Chapter 1.5.3*. This important finding had set a new stage for other pivotal findings, such as solving of ACE2 crystal structures, provided insights to the interaction of ACE2 with its inhibitor and substrates, and a potential therapeutic target for the treatment of SARS (all will be discussed under *Chapter 1.5.3*).

1.5.3 Characterisation of ACE2 ectodomain shedding

Membrane-bound ACE2 was recently shown to undergo an endogenous proteolytic cleavage at its ectodomain to release a soluble active form (*46*). Hence, it is not surprising that other studies have detected the presence of ACE2 in body fluids such as in blood plasma and in urine(*167, 172*). The overall mechanism and regulation for ACE2 ectodomain shedding is not defined. Unlike ACE, the site of cleavage-secretion of soluble ACE2 has not been identified. Preliminary studies of ACE2 ectodomain shedding suggested that the sheddase(s) responsible for ACE2 ectodomain shedding is different from that of ACE (unpublished data by our laboratory, in collaboration with Daniel W. Lambert, Nigel M. Hooper and Anthony J. Turner, University ofLeeds, UK). Subsequent studies showed that when ACE2-expressing cells were stimulated by phorbol esters (an activator of protein kinase C (PKC) signaling pathway that is known to induce ectodomain shedding in diverse range of membrane-bound proteins), ADAM17 is one of the responsible sheddase(s) (*46*).

Studies in both rats and humans have previously demonstrated that ACE2 undergoes an increased level of proteolytic cleavage to release its active soluble form as a result of myocardial infarction (*3*). As mentioned previously, ACE2 has also been identified as the functional receptor for the SARS-CoV. In this study, the authors successfully cultured SARS-CoV permissive Vero E6 cells, and showed that ACE2 can effectively bind to the S1 domain of the SARS-CoV (*182*). The latter study also revealed that recombinant soluble ACE2 could effectively block the association of SARS-CoV S1 protein from binding to its cellular receptor. Very little has been done in investigating the importance of ACE2 ectodomain shedding thus far. Moreover, the identity of all the ACE2 sheddase(s) is not known. Since the discovery of ACE2, studies have mainly focused on the membrane-associated form, and its implication in cardiovascular regulation through RAS. Considering that soluble ACE2 was found to inhibit the infectivity of SARS-CoV, the importance of ACE2 ectodomain shedding nonetheless is a process of great significance. One of the impacts from this discovery in the medical field is the recently solved crystal structure of ACE2-SARS-CoV complex (*183*), which highlighted the

value of soluble ACE2 as an important tool in understanding the pathogenesis of this new and sometimes fatal coronavirus. Following the SARS epidemic, ACE2 has now become a new therapeutic target for vaccine development and potentially a biomarker for cardiac regulation. Although these findings demonstrated an association of ACE2 in diseased states, it is clear that we are only barely scratching the surface in understanding the precise physiological role of ACE2 and its regulation.

1.6 Aims of the Current Research

Previous studies in our laboratory have shown that an active soluble form of the carboxypeptidase ACE2 is shed from the plasma membrane by two separate mechanisms: a low level constitutive secretion, and a regulated secretion stimulated by phorbol ester, phorbol-12-myristate 13-acetate (PMA) (*46*). Furthermore, our laboratory also successfully demonstrated that PMA-regulated shedding was significantly decreased in the presence of the ADAM17 inhibitor, TAPI-1, and the less specific MMPs inhibitor, GM6001 (Dr. Michael A. Yarski, and Dr. Fiona J. Warner – personal communication). These findings together with those of our collaborating group (Turner et. al., University of Leeds, United Kingdom) indicated that in PMA-stimulated ACE2 secretion, ADAM17 and perhaps MMPs are responsible for the shedding of ACE2 from the plasma membrane (*46*). However, the precise cleavage site of the ACE2 for both regulated and constitutive ectodomain secretion has yet to be identified. Moreover, the regulation by which ACE2 undergoes ectodomain shedding is not known. Therefore, the global aim of the current research was to characterise the ectodomain shedding of ACE2, specifically to gain a better understanding of the molecular mechanism underlying ACE2 ectodomain shedding and regulation.

Over the last 5 years, there have been many reports in the literature describing a role for calmodulin (CaM) in protein ectodomain shedding. For example, the shedding of membrane-bound proteins such as APP, TGF-α and TrK A is enhanced by inhibitors of CaM (*184*). Furthermore, CaM-inhibitor (CaMI)-induced proteolytic release of all of these proteins is metalloprotease-dependent. We have currently observed that the juxtamembrane of cytoplasmic sequence of human ACE2 contains a sequence analogous to the juxtamembrane of CaM-binding proteins. Therefore, the first aim of the current research is to determine the possible role of CaM in regulating ACE2 ectodomain shedding. Briefly, CaM binding to ACE2 using gel-shift assays and *in vitro* binding assays will be used to investigate CaM-ACE2 association. Specific inhibitors and antibodies of CaM and ACE2 will also be used to identify quenched fluorescent substrate (QFS) degradation by soluble and membrane preparations from the human liver cell line, Huh-7, which both endogenously expresses and sheds ACE2. These studies will elucidate if CaM can specifically bind to ACE2 and thus play a role in regulating ACE2 ectodomain shedding. The significance lies in understanding the potential signaling pathways that mediate ACE2 shedding.

Although the catalytic ectodomains of ACE and ACE2 share ~42% sequence similarity, the carboxyl-terminal cytoplasmic tail, transmembrane region and immediate extracellular amino acid sequences juxtaposed to the cell membrane share no recognisable homology. Thus, the comparisons are not useful for prediction of either the likely cleavage sites in ACE2 or the proteases that mediate the shedding (as yet unidentified for ACE). The second aim of the current research is to determine the precise cleavage site(s) of ACE2 ectodomain shedding and investigate the protease(s) involved in the shedding event. Briefly, secreted ACE2 will be purified and, using proteomics approaches, we will investigate the potential cleavage site of ACE2 ectodomain shedding. These studies will determine the site(s) of cleavage in the proximal membrane region of ACE2 and investigate the proteases

responsible for ACE2 shedding. The significance lies in understanding (and perhaps therapeutic blocking) of ACE2 shedding.

Very little is known about the regulation of ACE2 expression *in vivo*. We propose that AngII, acting via the AT1R, may stimulate ACE2 shedding though a biochemical feedback loop, leading to reduced peptide degradation. Thus, the final aim of the current research is to explore the above-mentioned hypothesis, and perhaps explore the effects of other angiotensin peptides in regulating ACE2 shedding. Briefly, specific inhibitors and antibodies of ACE2 and various angiotensin peptides will be used to investigate shedding activity by soluble and membrane preparations from ACE2-transfected cells. These studies will determine if AngII and/or Ang(1-7) (AngII cleavage product by ACE2) are able to directly and specifically modulate ACE2 shedding from the liver cells, thus identifying a possible positive or negative regulating feedback loop.

2 MATERIALS AND METHODS

2.1 Cell Culture

Chinese hamster ovary cells (CHO) stably expressing polyoma LT antigen (CHO-P), CHO-K1 (a subclone of the parental CHO cell line), human embryonic kidney cells (HEK 293) and a derivative of HEK 293 cells that stably express the large T-antigen of SV40 (HEK 293T), were obtained from the American Tissue Culture Collection. CHO-K1 and CHO-P cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen Corp., Mulgrave, VIC, Australia) containing 8% (v/v) foetal bovine serum (FBS) (JRH Biosciences, KS, USA), antibiotics (100 units/mL penicillin G sodium, 68.63 mM streptomycin sulphate, 2 mM L-glutamine, 100μM sodium citrate in 0.14% NaCl) (Invitrogen Corp., Mulgrave, VIC, Australia). HEK 293 and HEK 293T cells were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen Corp., Mulgrave, VIC, Australia) 8% (v/v) FBS and antibiotics. Mycoplasma infected human hepatoma cell line, Huh-7, that endogenously expressing full-length ACE2, were gifts from Professor Stephen Locarini (Victorian Infectious Diseases Reference Laboratory, Melbourne, VIC, Australia). Huh-7 cells were maintained in minimum essential medium alpha (α-MEM) (Invitrogen Corp., Mulgrave, VIC, Australia) containing 10% (v/v) FBS and antibiotics at pH 7.2. HEK 293 cells that stably expressing hemagglutinin antigen (HA) tagged AT1R, dubbed HEK-NHA-AT1 cells, were gifts from Dr. Walter Thomas (Baker I. D. I., Prahran, VIC, Australia). HEK-NHA-AT1 cells were maintained in DMEM growth medium containing 8% (v/v) FBS, antibiotics and 0.5 mg/mL Geniticin® selective antibiotic (Invitrogen Corp., Mulgrave, VIC, Australia). All cells were grown and maintained in BD Falcon™ cell culture dishes (6-well, 100 mm and 150 mm) and flasks (75 cm^3 and 175 cm^3)

with vented caps (Becton, Dickinson and Co., North Ryde, NSW, Australia) at 37°C in 5.0% CO_2.

2.2 DNA Plasmid Preparations

pcDNA3.1/V5-His-TOPO (Invitrogen Corp., Mulgrave, VIC, Australia) sub-cloned full-length ACE2 constructs encoding FLAG sequence in the carboxyl-terminal end, and pcDNA3.1/V5-His-TOPO sub-cloned soluble ectodomain form of ACE2 encoding FLAG sequence tag in the amino-terminal end and hexahistidine sequence tag in the carboxyl-terminal end, were engineered by Dr. Michael A. Yarski (Monash University, Clayton, VIC, Australia). The pcDNA3.1/V5-His-TOPO empty vector, pEGFP-N3 green fluorescent tag vector (Becton, Dickinson and Co., North Ryde, NSW, Australia), pRcNHA/AT1 plasmid, full-length and soluble ectodomain form of ACE2 subcloned plasmids DNA preparations were completed using Magic Miniprep™ purification kit (Promega Corp., Hawthorn, VIC, Australia) and QIAfilter™ Maxi Plasmid (QIAGEN Inc., Doncaster, VIC, Australia) purification kit, each according to manufacturer's instruction. DNA concentrations were determined using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia).

2.3 Plasmid Constructions

2.3.1 Engineering FLAG-tagged full-length ACE2 and soluble ectodomain form of ACE2

An expression construct for full-length human recombinant ACE2 containing a carboxyl-terminal FLAG peptide sequence was made by polymerase chain reaction (PCR) amplification of the cDNA from a human testis quick-clone cDNA (catalog number 711-7; CLONTECH, Palo, Alto, CA, USA). Using forward (5'-GGT-ACCATGTCAAGCTCTTCCTGGCTCC-

3') and reverse (5'-CGCTCGAGTCACTTGTCA-TCGTCGTCCTTGTAGTCAAAGGAGGTCTGAACATCATC-3') primers. The PCR product was digested with KpnI and XholI and ligated into these sites of the pcDNA3.1/V5-His-TOPO according to the manufacturer's instructions. The expression construct for secreted soluble ACE2 was generated by fusing IL-3 signal sequence and a FLAG tag to the amino-terminus of ACE2 (amino acids 2-738) and a hexa-histidine tag on the carboxyl-terminus, as previously described in (*173*). The cDNA was ligated into pcDNA3.1/V5-His-TOPO vector for transient transfection into HEK 293T cells by the calcium phosphate method (*185*), as described under 'Plasmid Transfections'. A schematic of the engineered recombinant full-length human ACE2 and soluble ectodomain form human ACE2 plasmid constructs in pcDNA3.1/V5-His-TOPO vector are illustrated in Figure 2-1 *(top panel)*.

2.3.2 Engineering ACE2 mutants

CHO-K1 ACE2 mutants' constructs were engineered PCR amplification. 100-200 ng of pcDNA3.1/V5-His-TOPO sub-cloned full-length ACE2 constructs encoding FLAG sequence in the carboxyl-terminal end was extended using 0.2-0.3 mM PCR Nucleotide Mix (Promega Corp., WI, USA), 0.25-0.30 µM of each primers (forward and corresponding reverse) (Geneworks, Hindmarsh, SA, Australia) as listed on Table 2-1, 1X Platinum® Pfx Amplication buffer (Invitrogen Corp., Mulgrave, VIC, Australia), or 1X *Pfu* DNA Polymerase buffer with $MgSO_4$ (Promega Corp., Hawthorn, VIC, Australia), or 1X KOD DNA Polymerase buffer (MERCK KGaA, Kilsyth, VIC, Australia); together with 1.0-2.5 U Platinum®*Pfx* DNA Polymerase (Invitrogen Corp., Mulgrave, VIC, Australia), 1.25 U Pfu DNA Polymerase (Promega Corp., Hawthorn, VIC, Australia) or 0.02 U KOD DNA Polymerase (MERCK KGaA, Kilsyth, VIC, Australia). Reactions were initiated in a Mastercyler gradient® (Eppendorf AG, North Ryde, NSW, Australia) thermal cycler according to manufacturers' instructions (1 cycle of 95°C for 1-10 minutes; 20-40 cycles of 95°C for 30-60 seconds, 42-65°C for 30-60 seconds and 72-

74°C for 15 minutes; maintaining at 4°C).Once the cycles had come into completion, the PCR products were digested with DpnI restriction enzyme as described in 'Restriction Digests and Agarose Gel Electrophoresis'. Following digestion, the samples were then transformed into *Escherichia coli* strain NM522 and plated on a LB agar plate containing 100 mM ampicillin (Sigma-Aldrich Inc., Castle Hill, NSW, Australia). Resulting positive colonies were picked for plasmid DNA preparation using Magic Miniprep™ purification kit as described previously. Purified plasmid DNA was sequenced to verify for respective ACE2 mutant strains. A schematic of the engineered recombinant full-length human ACE2 containing mutated sequence(s) plasmid constructs in pcDNA3.1/V5-His-TOPO vector are illustrated in Figure 2-1 *(top panel)*.

Table 2-1:Primers used to engineer ACE2 mutants in pcDNA3.1/V5-His-TOPO subcloned full-length ACE2 construct encoding FLAG peptide sequence in the carboxy-terminal end of the cytoplasmic tail.

Primer Name	Sequence	cDNA Position
ACE2-R708Afor	GCCATCAGGATGTCCGCCAGCCGTATC	2410 – 2436
ACE2-R708Arev	CGGTAGTCCTACAGGCGGTCGGCATAG	2410 – 2436
ACE2-S709Afor	GCCATCAGGATGTCCCGGGCCCGTATC	2410 – 2436
ACE2-S709Arev	CGGTAGTCCTACAGGGCCCGGGCATAG	2410 – 2436
ACE2-R708A/S708Afor	GCCATCAGGATGTCCGCCGCCCGTATC	2410 – 2436
ACE2-R708A/S709Afor2	ATGTCCGCCGCCCGTATCAATG	2419 – 2440
ACE2-R708A/S709Arev	CGGTAGTCCGACAGGCGGCGGGCATAG	2410 – 2436
ACE2-R708A/S709Arev2	GATACGGGCGGCGGACATCCTG	2419 – 2440
ACE2-R708Efor	GGCCATCAGGATGTCCGAGAGCCGTATC	2410 – 2436
ACE2-R708Erev	CCGGTAGTCCTACAGGCTCTCGGCATAG	2410 – 2436
ACE2-R710Efor	GGCCATCAGGATGTCCCGGAGCGAGATC	2410 – 2436
ACE2-R710Efor2	TCCCGGAGCGAGATCAATGATGCTTTCCGTCTG	2422 - 2454
ACE2-R710Efor3	CGGAGCGAGATCAATGATGCTTTC	2425 - 2448
ACE2-R710Erev	CCGGTAGTCCTACAGGGCCTCGCTCTAG	2410 – 2436
ACE2-R710Erev2	ATCATTGATCTCGCTCCGGGACATCCTGATGGC	2422 – 2454
ACE2-R710Erev3	ATCATTGATCTCGCTCCGGGACAT	2425 - 2448
ACE2-R710Dfor	TCCCGGAGCGATATCAATGATGCTTTCCGTCTG	2422 – 2454

ACE2-R710Drev	ATCATTGATATCGCTCCGGGACATCCTGATGGC	2422 – 2454
ACE2-R710Gfor	TCCCGGAGCGGTATCAATGATGCTTTCCGTCTG	2422 – 2454
ACE2-R710Grev	ATCATTGATACCGCTCCGGGACATCCTGATGGC	2422 – 2454
ACE2-R708E/R710Efor	GGCCATCAGGATGTCCGAGAGCGAGATC	2410 – 2436
ACE2-R708E/R710Efor2	TCCGAGAGCGAGATCAATGATGCTTTCCGTCTG	2422 – 2454
ACE2-R708E/R710Erev	CCGGTAGTCCTACAGGCTCTCGCTCTAG	2410 – 2436
ACE2-R708E/R710Erev2	ATCATTGATCTCGCTCTCGGACATCCTGATGGC	2422 – 2454

2.3.3 Engineering pIRESpuro-ACE2 construct

ACE2 gene from full-length pCI-neo-ACE2 (construct generated by Dr. Michael A Yarski, Monash University, VIC, Australia) was excised at NheI and NotI restriction sites at position 1085 and 3531, respectively, within the multiple cloning site regions following the methods described under 'Restriction Digests and Agarose Gel Electrophoresis'. ACE2 gene was then prepared with QIAquick Gel Extraction Kit (QIAGEN Inc., Doncaster, VIC, Australia) according to manufacturer's instruction, prior to ligation into pIRESpuro3 (a gift from Dr. John T. Price, Monash University, VIC, Australia) at position 927 and 983. Both excised ACE2 gene and pIRESpuro3 vector was digested with T4 DNA Ligase (New England Biolabs, Arundel, QLD, Australia) in 1X T4 DNA Ligase reaction buffer (New England Biolabs, Arundel, QLD, Australia) for 40 minutes at room temperature. Ligated products weretransformed into *Escherichia coli* strain NM522 and plated on a LB agar plate containing 100 mM ampicillin. Resulting positive colonies were picked for plasmid DNA preparation using Magic Miniprep™ purification kit as described previously. Purified plasmid DNA was sequenced to verify for respective ACE2 mutant strains. A schematic of the engineered recombinant full-length human ACE2 plasmid constructs in pIRESpuro vector are illustrated in Figure 2-1 *(bottom panel)*.

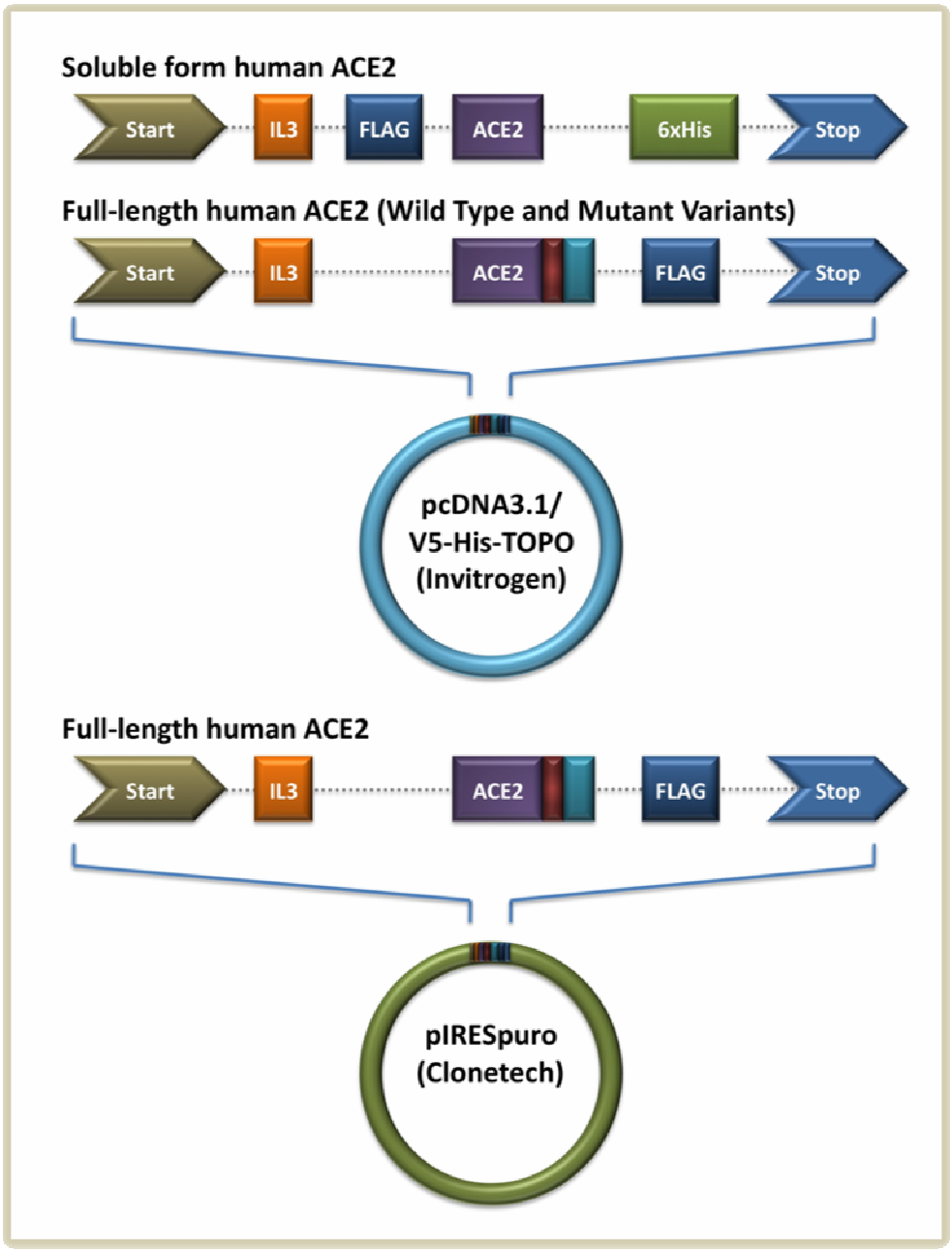


Figure 2-1: A schematic of constructed plasmid DNA. *(Top panel)* Soluble ectodomain form human ACE2 and full-length human ACE2 constructs were subcloned into the multiple clonding site of pcDNA3.1/V5-His-TOPO vector. *(Bottom panel)* Full-length human ACE2 construct were subcloned into the multiple cloning site of pIRESpuro vector.

2.4 DNA Sequencing

Plasmid DNA of full-length wild type of ACE2 and ACE2 mutants were sequenced using BigDye® Terminator (Applied Biosystems Inc., Mulgrave, VIC, Australia) reaction setup protocol. Plasmid DNA (300 ng) were mixed with 1 µL BigDye® Terminator Ready Reaction Mix (Applied Biosystems Inc., Mulgrave, VIC, Australia), 3.5 µL of BigDye® Terminator v3.1 Sequencing Buffer (5X) (Applied Biosystems Inc., Mulgrave, VIC, Australia), 5.0 pmoles of primers (see Table 2-2 for details) (Geneworks, Hindmarsh, SA, Australia) to a final volume of 20 µL. Cycle sequencing is initiated in a Mastercyler gradient® thermal cycler according to manufacturer's instruction (1 cycle of 96°C for 1 minute; 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes; maintaining at 4°C). Once the cycles had come into completion, the reaction was added to a sodium acetate/ethanol mixture containing 112.5 mM sodium acetate pH 5.2, and 78.13% (v/v) ethanol). The samples were vortexed and incubated for 15 minutes at room temperature before centrifugation for 15 minutes at 16,000 x g. Following centrifugation, the supernatants were carefully removed, and replaced with 70% (v/v) ethanol. The samples were vortexed once more and centrifuged at 16,000 x g for 5 minutes. Again the supernatant were carefully removed and the samples were dried down. The samples were sequenced using Applied Biosystems 3730S Genetic Analyser (Applied Biosystems Inc., Mulgrave, VIC, Australia) fitted with a 50 cm array. The proprietary Kb basecaller (Applied Biosystems Inc., Mulgrave, VIC, Australia) were used during data analysis.

Table 2-2: Primers used for sequencing of ACE2 plasmids.

Primer Name	Sequence	ACE2 Position
ACE2seq1	5' CTT GAA CCA GGT TTG AAT 3'	436-454 on ACE2
ACE2seq2	5' AT ATG ACT CAA GGA TTC TGG 3'	971-990 on ACE2
ACE2seq3	5' CA TAC TGT GAC CCC GCA TCT CT 3'	1493-1514 on ACE2
ACE2seq4	T' ATG ATT CTT TTT GGG GAG GAG G 3'	1990-2011 on ACE2

2.5 Restriction Digests and Agarose Gel Electrophoresis

0.5 µg of plasmid DNA, 1X reaction buffer (New England Biolabs, Arundel, QLD, Australia), 1X Bovine Serum Albumin (BSA) (New England Biolabs, Arundel, QLD, Australia), 1 U of XbaI, or XhoI or DpnI, or NheI, or NotI, or KpnI (New England Biolabs, Arundel, QLD, Australia) were combined and then incubated at 37°C for 1-hour. Following incubation, 1X Blue/Orange Loading Dye (Invitrogen Corp., Mulgrave, VIC, Australia) was added to each reaction mixture and separated on 0.8% (w/v) agarose gels made in TBE buffer (90 mM Tris-base, 90 mM boric acid, 2 mM disodium ethylenediaminetetraacetic acid (EDTA).$2H_2O$ (Sigma-Aldrich Co., Castle Hill, NSW, Australia), pH 8.3) containing 5% ethidium bromide. Agarose gels were run in TBE buffer at 100 V/50 mA (Hoefer EPS 2A200 power pack; Amersham Pharmacia Biotech, CA, USA) for 10-hour, followed by visualisation using a UVP BioDoc-It® UV Transiluminator (UVP LLC, CA, USA).

2.6 Plasmid Transfections

2.6.1 Calcium phosphate transfections

Cells were grown to 80% confluence in cell culture dishes in growth medium. Transient transfections using calcium phosphate transfection method were achieved by mixing 1.75 µg, 10.0 µg or 15.0 µg plasmid DNA in 6-well, 100 mm or 150 mm cell culture dishes, respectively, in HBS buffer (25 mM HEPES, pH 7.1; 140 mM NaCl, 1.5 mM Na_2HPO_4) containing 125 mM $CaCl_2$. 16-hours post-transfections, cells were washed, replaced with growth medium and maintained for a further 44-hours at 37°C in 5.0% CO_2.

2.6.2 DEAE-Dextran transfections

Cells were grown to 80% confluence in cell culture dishes in growth medium. Cells were then rinsed with reduced serum media, Opti-MEM® (Invitrogen Corp., Mulgrave, VIC, Australia). Transient transfections using DEAE-Dextran transfection method were achieved by mixing 1.75 µg, 10.0 µg or 15.0 µg plasmid DNA 6-well, 100 mm or 150 mm cell culture dishes, respectively, in a Opti-MEM® mixture containing 1.4 mg DEAE-Dextran (Sigma-Aldrich Co., Castle Hill, NSW, Australia), 10% (v/v) FBS and 200 mM Tris-HCl, pH7.3). Cells were incubated for 2-hours at 37°C in 5.0% CO_2. Following incubation, cells were rinsed twice with Opti-MEM® and exposed to a 1-minute dimethyl sulfoxide (Me_2SO) shock treatment using 10% (v/v) tissue culture-grade Me_2SO (Sigma-Aldrich Co., Castle Hill, NSW, Australia) in Dulbecco's phosphate buffered saline (PBS) (Invitrogen Corp., Mulgrave, VIC, Australia). Cells were then rinsed twice with PBS and maintained in growth medium for further 58-hours at 37°C in 5.0% CO_2.

2.6.3 Lipofectamine™ 2000 transfections

One day prior to transfection, 2.5 x 10^5 cells were plated in 6-well cell culture dishes containing 2.0 mL of growth medium without antibiotics. On the day of transfection, 4.0 µg of plasmid DNA was pre-diluted in 50 µL of Opti-MEM® before combining it with Opti-MEM® diluted Lipofectamine™ 2000, in DNA (µg): Lipofectamine™ 2000 (µL) ratios of 1:2, 1:3, and 1:4. The mixtures were added to cells containing pre-existing growth medium. Cells were incubated at 37°C in 5.0% CO_2 for 24-hours. Following incubation, cells were passaged at a 1:100 into fresh growth medium containing selective antibiotic. Cells were further passaged twice weekly for 6 weeks prior to testing for transgene expression.

2.7 Isolation and Purification of Protein

2.7.1 Full-length ACE2

72-hours post-transfections, cells were harvested from the cell culture dishes in PBS and were sonicated 10 seconds 3 times, using a Sonifier-B-30 cell disruptor (duty cycle 30%, output control: 3) in 50 mM HEPES, pH 7.5 containing 20 mM $CaCl_2$. Cell lysates were then centrifuged at 100,000 x g for 1-hour at 4°C using a Beckman Coulter Ti55 centrifuge rotor (Beckman Coulter Inc., Gladesville, NSW, Australia). Following centrifugation, cell membranes were solubilised in 1X Lysis Buffer (50 mM Tris HCL, pH 7.4; 150 mM NaCl, 1% (v/v) Triton X-100) at 4°C with gentle inversion. Full-length ACE2 in the membrane lysates were purified on EZview Red ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) according to manufacturer's instructions. Samples were kept at 4°C for 16-hours with gentle shaking. After subsequent washing in 1X TBS buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4) resin-bound full-length ACE2 proteins were subsequently eluted in 0.5 µg/mL 3X FLAG® peptides (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) in 1X TBS buffer. Beads were recovered by rinsing under acidic conditions using 0.1 M glycine-HCl, pH 3.5 for 10-15 minutes at room temperature with gentle shaking. Alternatively, resin-bound full-length was also eluted using a mixture of H_2O, isopropanol and formic acid in 4:4:1 ratio.

2.7.2 Shed/Secreted- and soluble ectodomain-form of ACE2

72-hours post-transfection, the medium from Huh-7, stable ACE2 and ACE2 mutant cells, and transfected with the soluble ectodomain form of ACE2, were harvested and transferred into a 15 mL or 50 mL BD Falcon™ Tubes (Becton, Dickinson and Co., North Ryde, NSW, Australia), or a 250 mL polypropylene high-performance high-speed bottle (Beckman Coulter Inc., Gladesville, NSW, Australia) and centrifuged at 6,000 x g for 20 minutes at 4°C.

Following centrifugation, the supernatant was concentrated using a 30 kDamolecular weight cut-off (MWCO) Nanosep®, 30 kDa MWCO Microsep®, 30 kDa MWCO 150 mL Stirred Cell Omega system centrifugal devices (all from PALL Corp, Cheltenham, VIC, Australia), Amicon® Ultra-4 centrifugal filter units 30 kDa MWCO (Millipore Corp., North Ryde, NSW, Australia). The final volume of concentrate was reduced to 30-50 fold concentration and the shed/secreted-and soluble ectodomain-form of ACE2 was purified on EZview Red ANTI-FLAG® M2 Affinity Gel, as described previously. Elution samples were concentrated to a final volume of approximately 500 µL using 30 kDa MWCO Nanosep® according to manufacturer's instructions.

2.8 Protein Assay

Protein assays were set up in BD Falcon™ 96-well Assay Plate with lid, standard, flat-bottom, sterile (Becton, Dickinson and Co., North Ryde, NSW, Australia) format, in triplicate wells. Concentrated medium (5 µL) containing shed or soluble ectodomain form of ACE2 from cells expressing full-length ACE2, soluble ectodomain of ACE2, or ACE2 mutants; and diluted cell lysates (5 µL) containing solubilised full-length ACE2 or ACE2 mutants in the ratio of 1:5 or 1:10. Bio-Rad DC reagent A (25 µL) and Bio-Rad DC reagent B (200 µL) was added to each protein/peptide containing wells. In determining protein concentrations in samples containing detergent, Bio-Rad DC reagent A' (25 µL) were used (Bio-Rad DC reagent A containing 0.5% (v/v) Bio-Rad DC reagent S) (all Bio-Rad DC reagents; Bio-Rad Laboratories Inc., Gladesville, NSW, Australia). Known concentrations (0 – 2.0 mg/mL) of BSA (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) were set up accordingly to determine the protein concentration in samples.

2.9 MCA Calibration Assay

Known concentrations of 7-methoxycoumarin-4-acetic acid (Mca) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) were made up in ACE2 Buffer (100 mM Tris-base, pH 6.5; 500 mM NaCl) and loaded onto a 96 MicroWell™ polypropylene 0.5 mL plates (Nunc GmbH, Thermo Fisher Scientific, Rochester, NY, USA) containing 0 µg, 25 µg or 100 µg of crude proteins extracted from the medium of the full-length ACE2 transfected HEK 293T cells. Fluorescence was read for one cycle using the filter set at 320 nm excitation and 405 nm emission at 37°C using BMG Labtech FLUOstar Optima plate reader (BMG Labtech GmbH, Mt. Eliza, VIC, Australia).

2.10 Quenched Fluorescent Substrate Assay for ACE2 Activity

ACE2 activities in the concentrated medium and cell lysates of ACE2 and ACE2 mutant expressing cells were determined by quenched fluorescent substrate (QFS) assay. 10 µg, 25 µg, 50 µg or 100 µg of protein were incubated with 50 µM ACE2 specific QFS (Mca-Ala-Pro-Lys(2,4-dinitrophenyl)-OH) (Auspep, Parkville, VIC, Australia), as shown in Figure 2-2 in ACE2 buffer. Specific activity of ACE2 was determined using 100 nM ACE2 specific inhibitor MLN 4760 ((S, S)-2-(1-carboxy-2-(3-(3, 5- dichlorobenzyl)-3 H-imidazol-4-yl)-ethylamino)-4-methylpentanoic acid), a gift from Dr. Natalie Dales (Millenium Pharmaceuticals, MA, USA). Samples were assayed on a 96 MicroWell™ polypropylene 0.5 mL plates. As the ACE2 QFS can also be cleaved by prolyl endopeptidase, 1 µM of Z-Pro-prolinal (Bachem A. G., Bubendorf, Switzerland), a specific inhibitor of this enzyme was incorporated in assay wells. Fluorescence was read at 320 nm excitation and 405 nm emission with selected gain of 912 for 4-hours at 37°C using BMG Labtech FLUOstar Optima plate reader. The reaction products

were quantified using a standard curve generated from known concentrations of Mca and expressed as pmoles of substrate hydrolysed/min/mg of protein.

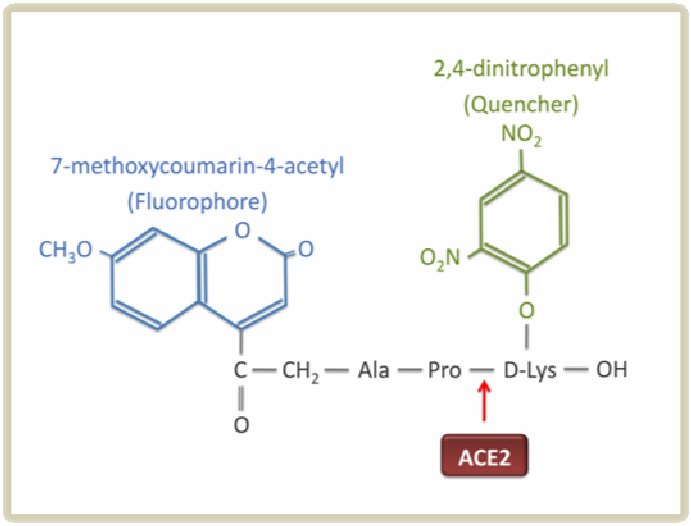


Figure 2-2: Structure representation of ACE2 quenched fluorescent substrate. (7-methoxycoumarin-4-yl)acetyl (Mca) on the amino-terminal end, and a quencher, dinitropheyl (Dnp) on the carboxy-terminal end, flanking a tripeptide backbone (Ala-Pro-Lys) that is specific for ACE2 cleavage.

2.11 Calmodulin Inhibitors Studies of ACE2 Activity

Huh-7 cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-free medium before equilibrated in Opti-MEM® for 16-hours prior to experimentation. All pharmacological agents (1 µM) phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), (2 µM) protein kinase C inhibitor Bisindolylmaleimide I (BIM) (MERCK KGaA, Kilsyth, VIC, Australia), and calmodulin specific inhibitors; (25 µM) trifluoperazine dimaleate (TFP) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), and (25 µM) calmidazolium chloride (CMZ) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), were diluted using serum-reduced media Opti-MEM®. All incubations were carried

out in a CO_2incubator at 37 °C with 5% CO_2 for 1-hour, or 16-hours. Following treatment of cells, the medium was harvested and concentrated 30-50 fold by centrifugation in Amicon® Ultra-4 centrifugal filter units 30 kDa MWCO, as discussed previously, to a final volume of 250 µL. Cells were scraped with ice-cold PBS, harvested by centrifugation, and solubilised in 500 µL of Lysis buffer, as discussed previously. Protein concentration was determined before analysed for specific ACE2 activity and western immunoblotting.

2.12 PMA Stimulation Studies of ACE2 Activity

Cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-free medium before equilibrated in Opti-MEM®for 16-hours prior to experimentation. Pharmacological agents PMA (1 µM),BIM (2 µM) and ADAM17 specific inhibitor, TAPI-1 (50 µM) (MERCK KGaA, Kilsyth, VIC, Australia),were diluted using serum-reduced media Opti-MEM®. All incubations were carried out in CO_2 chambers at 37 °C with 5% CO_2 for 4-hour. Following treatment of cells, the medium was harvested and concentrated 30-50 fold by centrifugation in Amicon® Ultra-4 centrifugal filter units 30 kDa MWCO concentrators, as discussed previously, to a final volume of 250 µL. Cells were scraped with ice-cold PBS, harvested by centrifugation, and solubilised in 500 µL of Lysis buffer, asdiscussed previously. Protein concentration was determined before analysis for specific ACE2 activity and western immunoblotting.

2.13 Angiotensin II Stimulation Studies of ACE2 Activity

Cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-free medium before equilibration in Opti-MEM®for 16-hours prior to experimentation. Pharmacological agents PMA (1 µM) and angiotensin I (100 nM) and

angiotensin II (100 nM) (both peptides from Auspep, Parkville, VIC, Australia); AT1R antagonist peptide losartan (1 μM), ACE specific inhibitor captopril (100 μM), AT2R antagonist PD123319 (1 μM) (inhibitors were gifts from Associate Professor Robert E. Widdop, Monash University, VIC, Australia); and AT1R agonist L-162,313 (1 μM) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia)were diluted using serum-reduced media Opti-MEM®. All incubations were carried out in CO_2 chambers at 37 °C with 5% CO_2 for 4-hours, 16-hours, or 24-hours. Following treatment of cells, the medium was harvested and concentrated 30-50 fold by centrifugation in Amicon® Ultra-4 centrifugal filter units 30kDa MWCO concentrators, as discussed previously, to a final volume of 250 μL. Cells were scraped with ice-cold PBS, harvested by centrifugation, and solubilised in 500 μL of Lysis buffer, asdiscussed previously. Protein concentration was determined before analysis for specific ACE2 activity and western immunoblotting

2.14 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, Parkville, Australia), and peptide mimetics consisting scrambled sequences (dubbed ACE2T2 mutant-1, -2, -3 and -4), were analysed by gel shift assay as described by Erickson-Viitanen and Degrado (*186*) and previously used to identify CaM-binding peptides (*187-190*). Reactions (30 μL) containing 300 pmol of bovine CaM (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) and increasing amounts (0 - 15 nmol) of the peptides in 100 mM Tris-HCl, pH 7.5, 4 mol/L urea and either 0.1 mM $CaCl_2$ or 1 mMethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetra-acetic acid (EGTA) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) were incubated at 25°C for 30 min. Following 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/L of urea in either 1 mM $CaCl_2$ or 1 mM EGTA. The gels were fixed and stained with Coomassie Blue (50% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie Blue R-250) for 1 minute. The gels were destained with 10% (v/v) acetic acids for approximately 16 – 24 hours.

2.15 In Vitro Binding Assay

Cell lysates (2 mg) from transiently transfected of ACE2 in CHO-P cells were incubated with 20 µg of glutathione-S-transferase (GST) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) or GST-CaM fusion protein (prepared and purified by Dr. Fi-Tjen, Mu as previously described in (*25*)) for 16-hours at 4°C with gentle shaking. Following incubation, the mixture was further incubated in the presence of 50% (w/v) glutathione sepharose resin(GE Healthcare Biosciences, Rydralmere, NSW, Australia) in the presence of 0.5 µL EDTA-free protease inhibitor cocktail (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) for 4-hours at room temperature. Bound resins were rinsed three times with Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) before separation by SDS-PAGE for western immunoblotting analysis, or analysis by ACE2 activity assay using ACE2 specific QFS in the presence of MLN 4760, as described previously.

2.16 *In Vitro* Cleavage Assay

Synthetic peptides corresponding to wild-type and mutant juxtamembrane region of ACE2 ectodomain (see Table 2-3), were incubated with 0.1 µg of recombinant human protein ADAM17 (MERCK KGaA, Kilsyth, VIC, Australia) in ADAM17 buffer (25 mM Tris, pH 9.0, 2.5 µM ZnCl2, 0.005% (v/v) Brij-35) for 0-hours, 4-hours or 8-hours, in with or without 50 µM ADAM17 specific inhibitor, TAPI-1 at 37°C. Cleavage reactions were terminated by addition

of 80% (v/v) acetonitrile containing 0.1% (v/v) trifuoroacetic acid. Cleavage products were analysed using MALDI-TOF-TOF mass spectrometry.

Table 2-3: List of peptide mimetics corresponds to juxtamembrane region of ACE2 ectodomain. Arginine^{708}, Serine^{709} and Arginine^{710} were substituted with alanine and glutamate (highlighted in red, underlined).

Peptide Name	Peptide Sequence
ACE2JM1	NH_2-INDAFRLNDNSLEFLGIQPT-COOH
ACE2JM2	NH_2-EKAIRMSRSRINDAFRLNDN-COOH
ACE2JM2-R708A	NH_2-EKAIRMS**A**SRINDAFRLNDN-COOH
ACE2JM2-S709A	NH_2-EKAIRMSR**A**RINDAFRLNDN-COOH
ACE2JM2-R708A/S708A	NH_2-EKAIRMS**AA**RINDAFRLNDN-COOH
ACE2JM2-R708E	NH_2-EKAIRMS**E**SRINDAFRLNDN-COOH
ACE2JM2-R710E	NH_2-EKAIRMSRS**E**INDAFRLNDN-COOH
ACE2JM2-R708E/R710E	NH_2-EKAIRMS**E**S**E**INDAFRLNDN-COOH

2.17 SDS-PAGE

Molecular weight markers Benchmark Prestained Protein Ladder (Invitrogen Corp., Mulgrave, VIC, Australia), purified full-length ACE2 (~100 µg protein) and soluble form of ACE2 proteins (~100 µg protein), and the cell lysates (~50 µg proteins) and concentrated media (~100 µg protein) harvested from Huh-7, ACE2 transfected cells, CHO-K1 cells stably expressing ACE2 mutants, and HEK-NHA-AT1 cells stably expressing ACE2, were run on NuPAGE® 4-12% Bis-Tris 10-well 1.5 mm or 15-well 1.0 mm gels (Invitrogen Corp., Mulgrave, VIC, Australia) in 1X NuPAGE® MES or MOPS SDS Running Buffer (Invitrogen Corp., Mulgrave, VIC, Australia) with NuPAGE® Antioxidant (Invitrogen Corp., Mulgrave, VIC, Australia) at

constant 200V (Hoefer EPS 2A200 power pack; Pharmacia Biotech, CA, USA) for 40-60 minutes or until the bromophenol blue marker reached the bottom of the gels.

2.18 Western Transfer and Immunodetection

NuPAGE® gels containing proteins from the SDS PAGE separation were washed in transfer buffer (120 mM Tris-base, 96 mM Glycine, and 20% (v/v) methanol), and the proteins were transferred to a polyvinylidene difluoride (PVDF) Immobilon™-P (Millipore Corp., North Ryde, NSW, Australia) membrane at 300 mA for 1-hour in cold (4°C) transfer buffer using Hoefer TE series Transfer Electrophoresis Unit (Amersham Pharmacia Biotech, CA, USA) and Pharmacia Biotech Multi Temp III cooling system (Amersham Pharmacia Biotech, CA, USA). Following transfer, PVDF membranes were washed in TST buffer (0.05% (v/v) polyoxyethlenesorbitan monolaurate (Tween-20) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) in TBS buffer). The membrane was then incubated with a blocking buffer (5% (w/v) skim milk powder in TST buffer) for 40 minutes with gentle shaking. PVDF membranes were then washed 3 times for 5 minutes each in TST buffer and then incubated with goat raised anti-human ACE2 ectodomain (amino acid 18 – 740) antibody (R&D Systems Inc., Gymea, NSW, Australia) at a dilution of 1:4000 in TST containing 3% (w/v) BSA, or mouse raised anti-FLAG antibody (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) at a dilution of 1:3000 in TST buffer containing 3% (w/v) BSA, or mouse raised anti-CaM antibody (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) at a dilution of 1:3000 in TST buffer containing 3% (w/v) BSA, or rat raised anti-HA-Biotin IgG antibody (Roche Diagnostics, Castle Hill, NSW, Australia) at a dilution of 1:3000 in TST buffer containing 3% (w/v) BSA; for 16-hours at 4°C. The blots were subsequently washed a further 3 times at 5 minutes each in TST buffer before incubation with affinity purified donkey raised anti-sheep/goat IgG conjugated to horse radish peroxidase (HRP) (Millipore Corp., North Ryde, NSW, Australia) at a dilution of 1:3000

in 5% (w/v) skim milk in TST buffer, or affinity purified sheep raised anti-mouse IgG conjugated to HRP (Millipore Corp., North Ryde, NSW, Australia) at a dilution of 1:3000 in 5% (w/v) skim milk in TST buffer, or affinity purified goat raised anti-rat IgG conjugated to HRP (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:3000 in 5% (w/v) skim milk in TST buffer, for 45 minutes at room temperature. The membranes were further washed 3 times for 5 minutes each in TST buffer and incubated with equal parts of enhanced luminol reagent and oxidizing reagent from the Western Lightning Chemiluminescence Reagent Kit (PerkinElmer Life Siences, Boston, MA, USA) for 1 minute. The membranes were then exposed to Kodak™ X-Omat™ Blue XB-1 photographic film (PerkinElmer Life Sciences, Boston, MA, USA) and developed on a Xerox film processor.

2.19 Edman Sequencing of Shed and Cleaved ACE2

Approximately 3 µg of purified full-length and soluble ectodomain form of ACE2 were incubated with 0.1 µg of recombinant human protein ADAM17 at 37°C for 5 hours in ADAM17 buffer. Following incubations, peptide samples were separated by SDS PAGE on a NuPAGE® 4 – 12% Bis-Tris 1.5 mm gel, as described previously. Subsequently, gels were washed and transferred to Immobilon™-P PVDF membrane at 50 V for 90 minutes in cold (4°C) 1X CAPS Buffer (10 mM CAPS, pH 10.5, 10% methanol). Following transfer, PVDF membranes were rinsed with distilled water and were then saturated with methanol for 10 seconds prior to staining with coomassie stain for exactly 1 minute. PVDF membranes were destained with 10% (v/v) acetic acids for approximately 48-hours and bands corresponding to cleaved stub of ACE2 were excised. The excised bands were then washed with alternating MilliQ water and 50% (v/v) methanol 3 times. These excised bands were further cut into smaller pieces and loaded into Procise™ Protein Sequencer cartridge (Applied Biosystems Inc., Mulgrave, VIC, Australia). Resulting peptide sequences were analysed with Procise 1.1

software (Method: PL PVDF Protein, Cycle: 08) (Applied Biosystems Inc., Mulgrave, VIC, Australia). Similarly, ACE2 peptide samples from EZview Red ANTI-FLAG® M2 Affinity Gel was directly loaded on the sequencing cartridge.

2.20 In Gel Tryptic Digestion

Gel bands were manually excised from the gel with a scalpel and destained with a solution of 50 mM ammonium bicarbonate, and 50% (v/v) acetonitrile. The gel pieces were washed and dehydrated with alternating washing cycles of 50 mM ammonium bicarbonate, and acetonitrile. Following the final dehydration of the gel pieces, it was again rehydrated with a solution containing 0.5 µg trypsin (Promega Corp., Hawthorn, VIC, Australia) and 20 nM ammonium bicarbonate. The gel pieces were incubated at 37°C for 16-hours and sonicated 10 minutes using Ultrasonic T1.9C sonicator (Health Sonics Corp., CA, USA) prior to mass spectrometry analysis.

2.21 Mass Spectrometry

2.21.1 MALDI-TOF and MALDI-TOF/TOF analysis

MALDI-TOF/TOF (matrix assisted laser desorption ionisation coupled with (tandem) time of flight analyser) was one of two mass spectrometers (MS) used for proteomics analysis of ACE2 cleavage site. Trysin digested samples were co-spotted onto the MALDI target plate with Matrix solution containing 10 mg/mL a-cyano-4-hydroxycinnamic acid (Laser Biolabs, Sopha-Antipolis, France) in 50% (v/v) 0.1% (v/v) TFA. The samples were analysed on an Applied Biosystems 4700 Analyser MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA) in reflectron mode with a mass range of 800 – 3,500 Da, focus mass of 1,400 Da, at 1500

shots per spectra. The 4700 Series Explorer software automatically selects 15 most intense peptides as precursor masses for tandem MS (MS/MS) analysis, and acquiring in the order of decreasing intensity. MS/MS analysis is carried out in reflector mode with a relative precursor mass window of 50 resolutions with metastable ion suppression on and spectra summed 2,500 shots per spectrum.

Peptide mass fingerprinting and MS/MS data is compiled by the GPS Explorer™ Software Ver. 3 (build 311) (Applied Biosystems, Forster City, CA, USA) and searched against the National Center for Biotechnology Information (NCBI) non-redundant and Swiss-Prot databases using the MASCOT search engine (version 1.9, Matrix Science Inc., London, UK) with all taxanomy selected. The following search parameters used: 0.1 Dalton; peptide charge, 1+; fixed modifications, carbamidomethyl; variable modification, oxidation (Met).

2.21.2 LC-MS/MS analysis

Trypsin digested ACE2 peptides that showed no match on MASCOT search engine were subjected for liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) analysis using the HCT ULTRA ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled online with a 1200 series capillary HPLC (Agilent technologies, Santa Clara, CA, USA). Samples injected onto a zorbax 300SB reversed phase column with Buffer A (5% (v/v) acetonitrile in 0.1% (v/v) formic acid) at a flow rate of 10 µL per minute). The eluant is nebulised and ionised using the Bruker electrospray source using the Low flow electrospray needle with a capillary voltage of 4,000 V dry gas at 300°C, flow rate of 8 L per minute and nebuliser gas pressure at 1,500 mbar. Peptides are selected for MS/MS analysis in autoMSn mode with smart parameter settings selected and active exclusion released after 1 minute.

Data acquired from LC-MS/MS run was exported in Mascot generic file format (*.mgf) and searched against NCBI non-redundant and Swiss-Prot databases using the MASCOT search engine (version 2.1, Matrix Science Inc., London, UK) with all taxonomy selected. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 0.4 Da; peptide fragment tolerance ± 0.2 Da, peptide charge, 2+ and 3+; fixed modification, carbamidomethyl; variable modification, oxidation (Met).

2.22 Circular Dichroism

Circular dichroism (CD) measurements were performed on a Jasco J-810 Circular Dichroism Spectropolariser (Jasco Inc., Tokyo, Japan) using quartz cuvettes of 1 mm path length. Scans between 190 and 260 nm were performed at a scan speed of 20 nm per minute, bandwidth of 1.0 nm, at resolution of 0.1 nm, a 1 second response time and with 3 scan accumulations. The quartz cuvette temperature was controlled at 37°C with a Peltier temperature controller and the CD instrument was calibrated with (+)-10-camphorsulphonic acid. CD spectra of peptide mimetics were measured in MiliQ-grade water or in 50% (v/v) methanol, and spectra were smoothed using the Jasco Fast Fourier (Jasco Inc., Tokyo, Japan) transform algorithm and then baseline corrected. Peptide concentration used was approximately 200 μM for each analogue.

2.23 Two-dimensional (2D) Gel Separation

Two-dimensional gel separation or referred to as 2D gel electrophoresis (2D-PAGE) were performed to separate proteins based on a combination of isoelectric focusing (IEF) partitioning and SDS-polyacrylamide gel electrophoresis. In the first dimension of separation (IEF), samples were added to standard rehydration solution containing 8 M Urea, 4% (w/v)

CHAPS (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), 2% (w/v)Dithiothreitol (DDT)(Sigma-Aldrich Inc., Castle Hill, NSW, Australia), 0.2% (v/v) ampholytes pH 3.0 – 10.0 (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), and 0.0002% bromophenol blue. Sample mixtures were loaded on immobilised pH gradient (IPG) strips, Amersham Pharmacia Biotech 7 cm Immobiline™ Drystrip gels pH 3.0 – 10.0 (linear)(GE Healthcare Biosciences, Rydralmere, NSW, Australia) and separated on IPGphor™ Isoelectric Focussing System (GE Healthcare Biosciences, Rydralmere, NSW, Australia) according to manufacturer's instructions. Separation condition as per following:

50 μA per IPG strip; 20°C for both rehydration and IEF

Step 1: Step-n-hold (gradient type), 50 V, 12 hours

Step 2: Step-n-hold (gradient type), 300 V, 1 hour

Step 3: Gradient (gradient type), 1000 V, 30 minutes

Step 4: Gradient (gradient type), 5000 V, 1.5 hour

Step 5: Step-n-hold (gradient type), 5000 V, 30 minutes

Step 6: Step-n-hold (gradient type), 50 V, 12 hours

Following first dimension partitioning, the IPG strips were removed from the holders and equilibrated in a solution containing 50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% (v/v) glycerol, 1% (w/v) DDT, 2% (w/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich Inc., Castle Hill, NSW, Australia) in trace of bromophenol blue for 15 minutes with gentle agitation. Following first equilibration step, the strips were further equilibrated in solution containing 50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% (v/v) glycerol, 1% (w/v) iodoacetamide (Sigma-Aldrich Inc., Castle Hill, NSW, Australia), 2% (w/v) SDS in trace of bromophenol blue for another 15 minutes with gentle agitation. Second dimension partitioning was achieved using Novex® 10% Tris-Glycine Gel 1.0 mm, 2D well (Invitrogen Corp., Mulgrave, VIC, Australia) in Hoefer™ miniVE tank (GE Healthcare Biosciences, Rydralmere, NSW, Australia) according to

manufacturer's instructions. The gels were stained with Coomassie staining for exactly1 minute. The gels were destained with 10% (v/v) acetic acids for approximately 16 – 24 hours for visualisation.

2.24 Silver Staining

Detection of low abundance proteins from SDS-PAGE and 2D-PAGE gels was achieved by silver staining technique using PlusOne™ Silver Staining Kits (GE Healthcare Biosciences, Rydralmere, NSW, Australia). Gels were rinsed twice in miliQ grade water followed by fixation for 30 minutes in solution containing 40% (v/v) ethanol, 10% (v/v) acetic acid. The gels were subsequently sensitised for 30 minutes in solution containing 30% (v/v) ethanol, 6.8% (w/v) sodium acetate (GE Healthcare Biosciences, Rydralmere, NSW, Australia), 0.2% (w/v) sodium thiosulphate (GE Healthcare Biosciences, Rydralmere, NSW, Australia), and 0.125% (v/v) glutaraldehyde (GE Healthcare Biosciences, Rydralmere, NSW, Australia). The gels were rinsed three times with miliQ grade water followed by staining in silver solution containing 0.25% (w/v) silver nitrate (GE Healthcare Biosciences, Rydralmere, NSW, Australia) and 0.015% (v/v) formaldehyde (GE Healthcare Biosciences, Rydralmere, NSW, Australia) for 20 minutes. The gels were rinsed twice with miliQ grade water prior to developing for 3 – 5 minutes in 2.5% (w/v) sodium carbonate (GE Healthcare Biosciences, Rydralmere, NSW, Australia) and 0.0074% (v/v) formaldehyde. The reactions were terminated using 1.5%(w/v) ethylenediaminetetraacetic acid, disodium salt (GE Healthcare Biosciences, Rydralmere, NSW, Australia) and prepared for visualisation.

3 CALMODULIN BINDS AND REGULATES ACE2 ECTODOMAIN SHEDDING

3.1 Introduction

Calmodulin (CaM) is an intracellular calcium (Ca^{2+}) regulatory protein found in eukaryotic cells. It is capable of regulating the biological activities of many cellular proteins and transmembrane ion transporters, and thus their associated physiological processes, mainly in a Ca^{2+}-dependent manner. CaM is a highly conserved protein across many species; with all vertebrates sharing the identical 148 amino acid of its primary sequence. Each CaM molecule contains four EF-hand '*helix-loop-helix*' motifs, each of which can bind one Ca^{2+} ion. The protein has two almost symmetrical domains, separated by an extended α-helix that functions as a flexible 'hinge' region(Figure 3-1A). The property of this flexible hinged region allows CaM to tightly bind to a wide range of different target proteins. When Ca^{2+} binds to CaM, the α-helix becomes rigid, adopting a compact, more stable globular structure (*191*) (Figure 3-1B). When CaM binds to target proteins, the conformation subsequently shifts to form an elongated structure, mediated by the partial unwinding of the α-helix (*192*) (Figure 3-1(C-F)). This change in its spatial properties is an important factor allowing CaM to play a role in binding to target proteins. In the globular form, domains of CaM contain hydrophobic binding pockets, and these may accommodate the indole rings of tryptophan side-chains (*193*), or for instance, CaMI such as W7, trifluoperazine or calmidazolium, that can all inhibit CaM activity by occupying these hydrophobic pockets and/or inhibiting the Ca^{2+}-dependent conformational changes (*194-196*). Hence it is not surprising that one of the key findings on the functional activity of CaM with its target proteins was confirmed by the use of these CaMI.

The unique nature of the CaM structure and the changes that occur when forming complexes with target proteins, provides the basis for specific regulation of CaM-dependent physiological processes.

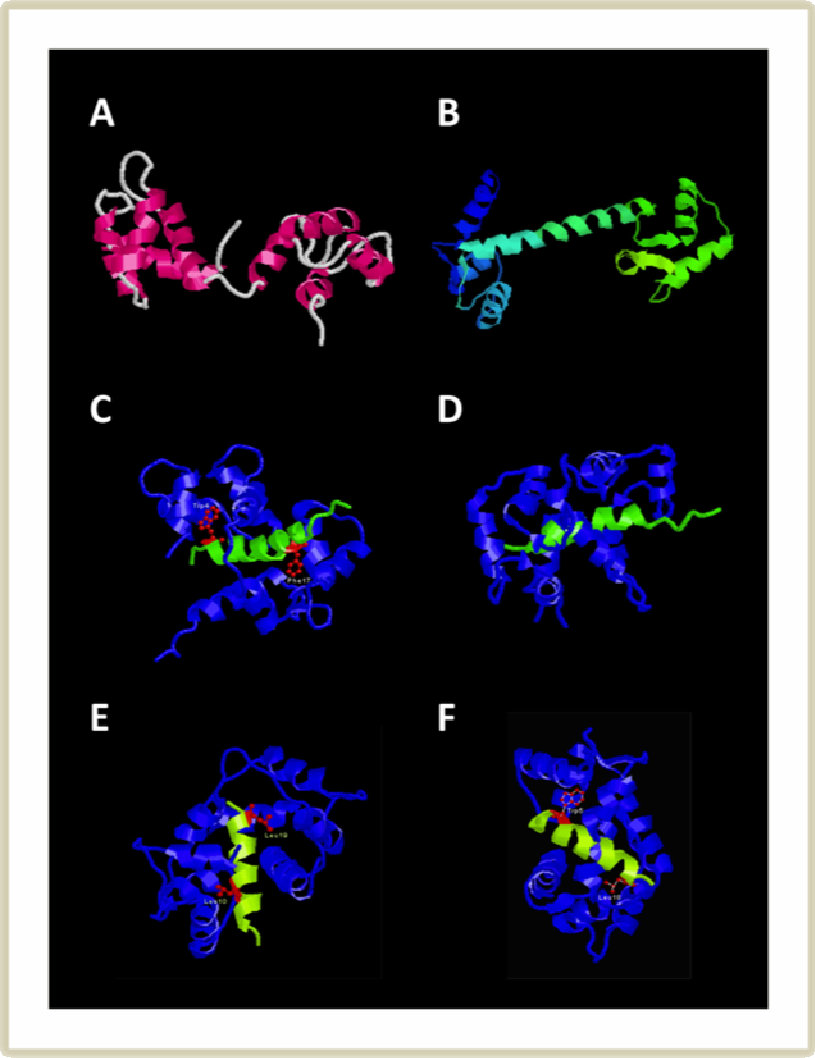


Figure 3-1: Structural conformations of calmodulin. *A*. Ca^{2+}-free CaM (Protein Database ID = 1DMO). *B*. Ca^{2+}-bound CaM, showing the elongated α-helix (Protein Database ID = 3CLN). *C*. Ca^{2+}-bound CaM (blue) binding to M13 target peptide (green) in skeletal muscle myosin light chain (Protein Database ID = 2BBM, 2BBN). *D*. Ca^{2+}-bound CaM binding to rabbit skeletal muscle myosin light chain kinase (Protein Database ID = 2BBM). *E*. Ca^{2+}-bound CaM binding to smooth muscle myosin light chain kinase (Protein Database ID = 1CDL). *F*. Ca^{2+}-bound CaM binding to CaM-dependent kinase II (Protein Database ID = 1CDM, 1CM1). Images were

created with RasMac v2.6 from Calmodulin Target Database.In *C.*, *E.* and *F.*, the Trp, Phe or Ile side-chains involved in hydrophobic interactions with CaM are shown in red.

One of the many roles that have been described for CaM is the regulation of ectodomain shedding of membrane-associated proteins. A previous study from our laboratory group demonstrated that GPVI is able to bind to CaM and this binding can be dissociated by the specific CaMI, W-7 (*25*). In the same study, it was also shown that dissociation of CaM from the platelet collagen receptor, GPVI, results in a time-dependent loss of GPVI from the platelet surface (*25*). Apart from GPVI, the role of CaM as an important regulator of ectodomain shedding was initially demonstrated for the leukocyte adhesion receptor, L-selectin, and has been proposed to play a role in shedding for other platelet receptors such as the glycoprotein that binds von Willebrand factor (GPIb-IX-V), and platelet-endothelial cell adhesion molecule-1 (PECAM-1). The ectodomain shedding of L-selectin mediated by CaM is the first shedding of an adhesion receptor to be characterised in detail. The cleavage-release of this receptor from unactivated leukocytes can be induced by inhibiting the binding of CaM to a membrane proximal amphipathic helix within the cytoplasmic tail of L-selectin (*197, 198*). In these studies, CaM association with L-selectin was demonstrated using two methods; 1) by treating leukocytes with CaMI, and 2) by site-directed mutagenesis of the CaM-binding region of L-selectin. For GPIb-IX-V, CaM was proposed to play role in ectodomain shedding, based on the known role of CaM associations with GPVI (*25*) and L-selectin (*197, 198*). GPIb-IX-V is made up of the GPIbα subunit, linked by disulfide bonds to GPIbβ, generating a non-covalent complex with GPIX and GPV subunits (*199*)(Figure 3-2). Recent studies have reported that CaM plays a role in ADAM17-mediated shedding of GPV, and this shedding can be upregulated by the use CaMI (*200*). Furthermore, GPIbα, and the GPV of the GPIb-IX-V complex themselves can both undergo shedding in aging platelets (*201, 202*). Lastly, studies have suggested that CaM plays a role in the membrane cleavage of PECAM-1. PECAM-1 has a six extracellular immunoglobulin domains, and like

GPVI and L-selectin, it contains a positively-charged, putative CaM-binding motif in the cytoplasmic juxtamembrane region (*203*). Studies have shown that the CaMI trifluoperazine induces proteolysis of PECAM-1 in human platelets (*203*). However, a soluble/shed fragment of this receptor was not detected, suggesting the possibility that the cleaved fragment remains membrane-anchored or the cleavage event is intracellular (*203*). In a completely different study, Naganuma et al have also suggested that the possibility of cleaved soluble ectodomain remained tethered to platelets thus could not be detected in the extracellular matrix (*204*). In addition, two latter studies have successfully detected the elevated level of cleaved soluble PECAM-1 in ischaemic stroke and myocardial infarct patients (*205, 206*).

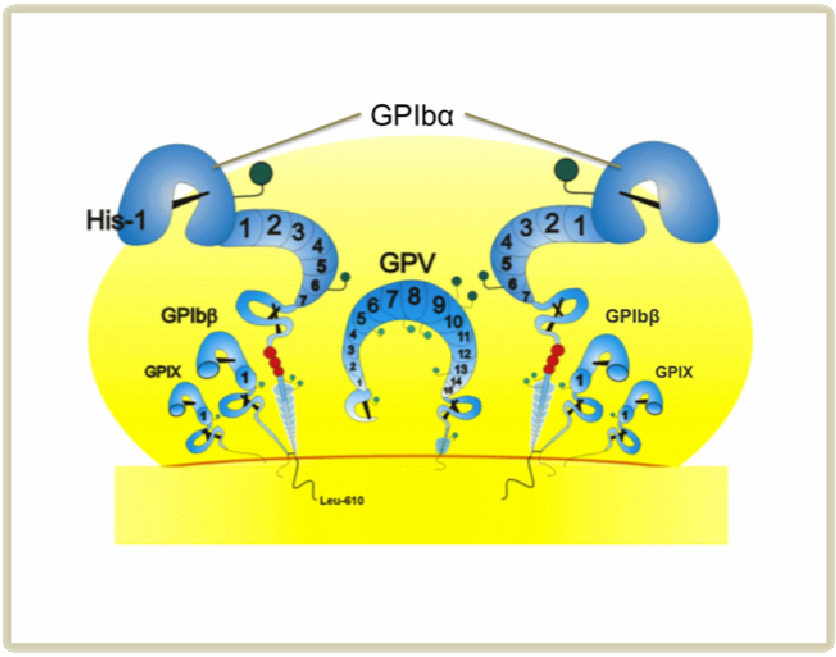


Figure 3-2: Schematic representation of GPIb-IX-V complex and its components (figure courtesy of Dr. Robert K. Andrews, Monash University).

In the context of my research program investigating the possible role of CaM in regulating the ectodomain shedding of ACE2, there has been a prior report describing a role for CaM in the ectodomain shedding of germinal form of ACE (not ACE2) on the cell surface of a stably transfected mouse epithelial cell line (*207*). Based on our understanding of the

putative calmodulin-binding motif, this highly conserved sequence, usually found in many of the CaM target proteins, is absent in the germinal form of ACE (*207*). Nevertheless, CaM binds to amino acids in the juxtamembrane region within the cytoplasmic tail of germinal ACE(*207*). The cytoplasmic region of ACE2 however, does not share homology with either germinal or somatic forms of ACE. Instead, ACE2 shares some sequence similarity with collectrin, as described in *Chapter 1.5.1*. Unlike ACE, ACE2 has a highly positively charged stretch rich in arginine and lysine residues in the juxtamembrane region, immediately after the hydrophobic membrane-spanning domain, resembling the typical CaM-binding sequence motif.

Since the putative CaM-binding motif has functional roles in regulating ectodomain shedding in many of its membrane-associated target proteins, such as in GPVI, L-selectin and the other receptors mentioned above (*25, 193*), the initial aim of my research program was to investigate whether CaM is able to bind to the proposed putative calmodulin-binding sequence within the cytoplasmic juxtamembrane region of ACE2. A subsequent aim was to investigate if CaM binding to this site plays any role in the regulation of ACE2 shedding and surface expression.

3.2 Experimental Procedures *(detailed in chapter 2)*

3.2.1 Cell culture and transfection

CHO-P and Huh-7 cells used were maintained as described in *Chapter 2.1*. Transient expression of a recombinant soluble form of human ACE2 in CHO-K1 cells was achieved using a calcium phosphate transfection protocol outlined in *Chapter 2.6.1*. Transient expression of recombinant full-length human ACE2 in CHO-P cells were achieved using a DEAE-Dextran transfection protocol outlined in *Chapter 2.6.2*. All cells were maintained at 37°C in 5% CO_2.

3.2.2 Plasmid construction

The expression constructs for full-length human recombinant ACE2 containing a C-terminal FLAG sequence, and secreted soluble ACE2 were both engineered as described in *Chapter 2.3.1*. Restriction digests and DNA extraction from gels were performed according to methods described in *Chapter 2.5*. The purification of plasmid constructs was achieved as described in *Chapter 2.6.1(calcium phosphate method for recombinant secreted soluble ACE2)* and *Chapter 2.6.2 (DEAE-Dextran for recombinant full-length human ACE2).* Purified plasmid constructs were sequenced as described in *Chapter 2.4*.

3.2.3 Cell treatment and protein extraction

Huh-7 and CHO-P cells expressing recombinant full-length human ACE2 cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-reduced medium prior to performing experiments. Huh-7 cells were treated with CaMI or PMA *(see Chapter 2.11 and Chapter 2.12; respectively, for detailed protocols)* or vehicle control. Growth medium from CHO-K1 cells expressing the recombinant soluble form of human ACE2 were harvested 72-hours post-transfection. Secreted proteins from the growth medium and proteins expressed on the cell surface were harvested according to methods described in *Chapter 2.7*. Protein concentrations were also determined, as outlined in *Chapter 2.8*.

3.2.4 ACE2 activity assay

The catalytic activity of recombinant full-length and shed forms of human ACE2 was measured using a highly specific quenched fluorogenic substrate, as detailed in *Chapter 2.10.*

Known amounts of protein were analysed by following the hydrolysis of a specific quenched fluorescent substrate monitored over time. Results were analysed against the mock-transfected cells or mock-treated cells as experimental controls, and the significance of data sets were statistically compared to controls using Student's *t* test.

3.2.5 Gel shift assay

Complexes formed between CaM and ACE2 peptide mimetics/mutants were analysed by gel shift assay as described in *Chapter 2.14* using a range of CaM to peptide molar ratios. The mixtures were separated on native polyacrylamide gels prior to fixing and staining with coomassie blue for visualisation (Chapter *2.14*).

3.2.6 *In vitro* binding assay

The ability of CaM to bind full-length recombinant human ACE2 was investigated using a CaM-binding assay, as described in *Chapter 2.15*. ACE2 bound to GST-CaM fusion protein was eluted from a glutathione affinity column for subsequent analysis. Competitive binding studies in the presence of peptide mimetics were performed in parallel experiments to confirm specificity *(see Chapter 2.15)*.

3.2.7 SDS-PAGE and immunoblotting

Samples of concentrated cell culture medium, cell lysates, and protein eluted from affinity resins were separated by SDS-PAGE and proteins electrotransferred to PVDF, as described in *Chapter 2.17*. The membranes were blocked, then incubated with antibodies to human ACE2 ectodomain, GST, or FLAG peptide, and visualised using a HRP-conjugated

secondary antibody and ECL chemiluminescence, according to the methods outlined in *Chapter 2.18*. Purified soluble recombinant human ACE2 was used as a positive control for anti-ACE2 blots.

3.2.8 Circular dichroism

Definitive secondary structures of the peptide mimetics (wild-type human ACE2 and mutant variants) were determined using circular dichroism according to the methods detailed in *Chapter 2.22*. The abilities of the 16-residue peptide mimetics to form a distinct α-helix were investigated under aqueous condition (miliQ grade H_2O), or in the presence of inorganic solvent (50% (v/v) methanol).

3.3 Results

3.3.1 Calmodulin binds to the cytoplasmic tail of ACE2

To initially address whether CaM could bind to the membrane proximal region in the carboxyl-terminal region of ACE2, a series of peptide mimetics were synthesised, corresponding to the 16-amino acid sequence (residues 762-777) of the ACE2 cytoplasmic region. An additional four different peptide variants (mutants 1-4) containing alanine-substitutions of different charged residues (Figure 3-5) were also synthesised. These synthetic peptides were incubated with CaM in the presence of either Ca^{2+} or EGTA, followed by separation on non-reducing native gels. We observed the formation of a CaM-peptide complex as a shift in the migration of the CaM band of the wild-type sequence (dubbed ACE2T2). Maximal shift was observed at synthetic peptide to CaM molar ratio of ~2.5:1, where a single band was observed migrating above the CaM band, corresponding to the Ca^{2+}-dependent CaM-peptide complex. The intensity of this band was gradually increased as the

molar ratio increased to 50:1 (Figure 3-4; *left panel*). This observation strongly suggests that the affinity of the interaction is within the range that has been previously reported for other calmodulin-binding peptides (*187-190*). The CaM-peptide interaction was Ca^{2+}-dependent, since the band shift was not observed in the presence of EGTA (Figure 3-4; *right* panel). Together, these data suggest that the cytoplasmic tail of ACE2 could potentially bind to CaM, implicating a possible role for CaM in ACE2 ectodomain shedding.

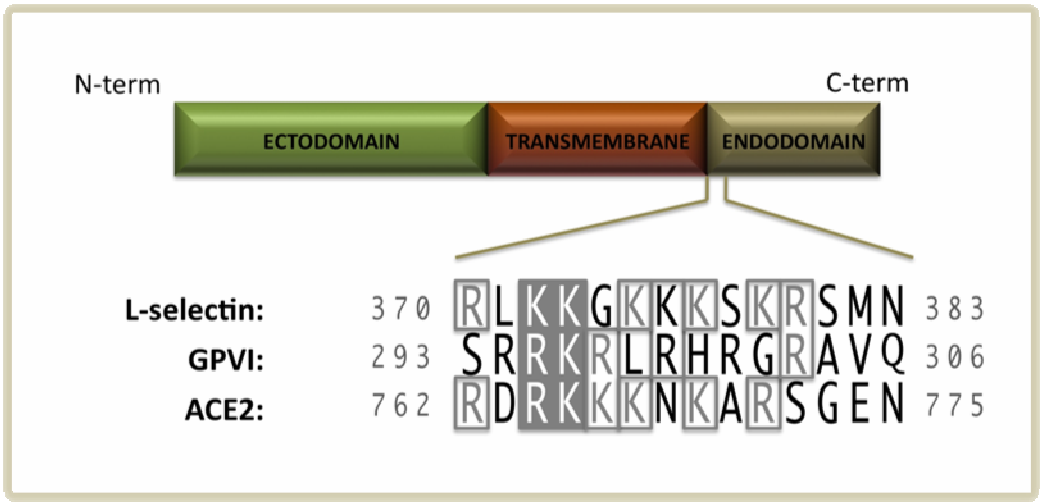


Figure 3-3:Sequence comparison of ACE2 to GPVI and L-selectin. Cytoplasmic juxtamembrane sequence of human ACE2 compared to functional CaM-binding juxtamembrane sequences that regulate metalloproteinase mediated ectodomain shedding of platelet GPVI and leukocyte L-selectin. Shown are alignments of residues in the cytoplasmic tails of ACE2, GPVI and L-selectin. Identical or conserved residues are *highlighted*.

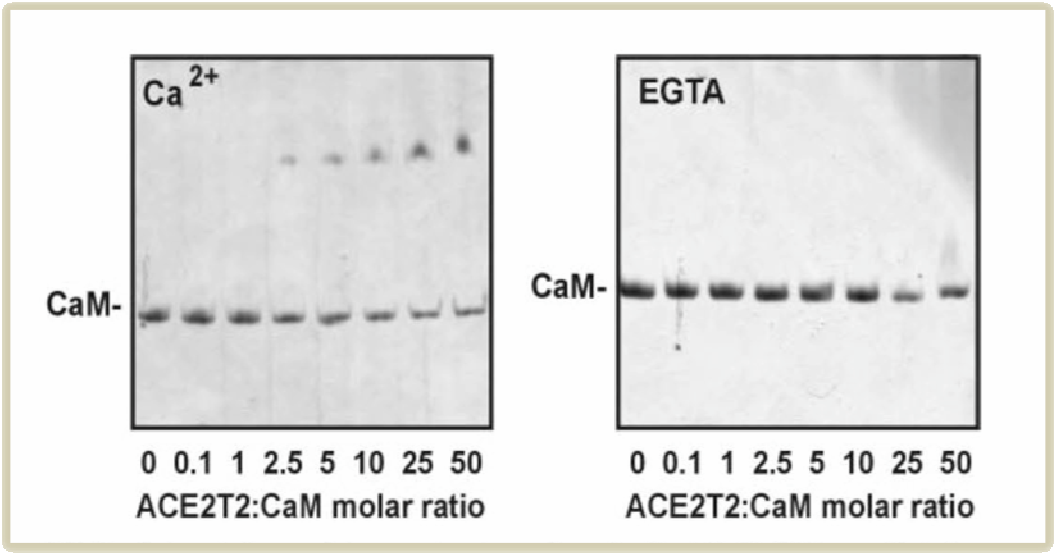


Figure 3-4:Calmodulin-peptide complex formation. Purified CaM (300 pmol/tube) was incubated with increasing amounts (0; 30; 300; 750; 1,500; 3,000; and 15,000 pmol) of peptide ACE2T2 in the presence of 4 M urea and either Ca^{2+} or EGTA for 30 min at room temperature. Complexes were run on gels containing 4 M urea and either Ca^{2+} or EGTA (to maintain the same conditions 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 Ca^{2+}*(left panel)*. No CaM shift was observed in the presence of EGTA at any concentration of the added peptide *(right panel)*.

3.3.2 Calmodulin binding to the cytoplasmic tail of ACE2 is sequence specific

To investigate the binding of CaM to the cytoplasmic region of ACE2 in more detail, a series of peptide mimetics were further synthesised to characterise the nature of this binding. The putative binding sequences recognised by CaM in target proteins are very often basic amphipathic helices, with a hydrophobic face and a positively charged face, or related to the 'IQ' consensus sequence found initially in L-type ion channels (*187, 188, 190, 208-214*). Hence our initial concept for the design of these 'mutant' peptides was based on neutralising the positively-charged face using a variety of alanine substitutions at different key residues,

as illustrated in Figure 3-5 and Figure 3-6. By utilising circular dichroism based techniques, we looked at the formation/ablation of this possible helical wheel. However, our analysis showed no significant secondary structure was formed in the wild-type sequence ACE2T2, nor in any of the peptide variants (mutants 1-4) (Figure 3-7). In addition to measurements under aqueous condition (miliQ grade H_2O), the peptides were also measured in organic solvents (50% (v/v) methanol), a condition known to have the advantage of inducing structure formation of proteins (beta-sheets and alpha helices), which may not form under aqueous conditions. However, this approach also failed to show any secondary structure formation in these peptides. Gel shift assay experimentation was repeated using mutant peptides, but no shifts in CaM bands were detected over a range of CaM:peptide molar ratios (0.1:1 – 50:1) in the presence of either Ca^{2+} or EGTA (Figure 3-8). These data suggest that CaM binding to cytoplasmic tail of ACE2 is sequence specific but may not require the presence of an amphipathic helical structure in the uncomplexed peptide.

Figure 3-5: Sequence comparison of ACE2T2 to mutant (1-4). Synthetic peptides corresponding to residues 762-777 of human ACE2 (ACE2T2) and peptide variants (mut 1-4) containing alanine substituted amino acid residues.

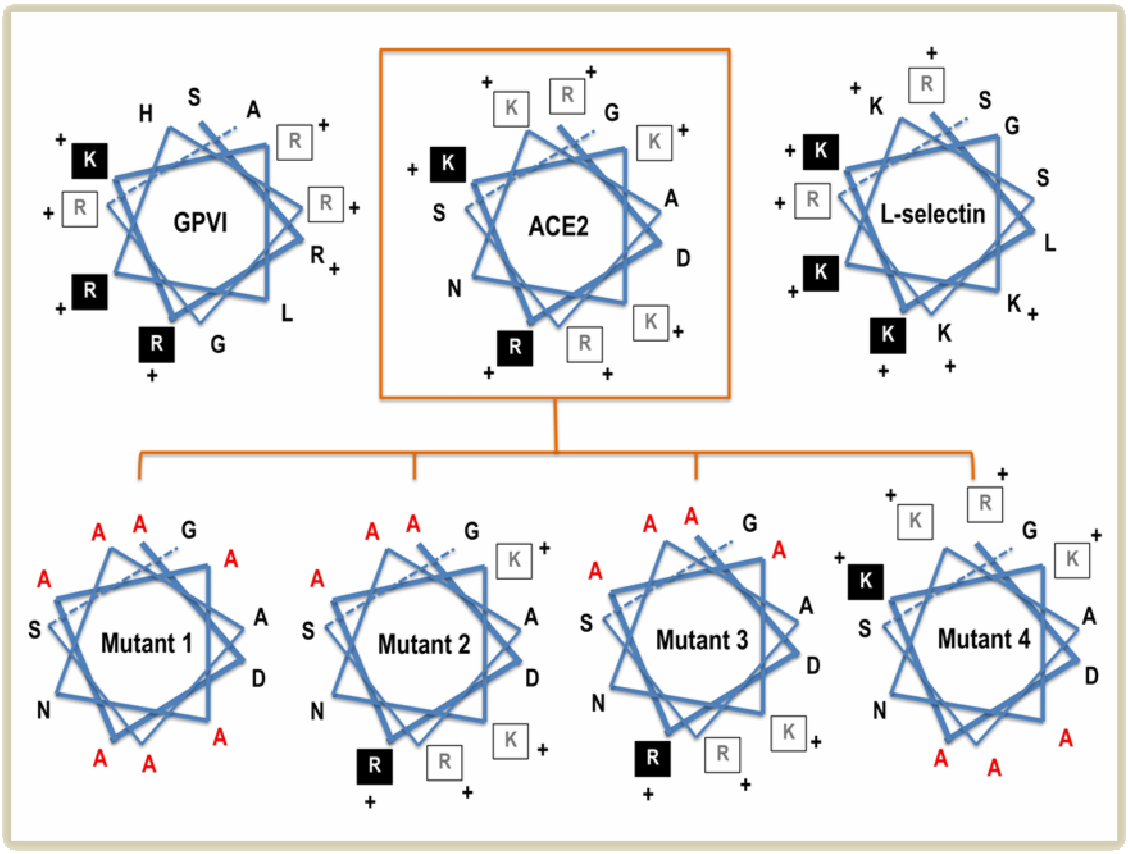


Figure 3-6:A scheme illustrating predicted alpha-helical wheels of the putative motif for calmodulin-binding to membrane-bound proteins. *Top panel*, Sequence similarity of charged arginine and lysine residues in GPVI, ACE2 and L-selectin. *Bottom panel*, Peptide mimetics consist of alanine substitution of the charged residues.

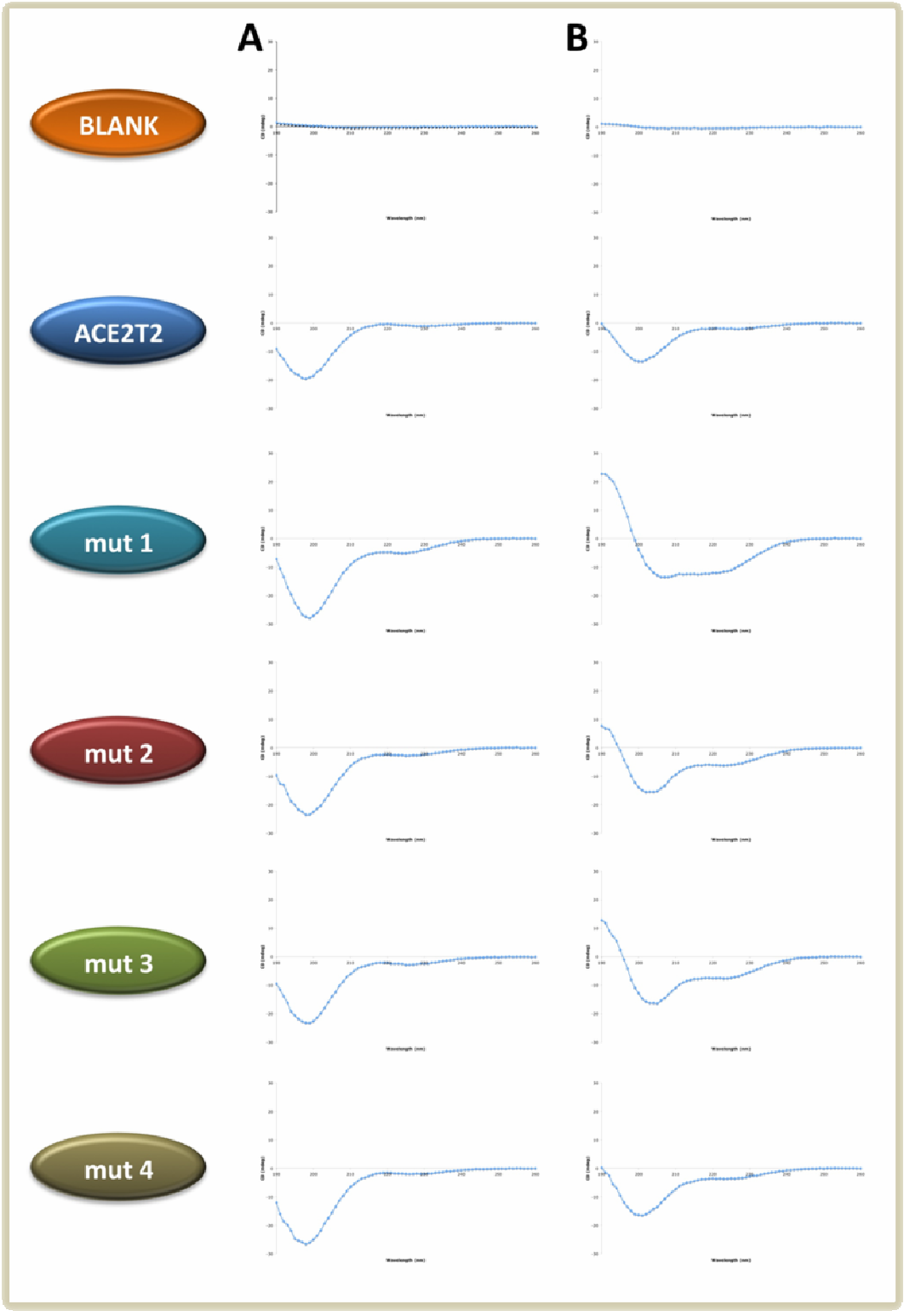


Figure 3-7: Circular dichroism spectral analyses of peptide mimetics corresponding to ACE2 residues 762-777. Peptide mimetics (200 μM) were measured in *A.* MiliQ-grade water, and *B.* 50% (v/v) methanol.Acquired spectra were smoothed using the Jasco Fast Fourier (Jasco Inc., Tokyo, Japan) transform algorithm and then baseline corrected. No secondary structure

formation was observed in both ACET2 and mutant peptides under both conditions (with and without calcium).

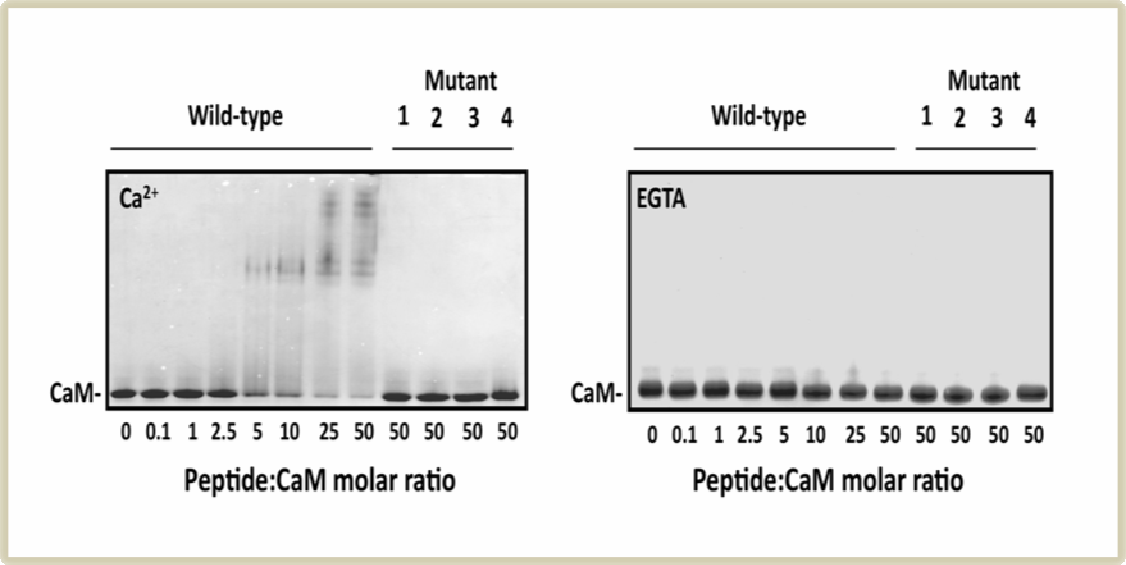


Figure 3-8:Calmodulin-peptide complex formation in mutant peptides. Purified CaM (300 pmol/tube) was incubated with 15,000 pmol of peptides consisting of scrambled sequences (mutant 1-4) in the presence of 4 M urea and either Ca^{2+} or EGTA. Complexes were run on gels containing 4 M urea and either Ca^{2+} or EGTA, reflecting the same condition as in the samples. The gels were stained with Coomassie blue and binding observed as a shift in the migration of CaM in the presence of calcium (Ca^{2+}) *(left panel)*, and EGTA *(right panel)*.No CaM shift was observed in the mutant peptides at 15,000 pmol concentration (50:1 peptide to CaM molar ratio).

3.3.3 Calmodulin binds to full-length human ACE2

After successfully demonstrating that CaM is able to bind to a synthetic peptide corresponding to a stretch of amino acids (residues 762-777) in the cytoplasmic tail region of human ACE2, the next step was to examine the ability of CaM to bind full-length human ACE2. Either purified recombinant GST-CaM fusion protein or GST alone (as a negative control)

were incubated with cell lysates prepared from CHO-P cells that were transiently expressing FLAG-tagged full-length ACE2. After affinity pull-down using glutathione-Sepharose beads, the samples were separated by SDS-PAGE and analysed by western blotting with anti-ACE2 antibody. From the western blot analysis, we observed that ACE2 was present in crude cell lysate, migrating as an ~120-kDa band and the purified soluble form of ACE2, was used as positive blotting control (migrating as an ~110-kDa band) (Figure 3-9 *left panel*). A band corresponding to ACE2 was detected in the GST-CaM pull-down. This indicates that 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 PVDF membranes were also re-probed with an anti-GST antibody to confirm that equivalent bait proteins were present in the appropriate lanes and running at the appropriate molecular mass (Figure 3-9 *right panel*). The binding of CaM to recombinant full-length human ACE2 was confirmed in separated experiments, where ACE2 pull-down by GST-CaM, in this case analysed using the ACE2 activity (QFS) assay, was significantly inhibited in the presence of a peptide mimetic corresponding to residues 762-777 of human ACE2, whereas a scrambled sequence of this peptide was unable to displace recombinant ACE2 (Figure 3-10). Pull-downs with GST alone was also used as a negative control in these experiments.

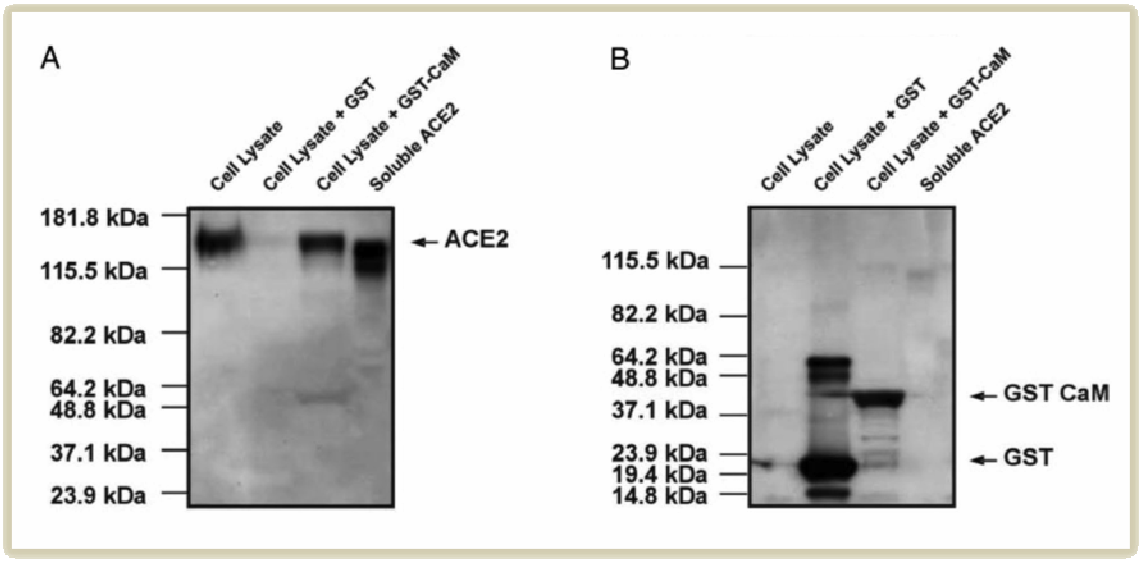


Figure 3-9:Binding of ACE2 from cell lysates by GST-CaM. Cell lysates (~2 mg protein) from full-length ACE2-transfected CHO-P cells were incubated with glutathione Sepharose affinity beads and GST (20 µg), or GST-CaM (20 µg) bait fusion protein for 16-hours. After incubation, samples were separated by SDS-PAGE and immunoblotted for ACE2 *(A)*, and GST *(B)* with appropriate corresponding antibodies. Cell lysates (transfected with full-length ACE2) and soluble forms of ACE2 were used as controls for western immunoblotting.

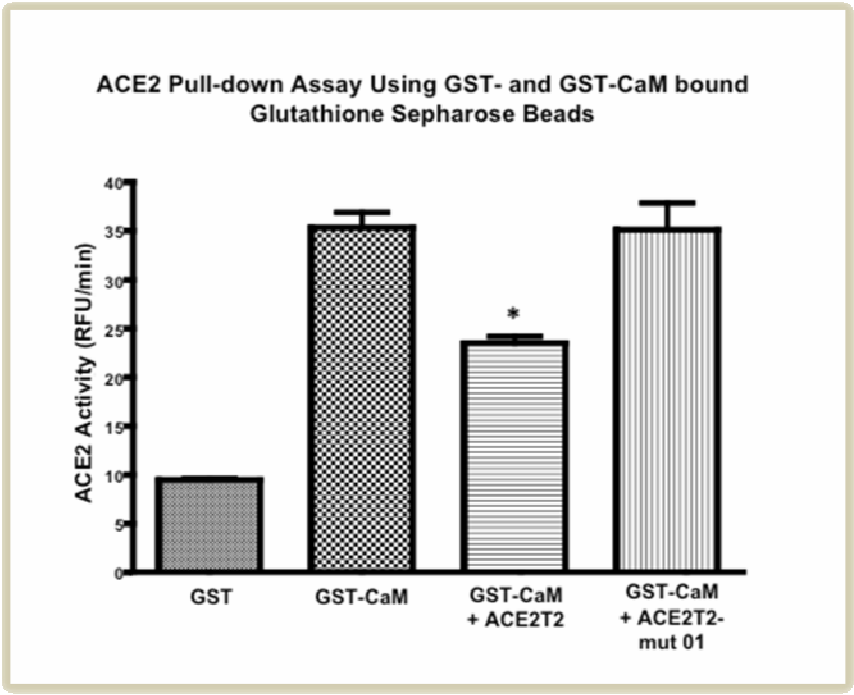


Figure 3-10: Cell lysates (~2 mg protein) from ACE2-transfected CHOP cells were incubated with GST (20 µg), or GST-CaM (20 µg) bait in the presence of the peptide ACE2T2 (corresponding to residues 762-777 in CaM) (20 µg) or equal amount of the scrambled sequence of ACE2T2 peptide for 16-hours. Following incubation, the samples were subject to pull down using 100 µl of 50% slurry of glutathione Sepharose affinity beads. The slurry mixtures were washed three times and sediment assayed for their abilities to cleave an ACE2 quenched fluorogenic substrate. The data are compared against the all treatments from at least three independent experiments (n ≥ 3, Student's *t* test; unpaired, two-tailed). *Asterisk* (*) denotes significant difference ($P< 0.05$)compared to against GST-CaM control.

3.3.4 Calmodulin inhibitors stimulate ACE2 ectodomain shedding

The demonstration that CaM is able to bind ACE2 from the gel shift and GST-CaM pull-down assays, raises the question of whether CaM could potentially regulate the ectodomain shedding and surface expression of ACE2. To address this possibility, Huh-7 cells, a cell line we have shown to have a significant level of ACE2 expression (*46*), were treated with the

specific CaMI, trifluoperazine and calmidazolium, to disrupt the CaM-ACE2 association. Analysis of the concentrated medium from cells treated with CaMI showed a significant increase in secreted/shed ACE2 activity compared to control medium (Figure 3-11). In cells that were treated with trifluoperazine (25 μM), a 44.1 ± 10.8% increase (P< 0.05 student's t tests; unpaired, two tailed) in ACE2 activity was observed after 16-hours (Figure 3-11 *A*). Subsequent experiments using a more potent CaMI, calmidazolium (25 μM), in the presence of PMA and Bisindolylmaleimide I, also showed a significant increase in ACE2 activity (51.1 ± 7.4%) in the medium compared with control after 1-hour treatment (P< 0.05, one-way ANOVA) (Figure 3-12). As demonstrated previously (*46*), PMA treatment is able to increase ACE2 activity in the medium (92.3 ± 5.3%). The increase in ACE2 activity in the medium after CaM inhibitor treatments suggests that CaM plays a role in the cleavage-release of the ACE2 ectodomain. Cell lysates from the trifluoperazine treatment showed minimal changes in ACE2 activity, reflecting that only a very small amount (possibly less than 5%) of ACE2 is shed from the cell surface under these conditions.

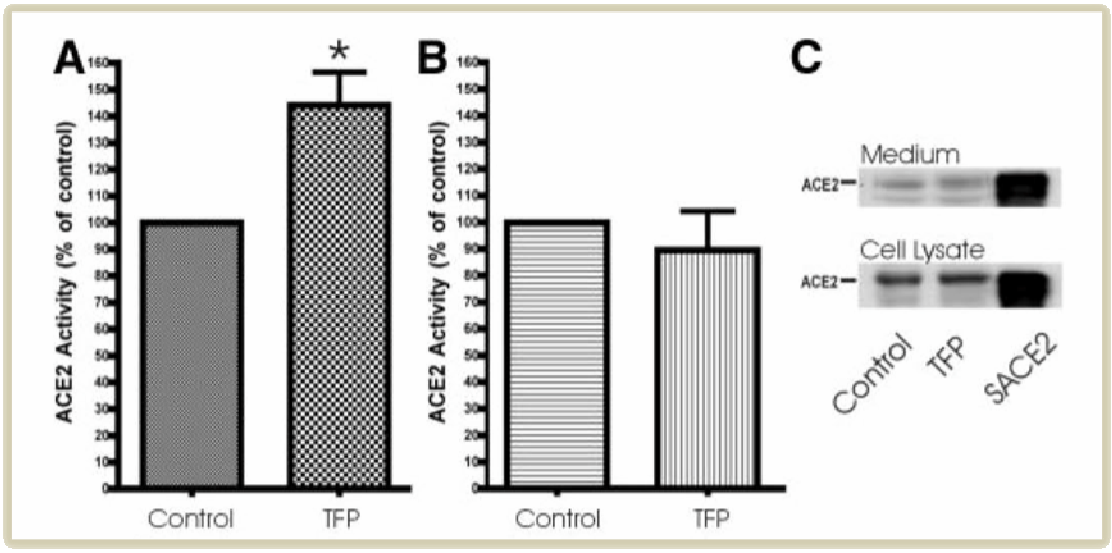


Figure 3-11:Trifluoperazine stimulated shedding of ACE2. Huh-7 cells that endogenously express ACE2 were incubated in serum-reduced media Opti-MEM containing 25 μM TFP, or in an equal volume of carrier (Me_2SO) for 16-hours. The medium was subsequently harvested

and concentrated 30-50 fold, while the cells were pelleted and detergent solubilised, as described in *Chapter 2.11.* Medium (25 µg protein) (*A*), and cell lysates (25 µM protein) (*B*) were assayed for their ability to cleave an ACE2 specific QFS. The data are normalised against the controls from at least four independent experiments (n ≥ 4, Student's *t* test; unpaired, two-tailed). Asterisk denotes significant different ($P < 0.05$) compared against control. *C*, 100 µg total protein from the concentrated medium and cell lysates were separated by SDS-PAGE and western blotted with a monoclonal anti-ACE2 antibody. A soluble ectodomain form of ACE2 (sACE2) served as positive control for immunoblotting.

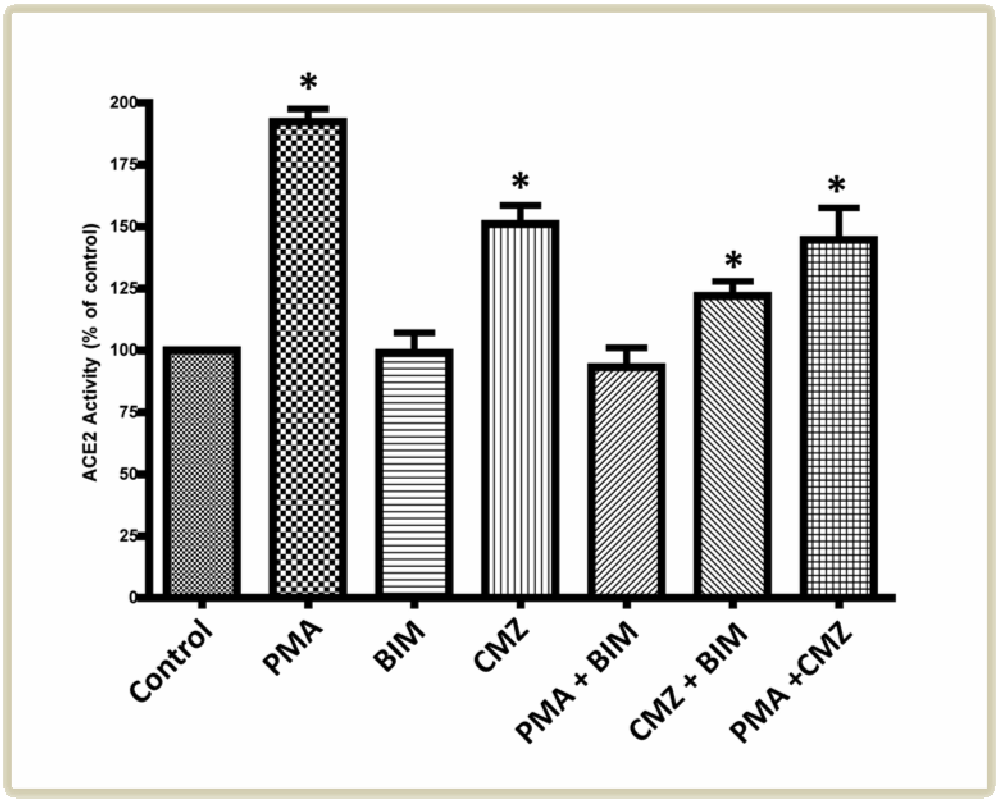


Figure 3-12:Huh-7 cells were incubated in Opti-MEM containing combinations of 25 µM CMZ, 2 µM BIM or 1 µM PMA, and equal volume of Me_2SO for 1-hour. The harvested medium was prepared and analysed as described under *Chapter 2.11.* The mean-control for ACE2 activity after calmidazolium chloride treatment in the medium was calculated. The data are normalised against the controls from at least four independent experiments (n ≥ 4, one-way ANOVA). Asterisks denote significant differences ($P < 0.05$) compared to control.

3.3.5 Sensitivity of the ACE2 quenched fluorescent assay

To determine whether subtle changes in ACE2 activity could be reliably detected, the sensitivity of the QFS assay was determined in both the concentrated medium and cell lysates, harvested from CHO-P cells transiently expressing full-length ACE2. In this experiment, it was confirmed that significant changes in ACE2 activity in the concentrated medium could be detected following PMA-stimulation. A minimal change (~5% in the total protein used) in the concentrated media is sufficed to demonstrate significant change in overall ACE2 activity (Figure 3-13 *top panel*). In contrast, a greater change (>15% in the total protein used) in the concentrated media of non-stimulated cells is required to detect any significant differences (Figure 3-13 *top panel*). This assay however, is inaccurate when used to compare the ACE2 shedding activity in the cell lysates, since no significant differences were observed in the ACE2 activity even with >15% of total protein used in the assay (Figure 3-13 *bottom panel*).

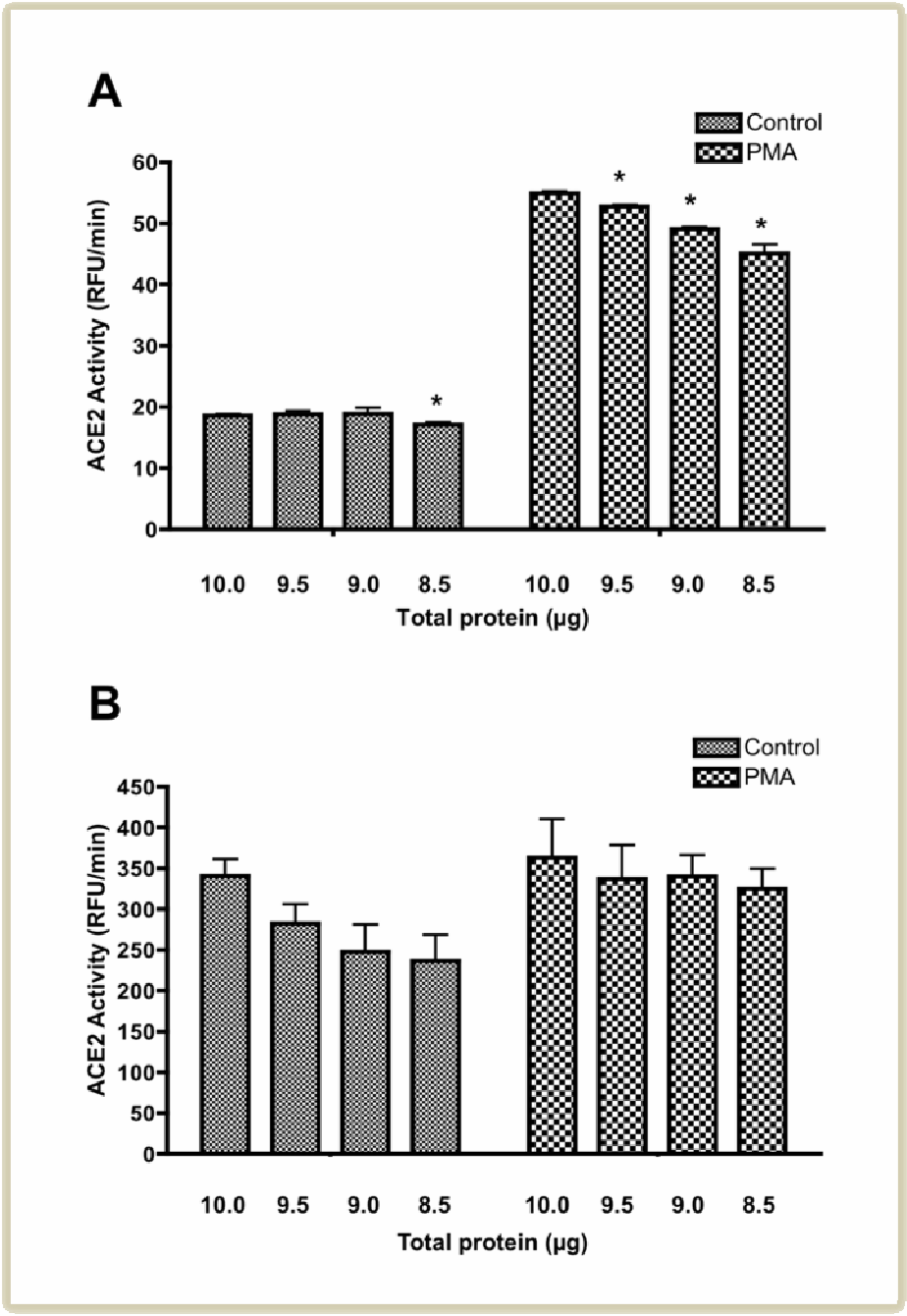


Figure 3-13:ACE2 transfected CHO-P cells were stimulated using 1 µM PMA, or in an equal volume of carrier (Me_2SO) for 1-hour. The medium was subsequently harvested and concentrated 30-50 folds, while the cells were pelleted and detergent solubilised. Varying amounts of total protein (8.5 µg,9.0 µg,9.5 µg, or 10.0 µg) from concentrated medium (*A*), and cell lysates (*B*) were assayed for their ability to cleave an ACE2 quenched fluorogenic substrate. The data are normalised against individual controls from three independent

experiments (n=3, Student's *t* test, unpaired, two-tailed). Asterisks denote significant differences ($P< 0.05$) compared to all data under the same category.

3.4 Discussion

The recent discoveries that CaM was involved in regulating the phosphorylation of ACE (*207*), and that both ACE and ACE2 (*207, 215*) underwent ectodomain shedding, led us to look into the mechanism of how CaM regulates ACE2 ectodomain shedding. Nevertheless, the CaM-binding site identified in the cytoplasmic region in ACE2 is structurally very different from the CaM-binding motif identified for ACE; thus, the mechanism by which calmodulin regulates ACE2 shedding might also be different. This is not surprising, given that the homology of between ACE and ACE2 resides primarily in the extracellular domain (*163*). Other than ACE, CaM is known to bind to a number of other membrane-bound proteins that are shed from the cell surface. Previous studies showed that CaM binds to the membrane-proximal cytoplasmic sequence of platelet GPVI (*25*). Similarly, CaM was shown to bind to the cytoplasmic domain of leukocyte L-selectin (*197*). The juxtamembrane cytoplasmic sequence of ACE2 is homologous to membrane-proximal sequences of GPVI and L-selectin (Figure 3-3), both previously shown to bind CaM (*190, 197*). This sequence similarity suggests, and results here confirmed, that CaM is able to bind to the proposed CaM-binding region of ACE2 (*215*). Study of the interaction between CaM and a synthetic peptide analogue of the putative CaM-binding site in ACE2 demonstrated that these proteins associate at a 2.5:1 molar ratio, within the range of values reported for other CaM-binding peptides in gel shift assays (*187-190*). One of the common characteristics for CaM-binding motifs in other proteins is a conserved region of positively charged amino acids, predicted to form amphipathic alpha helices (*210, 216*). Hence the design of the peptide variants illustrated in Figures 3-5 and 3-6 was based on the hypothesis that the charged residues in the wild-type sequence will form an amphipathic

alpha helical structure. However, none of the peptides, including the wild-type sequence, successfully adopted any defined secondary conformations when analysed by circular dichroism (Figure 3-7). These data indicate that the binding of CaM to the peptides is based on the amino acid sequence rather than the structural characteristic of the region in the unbound peptide. Although beyond the scope of the present work, it would be interesting to determine the structure of the peptide in complex with CaM.

CaM plays a pivotal role in the regulation of signal transduction for many biological processes, including regulating the function of integral membrane proteins (*217, 218*). The presence of a putative CaM binding site in ACE2 may thus indicate a functional role for CaM in 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 of Huh-7 cells endogenously expressing ACE2, following incubation 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 (Trk A) showed that the ectodomain of this integral membrane protein can be shed by both PMA and CaMI (*184*). PMA activates PKC signalling pathway, leading to increased cleavage-secretion of many membrane-bound proteins (*219*), including ACE2. In contrast, CaMI-induced ectodomain shedding of TrK A was reported to be independent of PKC activity, indicating that CaMI and PMA mediate cleavage-secretion by two distinct signaling pathways (*184*). CaMI-induced shedding was also observed for other cell surface molecules such as the adhesion molecule CD44 and the membrane-anchored precursor for TGF-α (pro-TGF-α) (*66, 220*). Two different sheddases, ADAM10 and ADAM17, have been reported to cause ectodomain shedding of CD44 by CaMI and PMA, respectively (*66, 220*), whereas CaMI and PMA were shown to mediate pro-TGF-α shedding through two distinctive signal transduction mechanisms (*66, 220*). Our laboratory previously reported that the ectodomain shedding of ACE2 can be stimulated by PMA, which could be inhibited using

either an ADAM17-selective inhibitor, TAPI-1 or small interfering RNA technology (*46*). These results suggest that ADAM17 is involved in the regulated, but not constitutive shedding of ACE2 (*46*). 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 demonstrate that CaMI-induced shedding were unaffected by the inhibition of PKC pathway.

A few studies have reported 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 (*221, 222*), is reduced after liver injury (*223*). Wang and Abdel-Rahman (*223*), were able to demonstrate that, although the level of CaM remained unchanged in the diseased liver, the binding of CaM to eNOS significantly decreased. Similarly in one of the studies by our laboratory, it was reported that ACE2 expression is significantly up regulated in human and rat liver after chronic liver injury (*224*). 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 shedding. In diseased tissues such as of the heart and liver, an increase in secreted ACE2 could counteract the effect of increased AngII production caused by other overexpressed components in the renin-angiotensin system (*225, 226*).

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 tightly 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.

4 CLEAVAGE SITE IDENTIFICATION OF ACE2 SHEDDING MEDIATED BY ADAM17

4.1 Introduction

ACE2 is a membrane type I integral protein, with a large extracellular catalytic domain, a single membrane spanning helix and a short carboxy-terminal cytoplasmic domain. As reviewed in earlier chapters, many membrane-bound protein families can undergo ectodomain shedding, including the ACE2 homologue ACE, releasing their biologically active ectodomain from the cell surface to the extracellular milieu. As ACE2 was only discovered relatively recently (compared to ACE), studies thus far have been largely focused on deducing its physiological functions, as well its relevance in diseases. However, a handful of reports have looked into the ectodomain shedding event of this enzyme, none of which have offered much insight into the precise regulation and mechanisms involved in this shedding event. Examining other membrane-bound protein families that can undergo ectodomain shedding, it would appear that many of these proteolytic events are mediated by zinc-dependant metalloproteases, including ADAMs and MMPs *(reviewed under Chapter 1.2.1 and 1.2.2)*.

To investigate this post-translational proteolysis of ACE2, there is a need to understand the regulation of its mammalian homologue, ACE. The study of ectodomain shedding in ACE began more than a decade ago, before the discovery of ACE2. Some of these earlier findings include the report of the ACE sheddase being a zinc-dependant metalloprotease that shares similar properties to, but is distinct from ADAM17 (*155, 159, 161*), and that the glycosylation in the juxtramembrane stalk region of ACE plays a critical

role, however, deletion of these sites do not inhibit ectodomain shedding (*227*). Like ACE2, ACE is also a type I integral membrane-bound protein. This enzyme is well characterised for its key role in blood pressure homeostasis, and inhibitors for this zinc-metalloprotease are used routinely in the treatment of hypertension (*228, 229*). The cleavage site of somatic ACE by its sheddase has been identified as the Arg^{1203}-Ser^{1204} bond (human somatic ACE numbering), 24 residues upstream on the extracellular side of the membrane-spanning domain (*153*). 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), also 24 residues proximal to the transmembrane domain (*156, 230*). Like most of the membrane-bound proteins that can undergo ectodomain shedding, the precise requirements for the sheddase recognition of the ACE ectodomain, are still not known. For example, TGF-beta receptor, betaglycan does not usually undergo ectodomain shedding. However, when 14 extracellular amino acids proximal to transmembrane domain were replaced with the juxtamembrane stalk sequence from TGF-α and the Aβ precursor protein,the rendered beta-glycan receptor were to be cleaved, thus suggesting that the juxtamembrane domain may be the determinant for ectodomain shedding (*231*). In contrast, a chimeric protein containing ACE ectodomain and juxtamembrane region, with a transmembrane and cytoplasmic domains of CD4, which is not subject to cleavage sectretion, was effectively cleaved off the cell surface; whereas a chimera containing the ectodomain and the juxtamembrane domains of CD4, and transmembrane and cytosolic domains of ACE was not cleaved, implying that the distal ectodomain is the primary determinant for shedding (*159*). Additionally, there have been a considerable number of studies aimed at elucidating the absolute structural requirements for the ectodomain shedding event in ACE.Studies utilisingvarious ACE2 mutant constructs; such as point mutation mutants, domain substituted mutants, and mutants containing deleted domains have showed that all these mutants were still able undergo ectodomain shedding (*232-237*). In terms of how ACE interacts with its sheddase, two studies have proposed the existence of a 'recognition motif' in the ectodomain of ACE (*157, 158*). Specifically, it is has

been previously shown that the ectodomain shedding of somatic ACE is less compared to testicular ACE (*153*). Somatic ACE consists of two homologous, N- and C- domains, whereas testis ACE consists of a single C domain. It has been shown that the N- and C- domains are flexible, and both domains come together in close proximity for the hydrolysis of its substrates (*158*). 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 (*158*). Subsequent studies showed that this recognition motif is localised to a region within the C- domain of testicular ACE, within amino acid residues 191-214 (human testis ACE numbering) (*238*).

Our current understanding of ACE2 ectodomain shedding however is very limited compared to 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 PKC-signaling pathway (*46*). In this study, a series of protease inhibitors in conjunction with siRNA knockdown were used to show that ADAM17 clearly plays a role in the phorbol ester stimulated shedding (*46*). Interestingly, we were unable to completely block the stimulated the shedding (*46*). Furthermore, although the ADAM17 preferring inhibitor TAPI-1 significantly reduced 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 plays a significant role in the constitutive release of ACE2 (*46*). 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 localised between amino acid 716 and 741 (human ACE2 numbering), residing within the juxtamembrane region (*239*). Furthermore, this study also showed using several mutant and chimeric ACE2 proteins, that the juxtamembrane, transmembrane and cytoplasmic domains

of ACE2 are not required for constitutive shedding (*239*). 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 (*240*). To date, there has only been one report detailing a specific 'recognition motif' for ACE2 ectodomain shedding; a leucine residue at position 584 in the ectodomain, 157 amino acids proximal to the transmembrane domain (*239*). Site-directed mutagenesis of this residue, replacing the aliphatic residue to an alanine has demonstrated a significant attenuation of shedding (*239*). Taken together, these data demonstrated 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 to the membrane-spanning helix. Although our current understanding of ACE2 ectodomain shedding is somewhat limited, there is a growing interest for researchers looking at this proteolytic shedding event for this particular enzyme. This is likely a consequence of ACE2, having been identified as the functional receptor for SARS-CoV following the near pandemic SARS outbreak in 2003 (*182*). Subsequent studies have now focused on examining the shedding of ACE2 as a potential target for controlling the infectivity of the virus (*241*). Furthermore, studies in both rats and humans have demonstrated that ACE2 undergoes an increased level of proteolytic cleavage to release its active soluble form as a result of myocardial infarction (*3*). Therefore, it is clear that the characterisation/measurement of the ectodomain ACE2 may have diagnostic and/or prognostic potential in cardiovascular disease(s).

Despite the high 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 recognisable homology. Indeed, although the consensus sequence Ser-Ala-Arg-Ser-Glu-Gly has been identified as the ACE cleavage site (*207*), 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, nor the proteases that mediate the shedding (as yet unidentified for ACE; ADAM17 as one of the prime mediator for ACE2 shedding). Hence our immediate aim is to investigate and thus identify the precise ADAM17 cleavage site in ACE2.

4.2 Experimental Procedures *(detailed in chapter 2)*

4.2.1 Cell culture and transfection

Huh-7, CHO-P and CHO-K1 cells used were maintained as described in *Chapter 2.1*. Transient expression of the recombinant soluble form of human ACE2 in CHO-K1 cells was achieved by the calcium phosphate transfection protocol outlined in *Chapter 2.6.1*. Transient expression of recombinant full-length human ACE2 in CHO-P and CHO-K1 cells were achieved by DEAE-Dextran transfection protocol outlined in *Chapter 2.6.2*. Stable expression of ACE2 mutant proteins in CHO-K1 cells were achieved by Lipofectamine™ 2000 transfection protocol outlined in *Chapter 2.6.3*. All cells were maintained at 37°C in 5% CO_2.

4.2.2 Plasmid construction

The expression constructs for full-length human recombinant ACE2 containing a C-terminal FLAG sequence, and secreted soluble ACE2 were both engineered as described in *Chapter 2.3.1*. Restriction digest and DNA extraction from gels methods were performed according to methods described in *Chapter 2.5*. The purification of plasmid constructs were achieved as described in *Chapter 2.6.1(calcium phosphate method for recombinant secreted soluble ACE2)* and *Chapter 2.6.2 (DEAE-Dextran for recombinant full-length human ACE2).*The expression constructs for ACE2 mutants were achieved using site-directed mutagenesis

protocols described in *Chapter 2.3.2*. Purified plasmid constructs were sequenced as described in *Chapter 2.4*.

4.2.3 Cell treatment and protein extraction

CHO-K1 cells expressing ACE2 mutants, CHO-P cells expressing recombinant full-length human ACE2, and Huh-7 cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-reduced medium prior to experimentation. CHO-K1 cells were treated with PMA, BIM and TAPI-1 *(see Chapter 2.11 and Chapter 2.12; respectively, for detailed protocols)* or vehicle control. Growth medium from CHO-K1 cells expressing recombinant soluble form human ACE2 were harvested following 72-hours post-transfection. Secreted proteins from the growth medium and proteins expressed on the cells' surface were harvested according to methods described in *Chapter 2.7*. Protein concentrations were also determined, as detailed in *Chapter 2.8*.

4.2.4 ACE2 activity assay

The catalytic activity of recombinant full-length and shed form of human ACE2 was measured using a highly specific fluorogenic substrate, as detailed in *Chapter 2.10.* Known amounts of protein were analysed by following the hydrolysis of a specific quenched fluorescent substrate monitored over time. Results were analysed against the appropriate controls and the significance of data sets were determined on normalised data using one-way ANOVA statistical analysis.

4.2.5 One- and two dimensional gel electrophoresis and immunoblotting

Concentrated media samples, harvested cell lysates, and extracted protein bound to affinity resins were separated by one- and two- dimensional gel electrophoresis and proteins electroblotted to PVDF, as described in *Chapter 2.17, Chapter 2.18* and *Chapter 2.23,* respectively. The membranes were incubated with antibodies to human ACE2 ectodomain, GST, or FLAG peptide, according to the methods outlined in *Chapter 2.18*. Bands (staining intensity and relative retention) were compared to soluble recombinant human ACE2, which served as a positive control. Silver staining on gels was performed according to methods described in *Chapter 2.24.*

4.2.6 *In vitro* cleavage assay

The ability of ADAM17 to cleave synthetic peptides corresponding to wild type and mutant juxtamembrane region of ACE2 ectodomain full-length recombinant human ACE2 was investigated using an *in vitro* cleavage assay, as described in *Chapter 2.16*. Cleavage products were analysed using MALDI-TOF-TOF mass spectrometry as outlined in *Chapter 2.21.1*.

4.2.7 Peptide mass fingerprinting analysis

Identification of proteins using proteomics technologies are achieved by first, one-or two-dimensional gel electrophoresis followed by gel staining. Stained gel bands or spots were excised and digested with trypsin according to procedures outlined in *Chapter 2.20.* Resulting peptide fragments were analysed by MALDI-TOF/TOF and LC-MS/MS mass spectrometry techniques as outlined in *Chapter 2.21.1* and *Chapter 2.21.2*, respectively.

4.3 Results

4.3.1 Peptide mass fingerprinting for the identification of ACE2

Our first aim was to investigate an observation made in a recent study, which proposed that the cleavage-site of ACE2 constitutive shedding lies within amino acid residues 716 – 741 (human ACE2 numbering) (*239*). Based on this report, we attempted to reproduce the experiments following the methods described by the authors. We stimulated the cleavage-secretion of full-length ACE2 using PMA, in Huh-7 cells that endogenously express ACE2. Cleaved protein was then deglycosylated followed by separation by SDS-PAGE. Gel bands were then excised followed by in-gel digestions using proteolytic enzymes (trypsin and chymotrypsin), and finally they were identified by fingerprint and sequence analysis by MALDI-TOF/TOF. Peptide mass fingerprinting results showed that the excised gel bands did not match to human ACE2 from SwissProt protein database (Figure 4-1). Trypsin digested bands were positively matched to bovine serum albumin (Figure 4-1 *A*) while chymotrypsin digested bands were positively matched to NADH-ubiquinone oxidoreductase protein (Figure 4-1 *B*). The experiment was repeated multiple times but none showed a positive match to human ACE2. In order to achieve a higher level of separation and detection, we repeated the experiment using full-length recombinant human ACE2, and soluble ectodomain form of recombinant ACE2, pre-isolated using affinity purification, prior to 2D-PAGE separation and silver staining techniques. To identify the spots corresponding to ACE2, we ran a duplicate gel for western immunoblotting using anti-ACE2 antibody. Silver stained gel spots were excised but once again, none of the spots were successfully matched to human ACE2 (Figure 4-2 *A*). The experiment was repeated multiple times, yielding similar results. We have also replaced full-length human ACE2 with soluble ectodomain form of human ACE2, but MALDI-TOF analysis showed that we were unable to match any spots to human ACE2 (Figure 4-2 *B*).

Thus unfortunately we were unable to reproduce the data published in the earlier study (*239*).

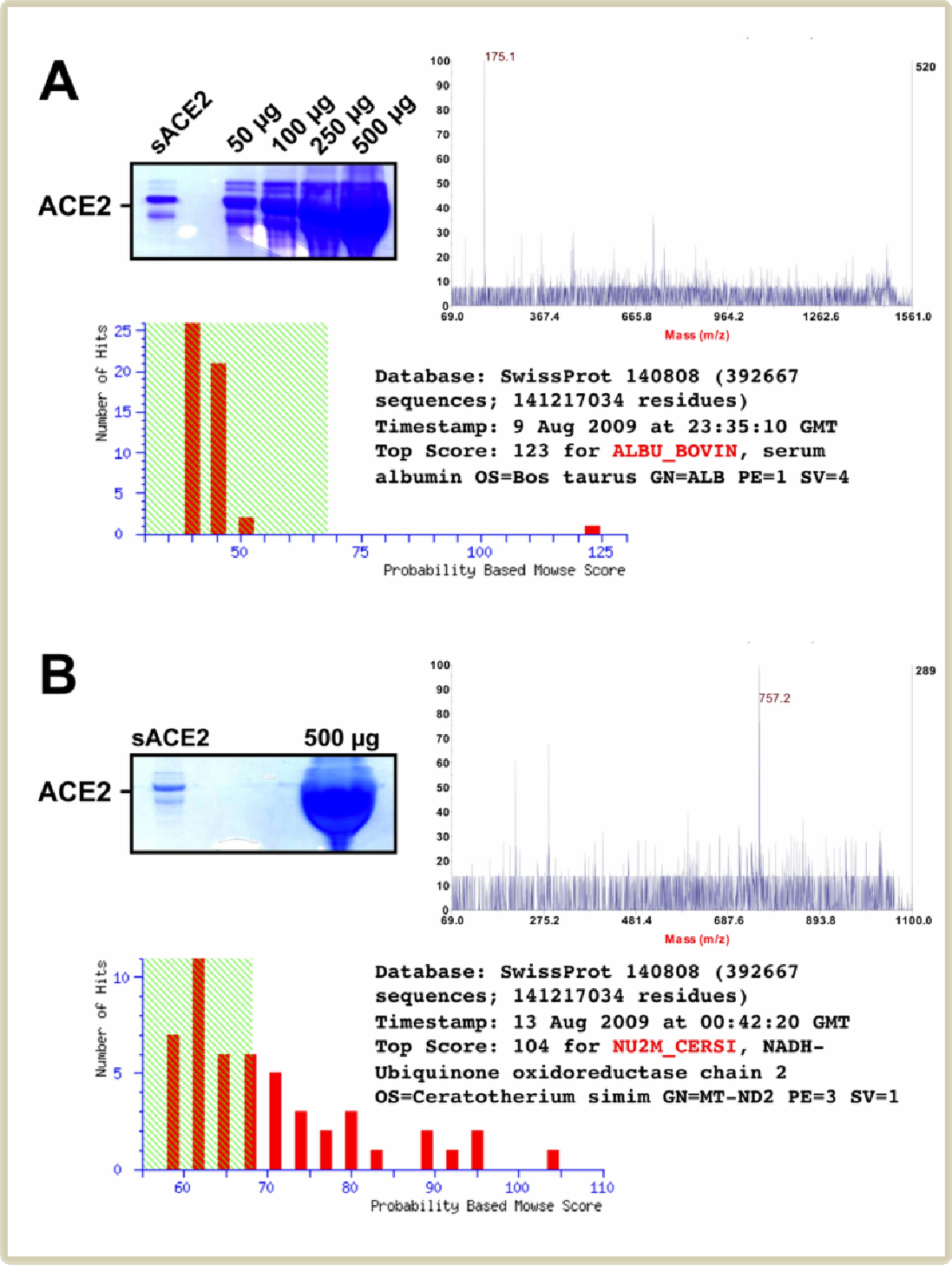


Figure 4-1: Peptide mass fingerprinting of human ACE2 by MALDI-TOF mass spectrometry. *A.* 500 µg (total protein) of ACE2 shed from concentrated medium of Huh-7 cells was

deglycosylated prior to SDS-PAGE gel separation, as described under 'Materials and Methods'. Bands corresponding to the molecular weight of ACE2 were subject to in-gel enzymatic digest using: *A.* Trypsin, or *B.* Chymotrypsin. Digested fragments were analysed by MALDI-TOF/TOF mass spectrometry and resulting fragments were searched against SwissProt protein database for a positive match. A soluble ectodomain form of ACE2 (sACE2) served as positive control for SDS-PAGE gel separation.

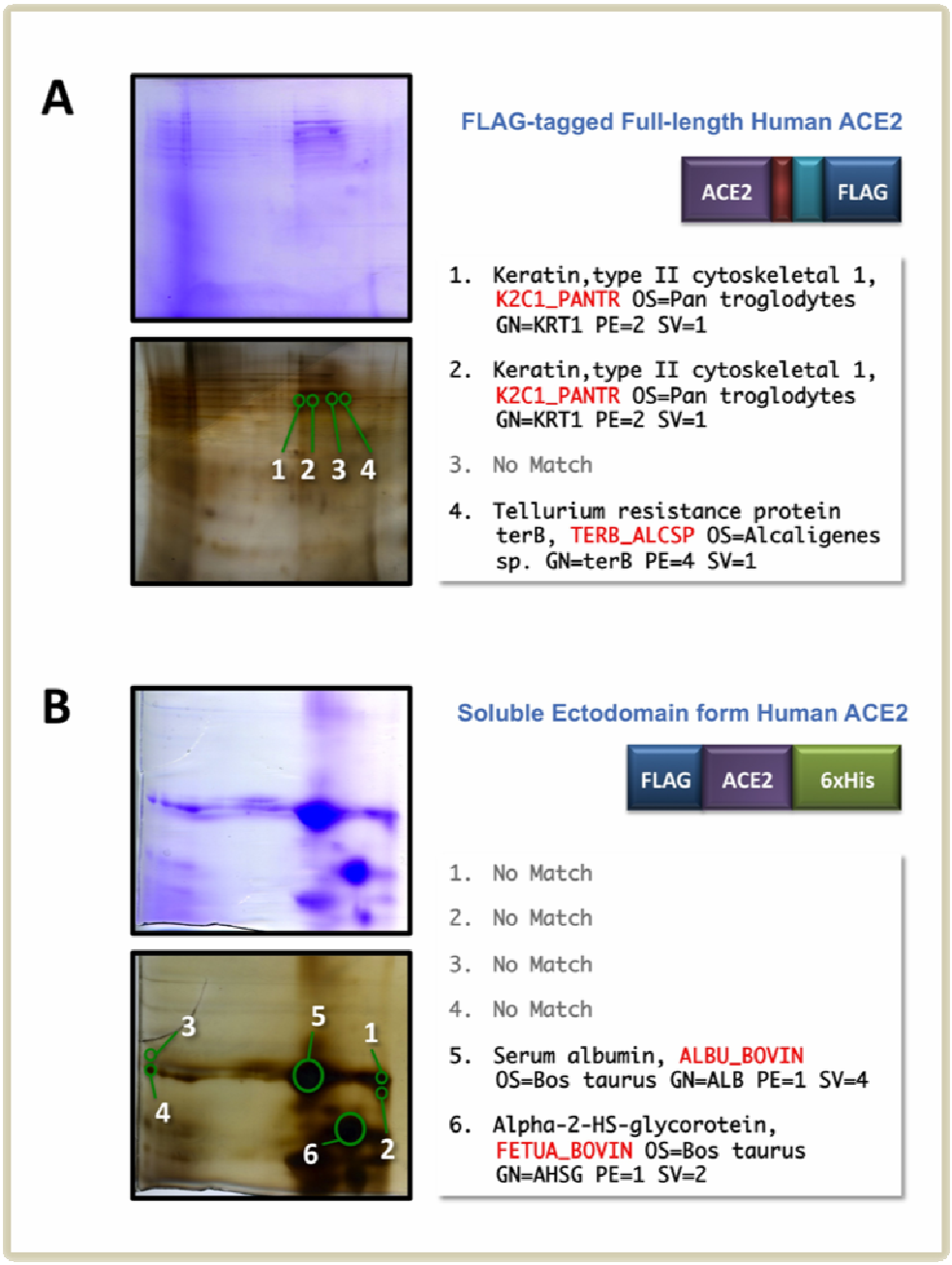


Figure 4-2: 2D-PAGE analysis of FLAG-tagged full-length human ACE2 and soluble ectodomain form human ACE2 from CHO-P cells. A. 100 µg (total protein) of affinity-purified FLAG-tagged full-length human ACE2, or B. 500 µg (total protein) of concentrated medium containing soluble ectodomain form of ACE2 were deglycosylated as described under 'Materials and Methods'. An equal amount of deglycosylated proteins were quantified on western immunobloting against ACE2, and separated using SDS-PAGE gels. Gels were subsequently stained with both Coomassie blue and silver staining. ACE2 corresponding

spots were excised and digested with trypsin for MALDI-TOF/TOF mass spectrometry analysis.

4.3.2 *In vitro* cleavage of ACE2 peptide mimetics by ADAM17

To determine the cleavage-site for ACE2 ectodomain shedding mediated by ADAM17, two overlapping peptide mimetic, corresponding to two 20-amino acid sequences; ACE2/JM1 (residues 701-720), and ACE2/JM2 (residues 711-730) of the human ACE2 juxtamembrane domain (Figure 4-3) were synthesised, These synthetic peptides were incubated with human recombinant ADAM17, followed by proteomics analysis using MALDI-TOF/TOF mass spectrometry. We observed that peptide ACE2/JM1 (MW. 2220.0 Da) was not cleaved by human recombinant ADAM17, even after 8-hours of incubation (Figure 4-4A). On the other hand, peptide ACE2/JM2 (MW. 2405.9 Da) showed cleavage by human recombinant ADAM17 after 4-hours (Figure 4-4B). The resulting cleaved peptides were identified as the 990.4 Da, and 1433.5 Da fragments (Figure 4-4*B*). Tandem mass spectrometry using MALDI-TOF/TOF analyser confirmed that the site for the peptide cleavage is between Arg^{708} and Ser^{709} (Figure 4-5). To validate this finding, the experiments were repeated in the presence of ADAM17 specific inhibitor, TAPI-1. As illustrated in Figure 4-6, in the absence of TAPI-1, we observed the identical cleavage of ACE2/JM2 peptide after 4-hours incubation *(left panel)*. However in the presence of TAPI-1 (50 μM), no cleavage was observed *(right panel)*.

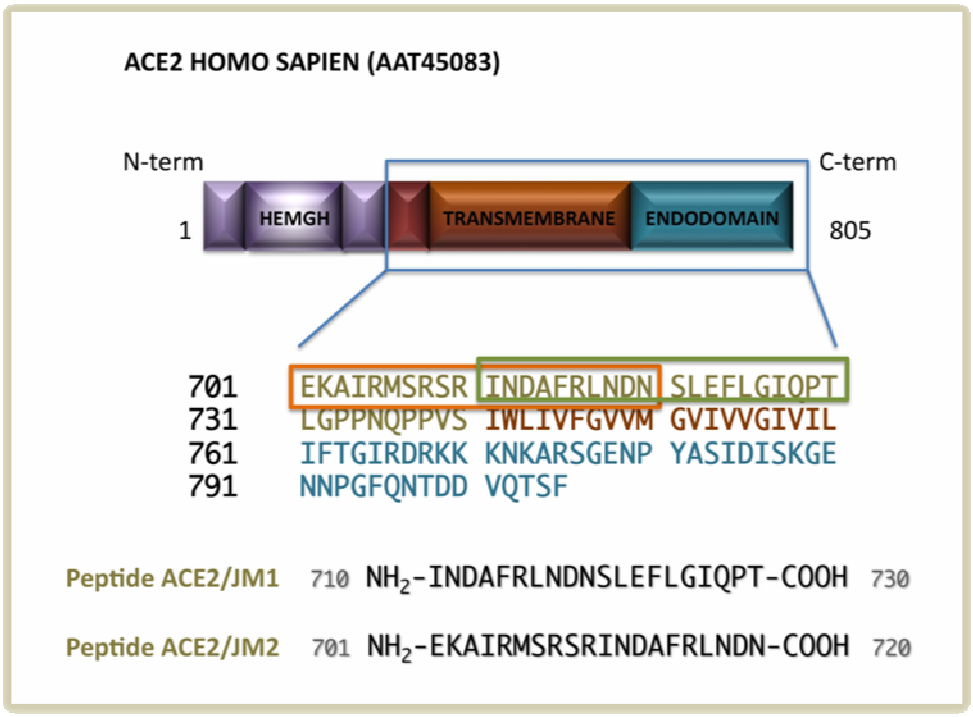


Figure 4-3: A schematic showing ACE2 domains and amino acid sequence of the juxtamembrane, transmembrane and cytoplasmic domains of human ACE2 (accession number AAT45083). Peptide sequence mimics corresponding to ACE2 juxtamembrane region (ACE2/JM1 and ACE2/JM2) were synthesised for *in vitro* cleavage assay by recombinant human ADAM17.

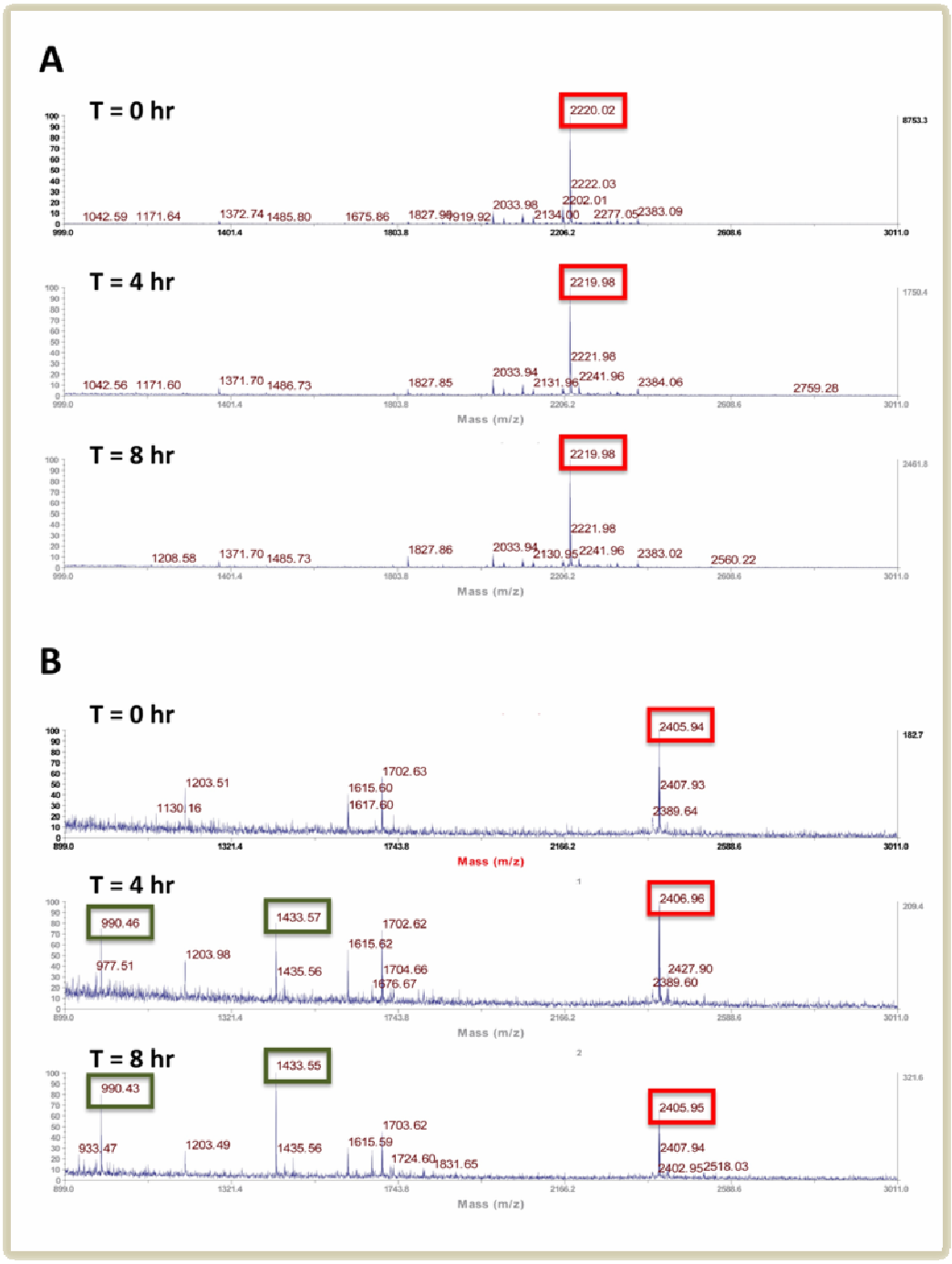
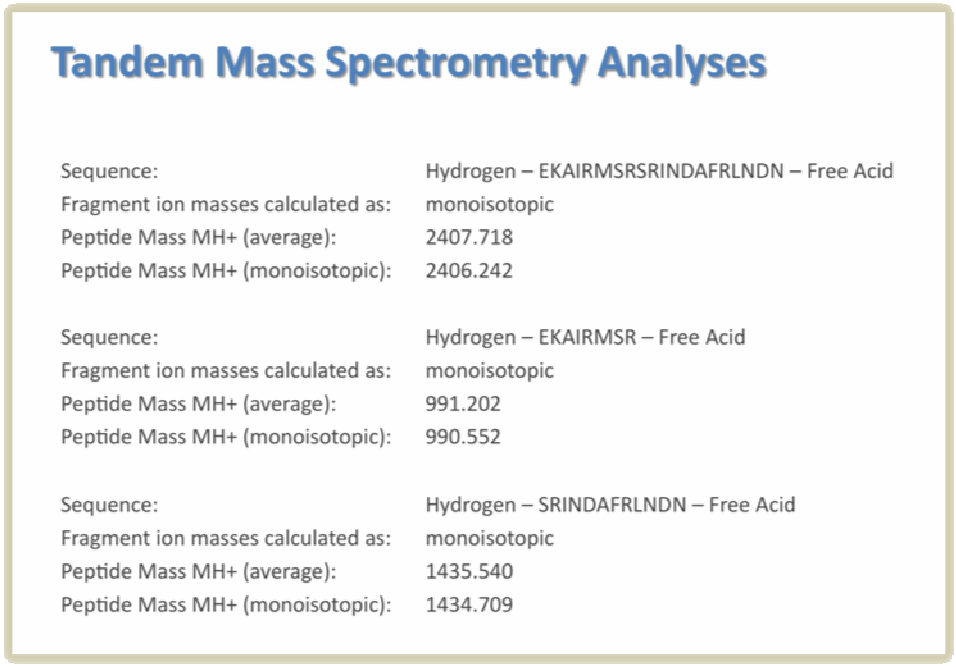


Figure 4-4: Mass spectrometry analysis of peptide fragments mimickingthe human ACE2 sequences: *A.*711-730 (ACE2JM1),and *B.*701-720 (ACE2JM2). Recombinant ADAM17 (0.1 µg) incubated with synthetic peptide (3 µg) for 8 hours in ADAM buffer containing 25 mM Tris, pH 9.0, 2.5 µM ZnCl2, 0.005% (v/v) Brij-35. Cleavage reactions were terminated by addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were

analysed using MALDI-TOF-TOF mass spectrometry. Peptide substrates (red) and cleavage products (green) are highlighted.



Tandem Mass Spectrometry Analyses

Sequence:	Hydrogen – EKAIRMSRSRINDAFRLNDN – Free Acid
Fragment ion masses calculated as:	monoisotopic
Peptide Mass MH+ (average):	2407.718
Peptide Mass MH+ (monoisotopic):	2406.242
Sequence:	Hydrogen – EKAIRMSR – Free Acid
Fragment ion masses calculated as:	monoisotopic
Peptide Mass MH+ (average):	991.202
Peptide Mass MH+ (monoisotopic):	990.552
Sequence:	Hydrogen – SRINDAFRLNDN – Free Acid
Fragment ion masses calculated as:	monoisotopic
Peptide Mass MH+ (average):	1435.540
Peptide Mass MH+ (monoisotopic):	1434.709

Figure 4-5:Tandem mass spectrometry (MALDI-TOF/TOF) analysis of peptide fragments corresponding to residues 701-720 of human ACE2. Resulting fragments (990.4 Da, 1433.5 Da and 2406.9 Da) fromADAM17 cleavage reactions were analysed for amino acid composition and sequence.

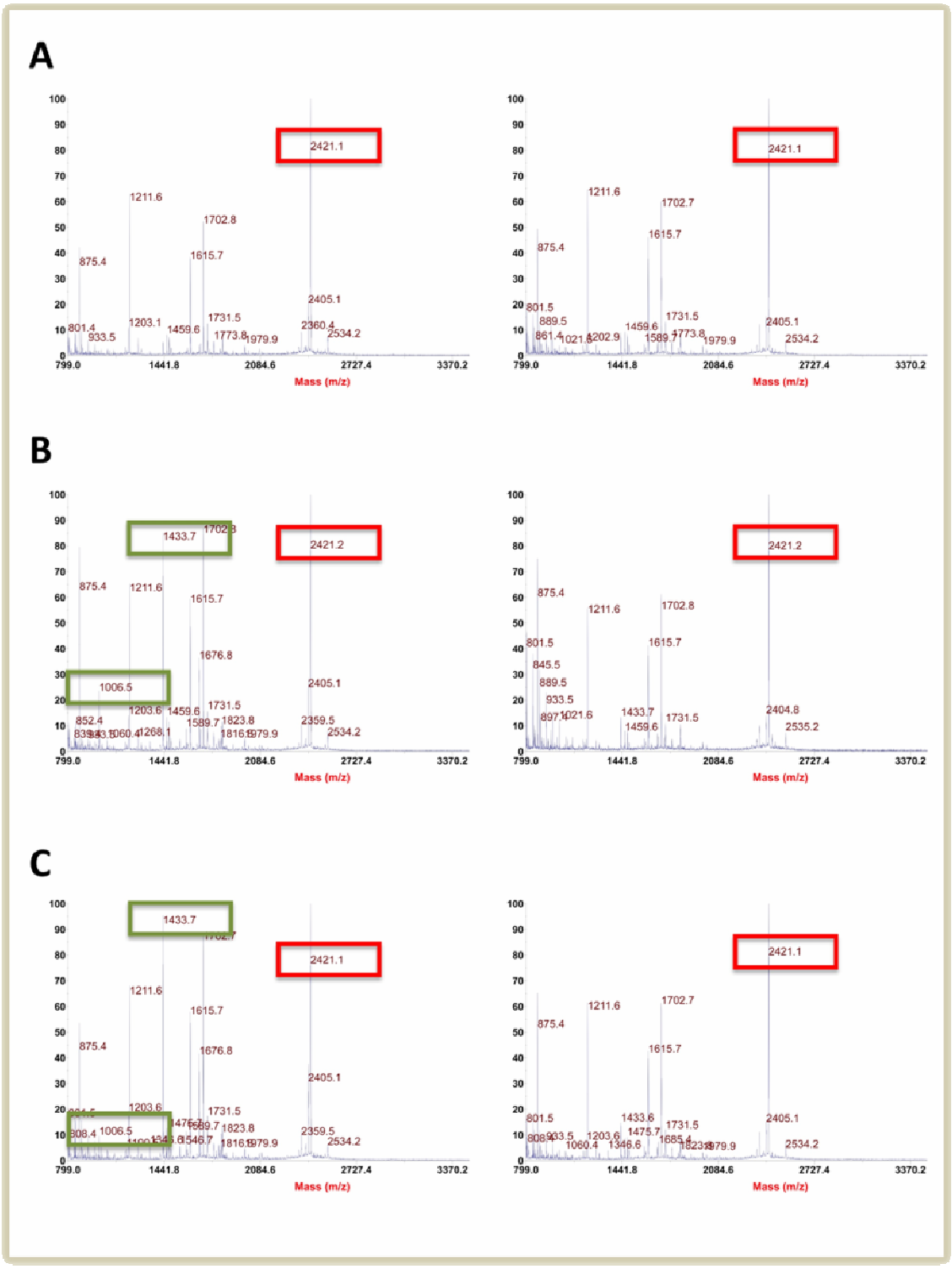


Figure 4-6:Mass spectrometry analysis of peptide fragments mimicking to human ACE2 sequences 701-720 (ACE2JM2) in the presence of ADAM17 specific inhibitor TAPI-1. Recombinant ADAM17 (0.1 µg) incubated with synthetic peptide (3 µg) for: *A*. 0-hour, *B.* 4-hours, or *C.* 8 hours in ADAM buffer in the absence (*left panel*), and presence of TAPI-1 (50 µM) (*right panel*). Cleavage reactions were terminated by addition of 80% (v/v) acetonitrile

containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analysed using MALDI-TOF-TOF mass spectrometry.Peptide substrates (red) and cleavage products (green) are highlighted.

4.3.3 *In vitro* cleavage of ACE2 mutant peptides by ADAM17

Having established that human recombinant ADAM17 is able to cleave peptides corresponding to the ACE2 juxtamembrane region, we next looked at which amino acid(s) around the proposed cleavage-site are important for the recognition and regulation of ACE2 ectodomain shedding. Thus, we further synthesised a series of peptide mimetics containing mutations around the proposed cleavage site for ACE2 ectodomain shedding. We introduced two sets of mutant peptides; alanine substitution mutants, and glutamate substitution mutants, as listed in Table 2-3 under 'Materials and Methods'. These peptides were investigated for their susceptibility to cleavage by human recombinant ADAM17. Mass spectrometry analysis showed that all three alanine-substituted mutations (Arg^{708}➔Ala^{708}, Ser^{709}➔Ala^{709} andArg^{708}/Ser^{709}➔Ala^{708}/Ala^{709} double mutants) had no effect on ADAM17 mediated cleavage. All three peptides were successfully cleaved by ADAM17 after 4-hours of incubation, as illustrated in Figure 4-7. Notably, the pre-cleaved peptides (Figure 4-7, highlighted in red) and only one of the expected cleavage fragments was detected as intact peptide from mass spectrometry analysis (Figure 4-7, highlighted in green). However, we were unable to detect any changes in fragment profile in the glutamate-substituted mutant peptides (Arg^{708}➔Glu^{708}, Arg^{710}➔Glu^{710} and Arg^{708}/Arg^{710}➔Ala^{708}/Ala^{710}), suggesting none of these peptides were successfully cleaved by ADAM17 after 8-hours (Figure 4-8). These above-mentioned experiments were repeated several times and similar results were observed. A summary of peptide cleavages analysed by MALDI-TOF/TOF mass spectrometry is listed on Table 4-1.

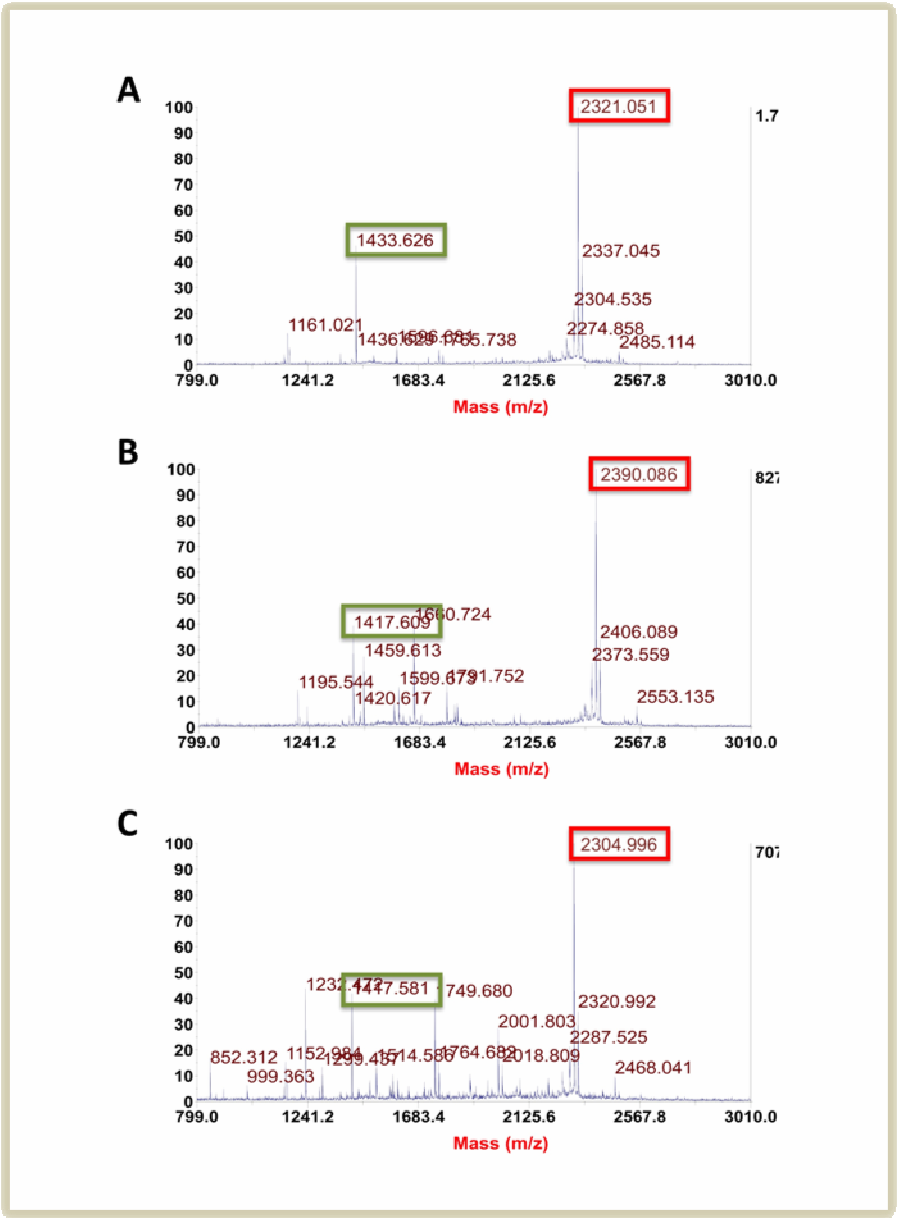


Figure 4-7:Mass spectrometry analysis of peptide fragments containing alanine substituted mutations inthehuman ACE2 residues 708 and 709. Recombinant ADAM17 (0.1 µg) incubated with synthetic peptides (3 µg)*A*. Arg^{708}➔Ala^{708} (R708A), *B.* Ser^{709}➔Ala^{709} (S709A), or *C.*Arg^{708}/Ser^{709}➔Ala^{708}/Ala^{709} (R708A/S709A), for 4 hours in ADAM buffer. Cleavage reactions were terminated by addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analysed using MALDI-TOF-TOF mass spectrometry.Peptide substrates (red) and cleavage products (green) are highlighted.

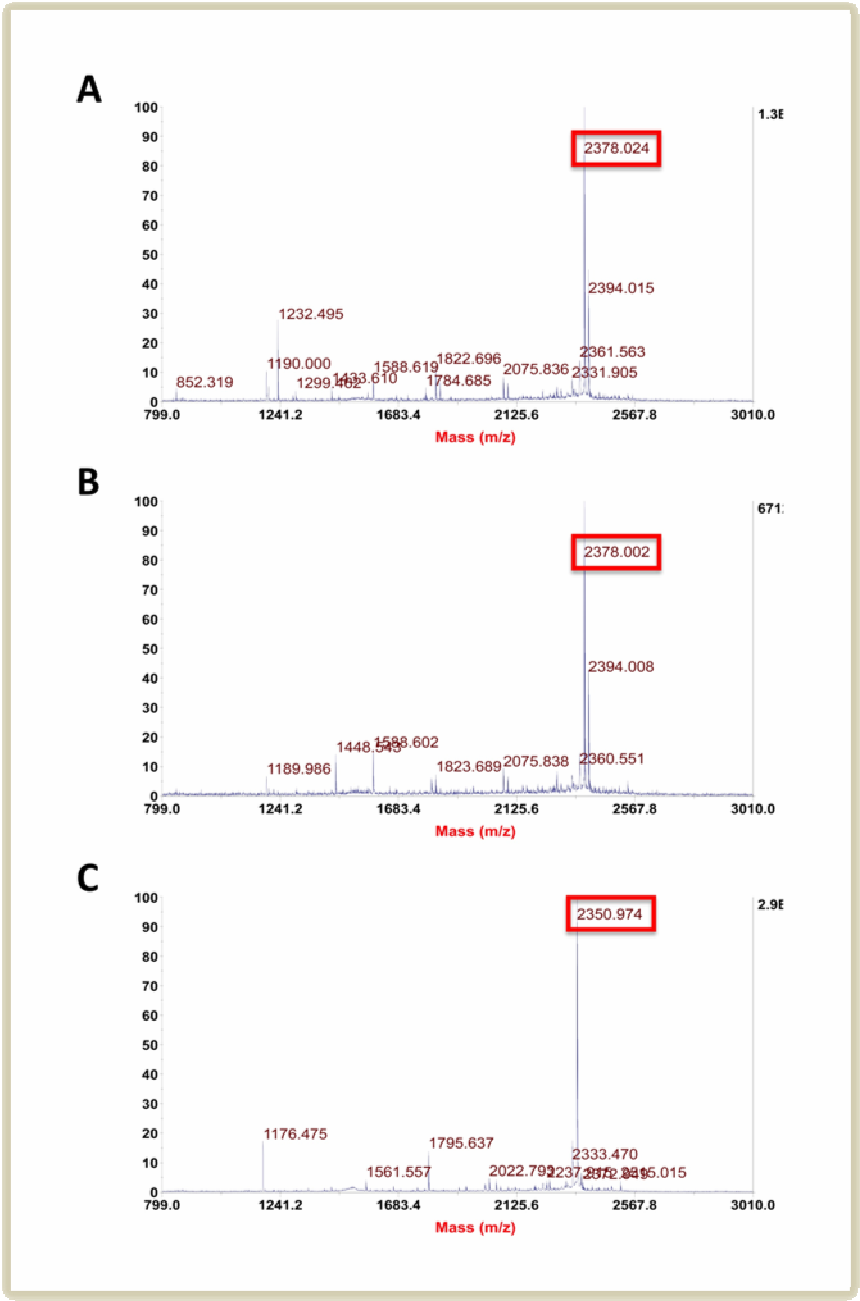


Figure 4-8: Mass spectrometry analysis of peptide fragments containing glutamate-substituted mutations in the human ACE2 residues 708 and 710. Recombinant ADAM17 (0.1 µg) incubated with synthetic peptides (3 µg) *A.* Arg^{708}➔Glu^{708} (R708E), *B.* Arg^{710}➔Glu^{710} (S709E), or *C.* Arg^{708}/Arg^{710}➔Ala^{708}/Ala^{710} (R708E/S709E), for 4 hours in ADAM17 buffer. Cleavage reactions were terminated by addition of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Cleavage products were analysed using MALDI-TOF-TOF mass spectrometry. Peptide substrates (red) are highlighted.

Table 4-1: Summary of MALDI-TOF mass spectrometry analysis of peptide fragments containing mutated residues (Arg^{708}, Ser^{709} and Arg^{710}) of human ACE2.

Peptide Name	Peptide Sequence	Mass (Da)	Fragment	Cleaved
ACE2/JM2 (*181*)	EKAIRMSRSRINDAFRLNDN	2405.2	Observed	
	EKAIRMSR	989.5	Observed	**YES**
	SRINDAFRLNDN	1433.7	Observed	
ACE2JM2-R708A	EKAIRMSASRINDAFRLNDN	2321.6	Observed	
	EKAIRMSA	904.5	-	**YES**
	SRINDAFRLNDN	1433.7	Observed	
ACE2JM2-S709A	EKAIRMSRARINDAFRLND N	2389.2	Observed	
	EKAIRMSR	989.5	-	**YES**
	ARINDAFRLNDN	1417.7	Observed	
ACE2JM2-R708A/S708A	EKAIRMSAARINDAFRLND N	2304.2	Observed	
	EKAIRMSA	904.5	-	**YES**
	ARINDAFRLNDN	1417.7	Observed	
ACE2JM2-R708E	EKAIRMSESRINDAFRLNDN	2378.2	Observed	
	EKAIRMSE	962.5	-	**NO**
	SRINDAFRLNDN	1433.7	-	
ACE2JM2-R710E	EKAIRMSRSEINDAFRLNDN	2378.2	Observed	
	EKAIRMSR	989.5	-	**NO**
	SEINDAFRLNDN	1406.6	-	
ACE2JM2-R708E/R710E	EKAIRMSESEINDAFRLNDN	2351.1	Observed	
	EKAIRMSE	962.5	-	**NO**

	SEINDAFRLNDN	1406.6	-

4.3.4 ACE2 mutants expressed in CHO-P and CHO-K1 cells

From the investigation of ADAM17 in cleaving peptide mimics and mimetics, our next studies looked at the effects of these mutated residues have on the ectodomain shedding of full-length ACE2. Using site-directed mutagenesis technique, we managed to successfully clone four of the six constructs; namely the R708A, S709A, R708E and R708E/R710E double mutants. Despite many attempts such as using new primers or different combination of primers, and testing over a range of PCR amplification conditions and different polymerase enzymes, both R708A/S709A double mutant and R710E mutants were not able to be cloned. Our first preliminary experiment looked at ACE2 mutant proteins, transiently expressed in CHO-P and CHO-K1 cells, while investigating the quality of these mutant proteins expressed in these cells, and also the ability of ACE2 mutants to undergo ectodomain shedding. Concentrated medium harvested from CHO-P cells transiently expressing the R708E/R710E mutant showed a marked increase in ACE2 activity (216.9 ± 16.5%), while in the presence of ADAM17 inhibitor, TAPI-1, the activity is significantly decreased (-30.0 ± 2.7%) (Figure 4-9 *A*). As observed previously in wild-type ACE2 (*46*), the shedding of ACE2-R708E/R710E mutant protein is not completely abolished in the presence of TAPI-1, suggesting perhaps there is/are other sheddase(s) involved in the shedding of ACE2. A detailed qualitative analysis comparing each of the individual shedding profile, however, cannot be carried out in transient expressing cells reflecting the differences in transfection efficiencies. On the other hand, in six independent experiments (n=6), we observed that R708E transfected CHO-K1 cells exhibited insignificant levels of ACE2 activity (Figure 4-9 *B*), suggesting that perhaps CHO-K1 cells are not an ideal candidate for transient expression studies of the ACE2 mutant proteins.

The experiments were repeated in CHO-K1 cells that stably expressing both types of mutant proteins, displaying similar shedding profile compared to the transient system. PMA stimulation in CHO-K1 cells expressing ACE2-R708E/710E and ACE2-R708E mutant protein showed significant increase in ACE2 activity in the concentrated medium compared to controls (207.0 ± 15.6% and 185.8 ± 3.8%, respectively). In the presence of TAPI-1, both mutants showed significant decrease in ACE2 activity detected in the concentrated medium compared to controls (40.8 ± 3.5% and 74.3 ± 2.7%, respectively).

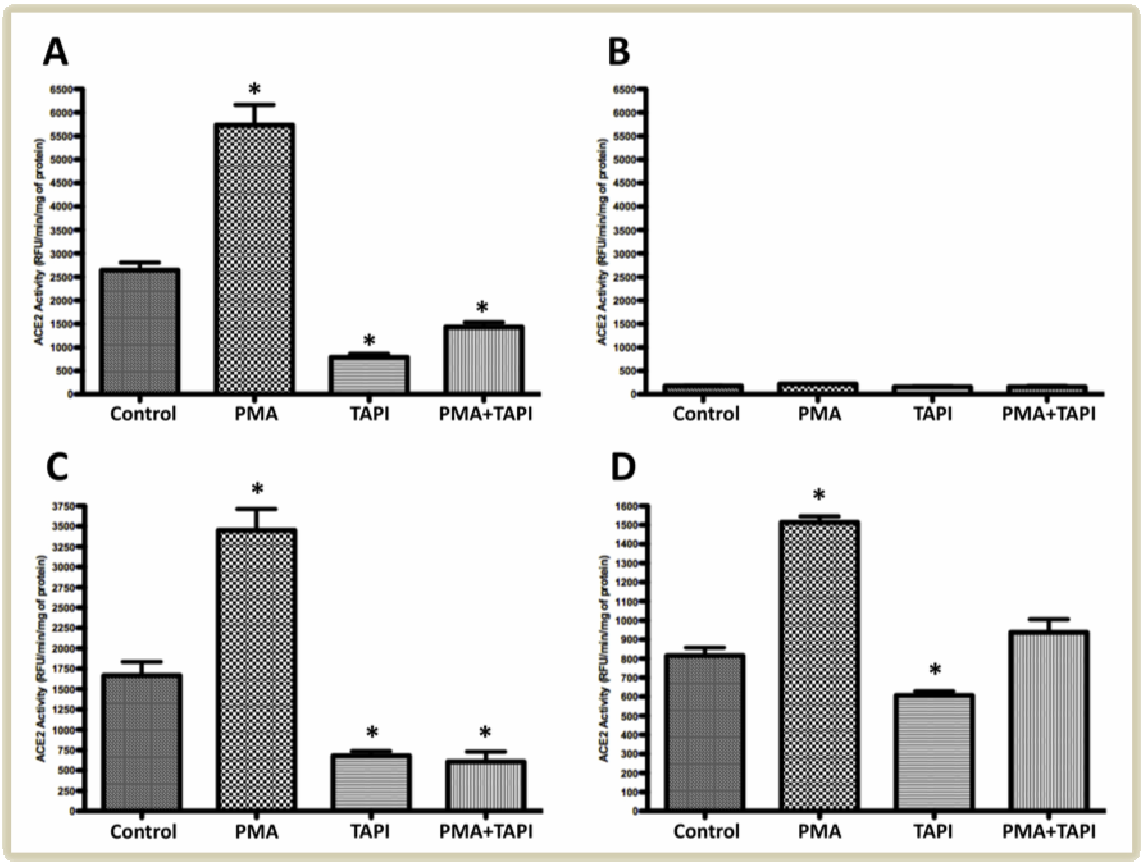


Figure 4-9: ACE2 activity from the concentrated medium of *A.*CHO-P cells transiently expressing ACE2-R708E/R710E mutant proteins, and *B.* CHO-K1 cells transiently expressing ACE2-R708E mutant proteins. 10 µg of plasmid DNA*(R708E/R710E,* and *R7080E,* respectively) were used to transfect 60% confluent cells in 100 mm cell culture dishes using the DEAE-Dextran method. 48 hours post transfection, cells were washed and replaced with serum free medium prior to further incubation for additional 16 hours. CHO-K1 cells that

stably expressing *C.* ACE2-R708E/R710E, and,*D.* ACE2-R708E mutant proteins were grown to 80% confluence prior to incubation with serum free medium for 16 hours. All cells were stimulated with the phorbol ester, PMA (1 μM) in the presence or absence of ADAM17 inhibitor, TAPI (50 μM) for 4-hours. Secreted protein (10 μg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from three independent experiments(n≥3, one-way ANOVA). Asterisks denote significant differences ($P< 0.05$) compared to other treatments.

4.3.5 ACE2 mutants undergo ectodomain shedding in CHO-P cells

Using the successfully cloned plasmids, we next looked at quantification analysis of ACE2 ectodomain shedding in CHO-P cells that were transiently expressing mutant ACE2. All of the expressing mutant proteins remained catalytically active in hydrolysing the ACE2 specific quenched fluorogenic substrate (Figure 4-10). Compared to the wild-type sequence (Figure 4-10 *B*), all of the mutants were able to undergo an increased ectodomain shedding in the presence of PMA (R708A 221.0 ± 45.2%, S709A 24.4 ± 8.7%, R708E 118.4 ± 39.6%, and R708E/R710E 37.6 ± 12.7%) compared to non-stimulated controls (Figure 4-10(*C*–*F*)). This effect was ablated in the in the presence of PKC-inhibitor BIM. As an experimental control, mock-transfected CHO-P cells using pcDNA3.1/V5-His-TOPO empty vector, showed only background fluorescent in control and treated cells (Figure 4-10 *A*). As mentioned previously, a detailed qualitative analysis comparing each of the individual shedding profiles, however, cannot be achieved in transient expressing cells due to the likely differences in transfection efficiencies.

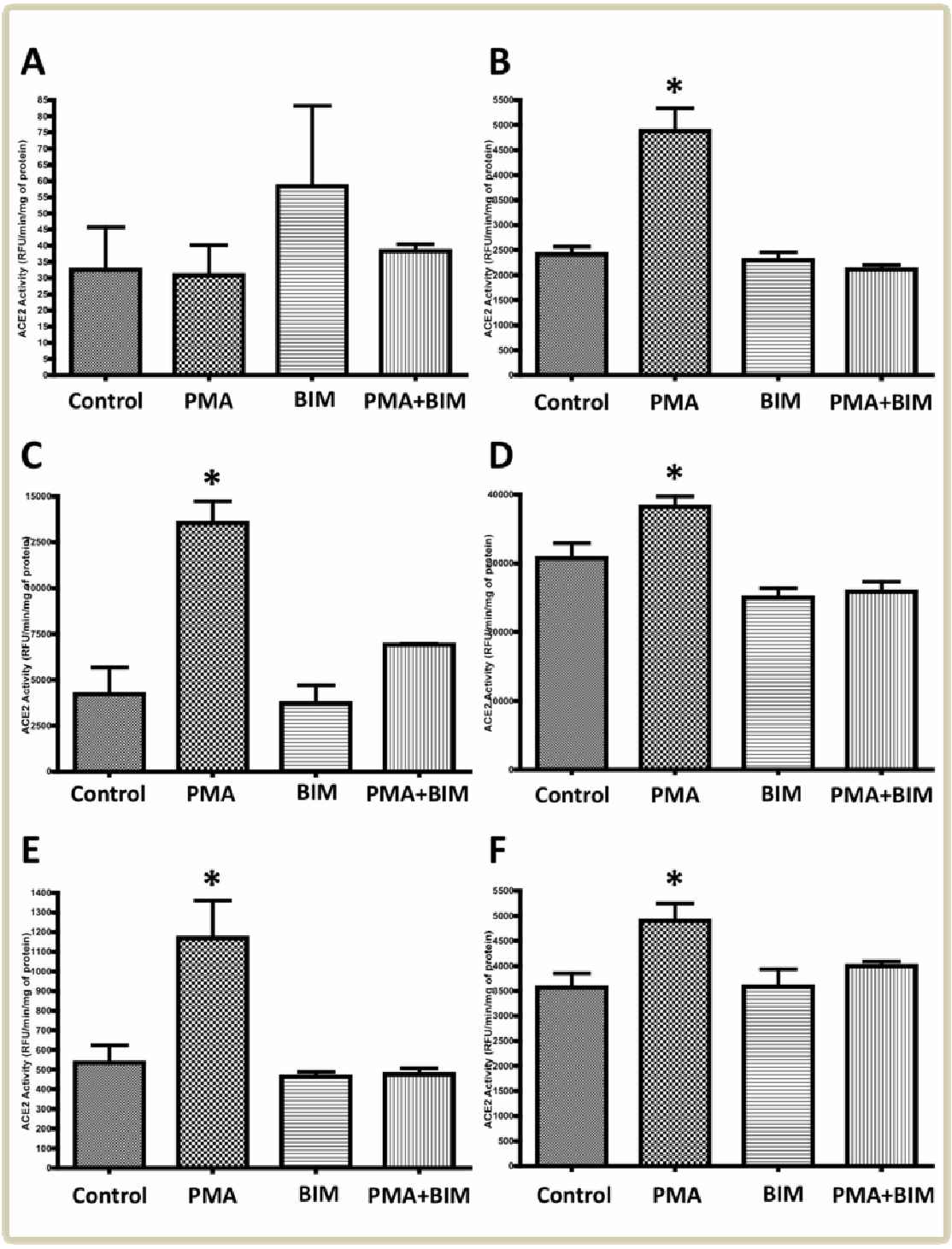


Figure 4-10:ACE2 activity from the concentrated medium of CHO-P cells transiently expressing ACE2 mutants. 10 µg of plasmid DNA; *A. mock, B. wild type ACE2, C. R708A, D. S709A*, *E. R708E, and F. R7080E/R710E,* respectively, were used to transfect 60% confluent CHO-P cells in 100 mm cell culture dishes using the DEAE-Dextran method. 48 hours post transfection, cells were washed and serum free media added prior to further incubation foran

additional 16 hours. Cells were stimulated with the phorbol ester, PMA (1 μM) in the presence or absence of PKC inhibitor, BIM (2 μM) for 1 hour. Secreted protein (10 μg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from three independent experiments(n=3, one-way ANOVA). Asterisks denote significant differences ($P< 0.05$) compared to all the data gathered under the same category.

4.3.6 Protein expression in CHO-K1 cells stably expressing vector plasmids

Using the mutant ACE2 plasmid constructs created in the previous experiments, we have stably transfected CHO-K1 cells to investigate the change in ectodomain shedding profile. The first step in creating a stably transfected cell line involves optimising the expression level of the protein of interest. To achieve this, we varied the ratio of transfecting agent to amount of plasmid DNA used; 2:1, 3:1, or 4:1 and maintained these transfected cells over six weeks in the presence of selecting antibodies. During the period of the selection process, it is important to ensure that the transfected cells express the protein(s) of interest. This was achieved by co-transfection of green fluorescence vector protein in native CHO-K1 cells. As illustrated in Figure 4-11, no green fluorescence was detected in native non-transfected, and pcDNA3.1/V5-His-TOPO transfected CHO-K1 cells. In pEGFPN3 transfected CHO-K1 cells however, a high proportion of the cells were emitting bright green fluorescence, reflecting the success of plasmid expression using the Lipofectamin™ 2000 transfection protocol.

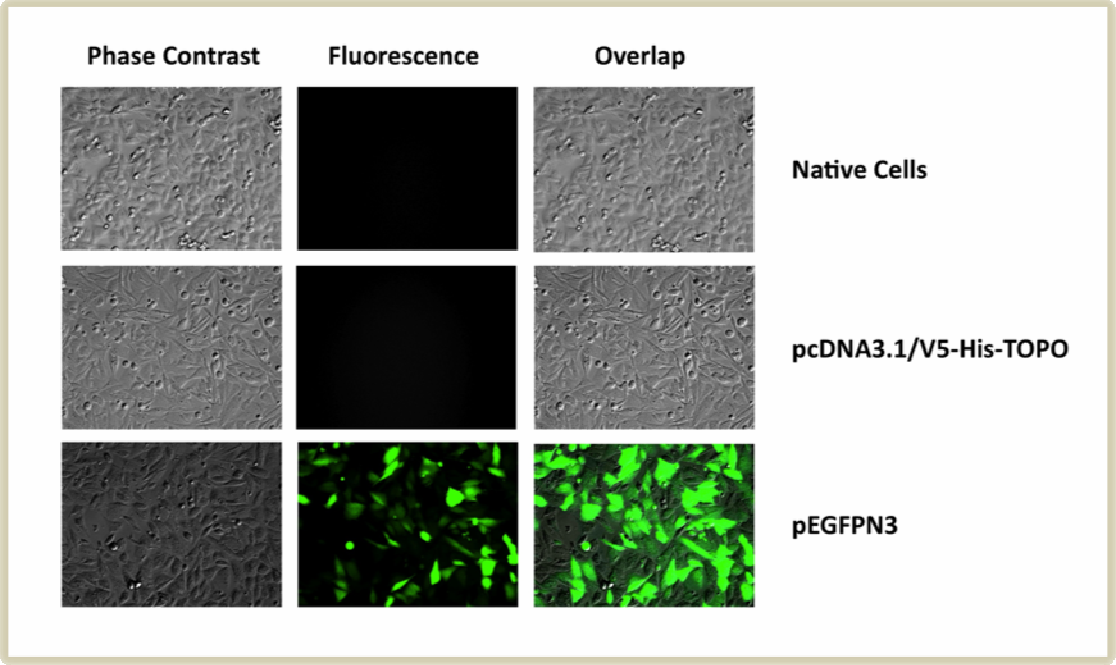


Figure 4-11: Detection of green fluorescence protein in CHO-K1 cells stably expressing plasmid vectors pcDNA3.1/V5-His-TOPO and pEGFPN3.4.0 µg of plasmid DNA:*pEGFPN3 and pcDNA3.1/V5-His-TOPO vector* were used to transfect 60% confluent CHO-K1 cells in 100 mm cell culture dishes using the Lipofectamine™ 2000 transfection method described under 'Materials and Methods'. Forty-two days post transfection, cells were screened for ACE2 level of expression. Cells were visualised by phase and fluorescence (GFP) microscopy.

4.3.7 ACE2 activity in CHO-K1 cells stably expressing mutant ACE2

Transfected CHO-K1 cells that stably expressing ACE2 mutants were screened for ACE2 activity at Day 42 (Figure 4-12 *A*), Day 49 (Figure 4-12 *B*) and Day 56 (Figure 4-12 *C*). On each of the screening days, cells were prepared separately in duplicates, and screened independently for consistency purpose (Screen 1, Figure 4-13 *left panel*; and Screen 2, Figure 4-13 *right panel*). As controls, cells were stably transfected with pcDNA3.1/V5-His-TOPO and pEGFPN3 empty vectors. Our statistical analysis showed that at transfection ratio of 3:1 (Lipofectamine™ 2000: plasmid DNA), both wild type ACE2 and all of the mutants have

consistent level of protein expression (Figure 4-13 *A*) (wild type ACE2, 485.1 ± 53.9; R708A, 607.2 ± 111.5; S709A, 461.1 ± 37.2; R708E, 539.2 ± 60.4; and R708E/R710E, 541.1 ± 64.1, measured as RFU/min/mg protein). As an additional specificity control, we also detected the presence of ACE2 in the cell lysate using western immunoblotting probing against ACE2 ectodomain (Figure 4.13 *B*).

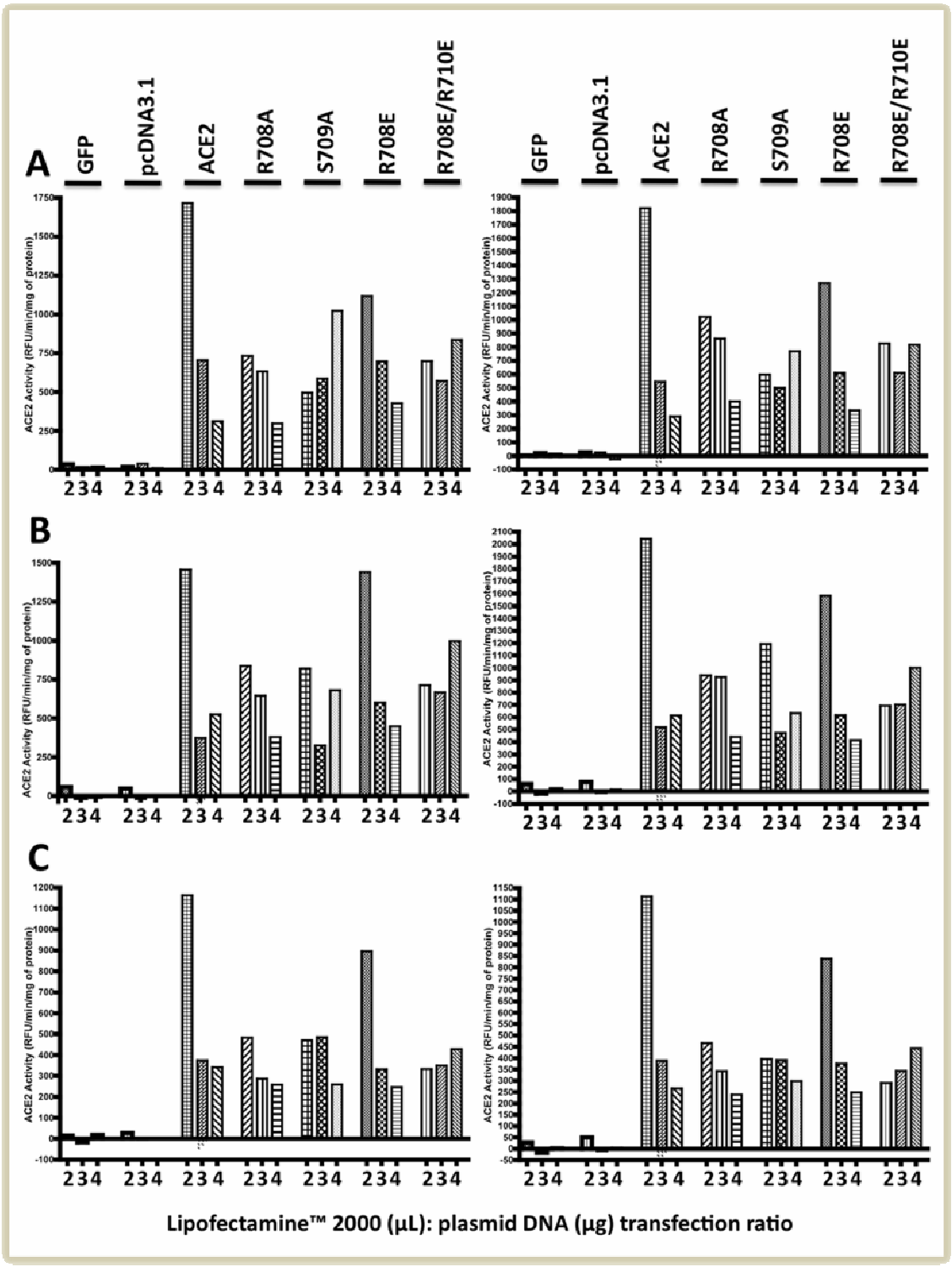


Figure 4-12: Screening of ACE2 activity from stably transfected CHOK1-ACE2 mutant cells under different transfection ratios. 4.0 µg of plasmid DNA:*pEGFPN3 (GFP), pcDNA3.1/V5-His-TOPO vector (pcDNA3.1), FLAG-tagged human recombinant ACE2 (ACE2), ACE2-R708A(R708A), ACE2-S709A(S709A), ACE2-R710E(R710E), orACE2-R708E/R710E(R7080E/R710E),* respectively, were used to transfect 60% confluent CHO-K1 cells in 100 mm cell culture

dishes using the Lipofectamine™ 2000 transfection method described under 'Materials and Methods'. Forty-two days post transfection, cells were screened for ACE2 expression levels. 10 µg (total protein) of cell lysate were assayed twice (First screening: *left panel*, and second screening: *right panel*) for ACE2 activity at, *A.* passage 7 *(Day 42)*, *B.* passage 8 *(Day 49)*, and *C.* passage 9 *(Day 56).*

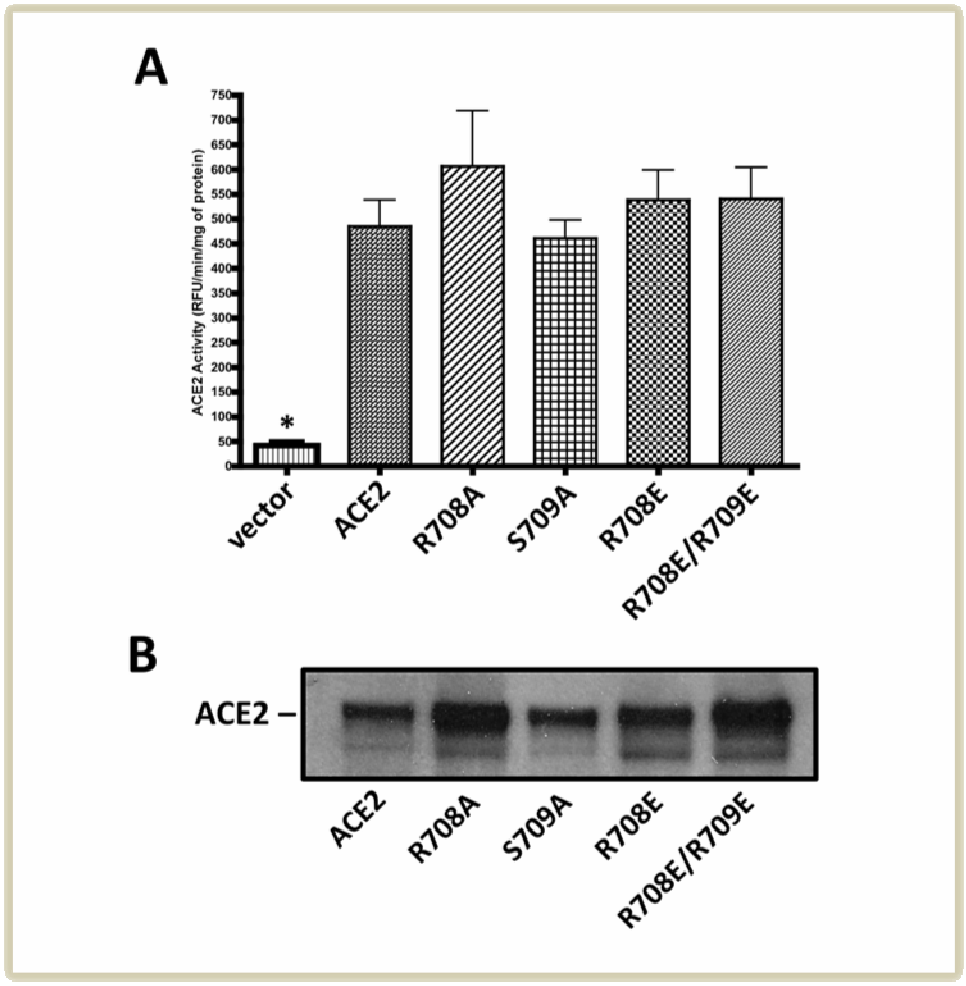


Figure 4-13: Optimised selection of CHO-K1 cells stably expressing ACE2 mutants. *A.* Lipofectamine™ 2000 (µL) to plasmid DNA (µg) ratio of 3:1 in each ACE2 mutants were selected for the level of ACE2 expression in cell lysate (10 µg total protein).The data compared from six independent experiments (consistencies within passage 7 – 9) (n = 6, one-way ANOVA) B. 100 µg (total protein) of the cell lysate from ACE2 mutant expressing CHO-K1

cells were separated by SDS-PAGE and western immunoblotted with a monoclonal anti-ACE2 antibody.

4.3.8 ACE2 shedding profile in CHO-K1 cells stably expressing mutant ACE2

After selecting the appropriate mutant ACE2 cell lines, the next investigation was to determine the ability of PMA in stimulating ectodomain shedding of these ACE2 mutants. Analysis of the concentrated media from cells treated with PMA showed a significant increase in secreted/shed ACE2 activity compared to control media (Figure 4-14 *A*, ACE2 activities are shown as % ACE2 activity against control media) (wild type ACE2, 167.4 ± 17.7%; R708A, 137.9 ± 7.4%, S709A, 196.0 ± 24.6%, R708E, 237.5 ± 18.3%, R708E/R710E, 248.5 ± 18.4%). In cells that were treated with Bisindolylmaleimide I, or a combination of both, we observed no significant changes in ACE2 activity in the concentrated media compared to control after 4-hour treatment (Figure 4-14 *A*). Statistical analysis of ACE2 activity in the concentrated media from both glutamate-substituted mutants were also significantly increased when compared to both wild-type ACE2 and alanine-substituted mutants in cells that were treated with PMA ($P < 0.05$, one-way ANOVA). For specificity control, we also detected the presence of ACE2 in the cell lysate using western immunoblotting probing against ACE2 ectodomain (Figure 4.14 *B*).

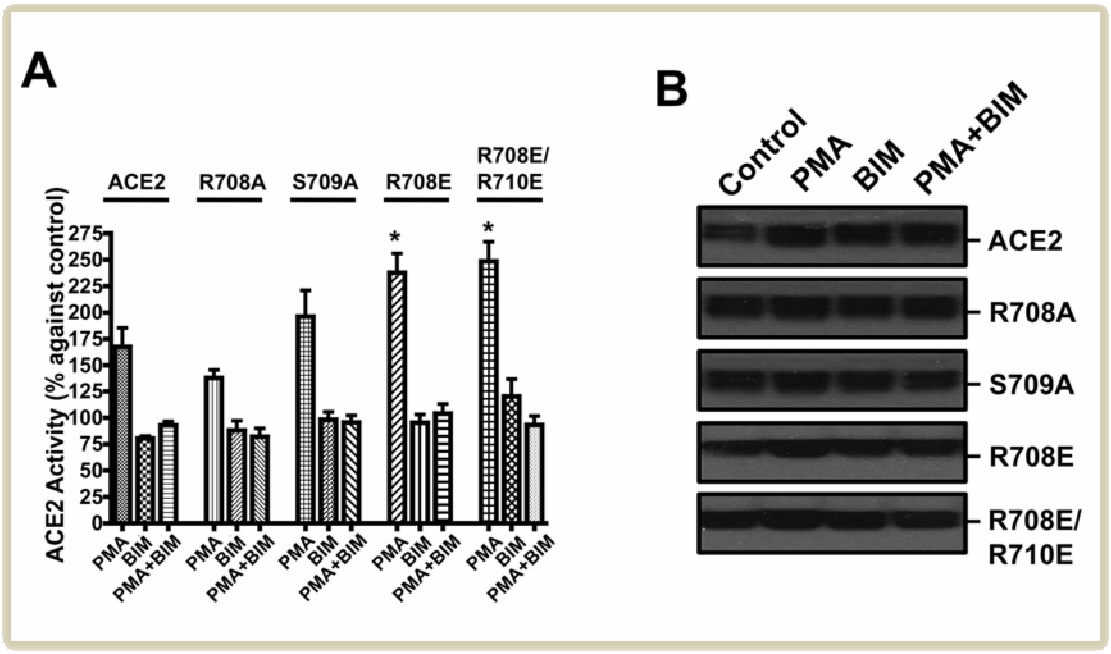


Figure 4-14: PMA stimulated shedding of ACE2 in CHO-K1 cells stably expressing ACE2 mutants. CHO-K1 cells stably expressing ACE2 mutants were incubated in serum-reduced media Opti-MEM containing PMA (1 μM), or BIM (2 μM) or and equal volume of Me_2SO for 4-hours as described under '*Materials and Methods*'. 25 μg (total protein) from the concentrated medium was assayed for ACE2 activity. The data are normalised against the controls from at least four independent experiments ($n \geq 4$, one-way ANOVA). Asterisks denote significant differences ($P < 0.05$) compared against control. *B.* 100 μg (total protein) of the cell lysate was separated by SDS-PAGE and western immunoblotted with a monoclonal anti-ACE2 antibody.

4.4 Discussion

ACE2 undergoes proteolytic release or ectodomain shedding when expressed in various eukaryotic cells in culture (*46, 215, 240, 242, 243*). An identical process has been observed in ACE2-expressing tissues in situ (*3, 239, 244-246*), and this mechanism is probably responsible for the generation of the soluble form of ACE2 that can be detected in bodily fluids *in vivo*, such as human blood plasma and urine (*3, 243*). However, the precise

mechanism(s) that confer both the specificity and that regulation of the ectodomain release of ACE2 are unresolved. The cleavage site in human ACE2 expressed in airway epithelia, Calu-3 cells, has been localised to a region within the juxtamembrane domain, predicted to be between amino acid residues 716 – 741 (human ACE2 numbering) (*239*). This was based on fragment analysis by linear ion trap mass spectrometry from secreted ACE2 separated using two-dimensional gel electrophoresis (*239*). The reported ACE2 detectable fragment that was the most proximal to the transmembrane domain corresponded to amino acids 711 – 716 (*239*). Our initial investigations were aimed at exploring the possibility of providing better fragments/sequence coverage utilising the different proteolytic enzymes and various mass spectrometry technologies available in the laboratory. It was not however to reproduce the data reported by these authors. Both one- and two-dimensional gel electrophoresis of ACE2 shed from PMA stimulation of Huh-7 cells failed to provide any significant match to the protein of interest (Figure 4-1). Furthermore, full-length recombinant human ACE2 and the soluble ectodomain form of human ACE2, transiently expressed in CHO-P cells, and pre-isolated using affinity purification and confirmed using western immunoblotting detection, also failed to yield any positive matches to human ACE2 following MALDI-TOF/TOF and LC-MS analysis (Figure 4-2).

To investigate and thus determine the precise cleavage site(s) within ACE2 that mediate ectodomain shedding, we resolved to an alternative approach, using synthetic peptides whose design was based on the extracellular membrane-proximal sequences of ACE2. This approach was recently and successfully used in our laboratory to demonstrate the cleavage site for GPVI ectodomain shedding mediated by ADAM10 (*247*). Peptide mimics corresponding to amino acid residues 701 – 720, and 711 – 730 were digested with recombinant ADAM17 and analysed by mass spectrometry. ADAM17 cleaved the ACE2-based peptide (residues 701 – 720) at Arg^{708} – Ser^{709} bond. Peptides where residues at the P1, P1', and P1 and P1' position were substituted by alanine had no effect on ADAM17-induced

cleavage, measured by MALDI-TOF/TOF. In contrast, glutamate subtitution in residues at P1, P2', and P1 and P2' position blocked ADAM17-dependent cleavage. Although there is a lack of a consensus cleavage motif by ADAM17, our proposed Arg^{708} – Ser^{709} cleavage-site resembles the one observed in its mammalian homologue, somatic ACE (Arg^{1203} – Ser^{1204})(*153*), and similarly, to other membrane-bound proteins such as the human L-selectin (Arg^{321} – Ser^{322})(*248*),and human amphiregulin (Lys^{177}– Ser^{178})(*249*). As ADAM17 clearly plays a role in the phorbol ester stimulated shedding, and had no detectable effect on constitutive shedding (*46*), it is not alarming to see our proposed cleavage site does not fall within stretch of amino acid residues 716 – 741 reported in (*239*). This potential cleavage site, 32 amino acids proximal to the membrane-spanning domain, is within the required juxtamembrane stalk length for cleavage-secretion by ADAM17 (*250*). However, there may be substantial conformational differences in the cleavage efficiencies on the shedding of full-length proteins on the cell surface and their peptide analogues (*251*). In native membrane-bound full-length TNF-α and its peptide analogues, both identical cleavage sites are readily hydrolysed by ADAM17, whereas peptide mimetics of other substrates are cleaved far less efficiently (*251*). Nonetheless, the differences in efficiency of ADAM17-mediated cleavage of synthetic peptides and full-length proteins have been comprehensively discussed in previous reviews (*252, 253*).

In CHO-P cells transiently expressing ACE2, mutating the residues at the P1, P1', and P1 and P1' positions to alanine, and P1, P2' and P1 and P2' positions to glutamate did not block PMA-induced shedding. The lack of effect on shedding by these mutations is not unexpected given previously published mutagenesis studies on other ADAM17 substrates, such as L-selection, GPVI and ACE (*234, 247, 254*). 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 an 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 on the topological parameters (*156*), we show here that in CHO-K1 cells stably expressing ACE2, that by reversing the positive charge of the arginine residues in P1, and P1 and P2' positions to negatively charged glutamate, we observed a significant increase in PMA-induced shedding (Figure 4-12). A similar shedding pattern was observed for the ACE2 homologue ACE, studies showing that mutation around the cleavage-site was able to increase ectodomain shedding (*232*). Leucine substitution at 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 (*232*). The molecular mechanism for the ACE2 cleavage-secretion and the spatial role of each of the amino acid within the proposed cleavage site will warrants further investigation. Furthermore, research looking into the structural conformation of ACE2 juxtamembrane region would perhaps be able to provide valuable data for the study of the regulated shedding of ACE2, and perhaps also contribute to our understanding of shedding of other membrane-bound proteins.

In conclusion, the present study demonstrates the possible cleavage-site for ADAM17 mediated shedding of ACE2 ectodomain. Although our preliminary experiments argue against a relevant report published recently, we nonetheless were able to demonstrate that ADAM17 is able to cleave peptide mimics at a position that corresponds to Arg^{708} – Ser^{709}, and this cleavage is attenuated in the presence of an ADAM17 specific inhibitor. Studies involving mammalian cells stably expressing ACE2 mutants provided no evidence that alanine substitution at P1 or P1' positions play a role in ADAM17-mediated shedding. In contrast, glutamate substitution at P1 and/or P2' positions significantly enhanced ADAM17-mediated shedding, possibly at an alternate cleavage site(s). Taken together, these data will set the stage for more comprehensive studies looking into this proposed cleavage-site and mechanisms to regulate ADAM17-mediated ectodomain shedding of ACE2.

5 ROLE OF ANGIOTENSIN PEPTIDES IN THE REGULATION OF ACE2 SHEDDING

5.1 Introduction

The renin angiotensin system generates peptide hormones that not only have significant impact on cardiovascular and respiratory regulation, but they also play a role in the pathogenesis of heart, lung and kidney disease. As mentioned in previous chapters, angiotensinogen mainly expressed in the liver is metabolised by renin from the kidney, to liberate AngI in the plasma. AngI is then further converted into AngII by ACE, which is primarily expressed on the outer surface of endothelial cells. The actions of AngII are transmitted via two main GPCR receptors, AT1R and AT2R (*255-257*). Most cardiovascular and respiratory effects of AngII can be ascribed to the AT1R receptor. Effects of the AT1R upon AngII binding are mediated by a complex array of intracellular signaling pathways, such as G-protein and phospholipase activation followed by an increase in intracellular inositol 1,4,5-trisphosphate (IP3) and calcium mobilisation (*122, 258, 259*). In addition, signaling cascades such as the PKC signaling pathway (*260*), small GTP-binding proteins (that act as molecular switches to regulate cellular responses) (*127*), and tyrosine kinase pathways are also activated and/or amplified, affecting a number of the mitogen-activated protein (MAP) kinase family, such as extracelullar regulated kinase 1 and 2 (ERK 1/2), p38 and Janus kinase-signal transducers and activators of transcription (STAT) (*130, 261, 262*). Furthermore, the AT1R also mediate transcription factors activation and initiates the expression of growth related genes (*132*). Finally, AT1R activation also causes the production of reactive oxygen species by NAD(P)H oxidase activation in a number of cells, which in turn leads to the

transactivation of other receptors such as EGFR and the mineralcorticoid receptor (*263*). In the heart, AngII-induced activation of AT1R ultimately leads to coronary vessel constriction, stimulation of aldosterone secretion, myocyte hypertrophy, non-myocyte proliferation, interstitial fibrosis, and presynaptic facilitation of noradrenaline release from cardiac sympathetic nerve terminals (*264, 265*). Thus, experimental and clinical studies have shown that AT1R antagonists are more effective than an ACE inhibitor and that AT1R antagonists attenuate most of the deleterious effects of AngII in the heart (*266*).

Compared to AT1R, AngII binds less extensively to AT2R. The actions of the AT2R are, in most cases appear opposite of those of the AT1R, and are partly mediated by the activation of the kallikrein-kinin system (KKS), a hormone system with two receptors for the effector peptides, the kinins, released from kininogen precursors by kallikrein enzymes (*267*). The interactions between the RAS and the KKS are numerous (*268*), however a detailed description of their actions is beyond the focus of this chapter. Nevertheless, a direct physical and functional interaction between AT1R and the kinin B2 receptor of the KKS has been previously described (*269*), though these data have been recently challenged (*270*). Unlike AT1R which is widely expressed in most cells, AT2R is only highly expressed in fetal tissues and its expression decreases significantly to a low level in adults and is restricted to certain cell types in the brain, adrenal, heart, vascular endothelium, kidney, myometrium and ovary (*122, 271-273*). There are three known major transduction mechanisms which appear responsible for AT2R signaling: the activation of a number of protein phosphatases causing protein dephosphorylation (*274-276*), activation of NO release causing a subsequent increase in intracellular cyclic GMP level (a signaling pathway shared with AT1R) (*277*), and activation of phospholipase A2 causing a subsequent release of arachadonic acid to regulate intracellular pH (*278-280*). Notably, AT2R activation is able to dephosphorylate ERK 1/2 (*274, 276, 281*), and other targets such as STAT and receptor tyrosine kinases (*282, 283*), thus opposing the AT1R-induced action. Apart from potential AT1R counter-regulatory

interactions in the cardiovascular and renal systems, AT2R also plays a role in cell growth and differentiation, which is closely connected to its other recently ascribed role in regeneration in the nervous system (for review see (*267, 284-286*)).

Another signaling mechanism involving the angiotensin receptor is the interaction between*mas receptor*(*287*). The *mas* receptor is a G-protein-coupled receptor, and binding of Ang(1-7) to the *mas* receptor triggers the release of NO from the vascular endothelium to mediate vasodilation(*137, 138*). Moreover, Ang(1-7)-mediated activation of this receptor stimulates the release of prostaglandins, which inhibit cardiac myocyte growth (*288*). Studies have shown that mice lacking the *mas* receptor proteins, exhibit marked cardiac dysfunction (*289, 290*). Interestingly however, a recent study, has provided evidence for the existence of another Ang(1-7) receptor. This putative receptor expressed in rat aorta was sensitive to the Ang(1-7) antagonist D-Pro^{7}-Ang(1-7), but not to another antagonist, D-Ala^{7}-Ang(1-7) (*291*). However, there are some discrepancies in the data in this study given that the authors have previously reported that both Ang(1-7) antagonists were able to block Ang(1-7) vasodilatory effects in these rats(*139*). This leads to two major questions that need to be addressed: (1) are the reported effects thus far of Ang(1-7) due to the activation of the *mas* receptor, rather than an alternate Ang(1-7)-mediated responses?, and (*157*) are the current Ang(1-7) antagonists solely specific to *mas receptor*? Nonetheless, the precise characterisation and role of newly reported Ang(1-7) receptor remains to be elucidated thus adding to the complexity of the RAS.

The renin angiotensin system like many other endocrine systems is regulated through a series of both positive and negative feedback loops/crosstalk (*292*). For example, the formation of AngII within the vasculature exerts both a negative action via the suppression of the renin secretion from the kidney (*293*), and a positive effect by the stimulation of angiotensinogen expression and secretion from the liver (*294*). However, in this context,

comparatively little is known about the regulation of ACE2 expression and even less about its shedding. Gallagher et al (*295*) have shown that in specific rat brain regions (cerebellar and medullary astrocytes), treatment with AngII down regulates both ACE2 mRNA and protein and that this down-regulation can be blocked by the AT1R antagonist losartan, but not by an AT2R receptor antagonist. In heart, prevention of AngII production by ACE inhibitors leads to an increase in cardiac ACE2 expression (though not tissue ACE2 protein), while in parallel studies, AT1R blockade increases both ACE2 mRNA and ACE2 activity (*296*). Thus, evidence to date suggests that AngII lowers ACE2 levels, an action which presumably results in less degradation, and hence, potentiation of the AngII effects. A positive feedback system such as this may contribute to the well-known cumulative detrimental effects of an activated RAS. Another well-established effect of AT1R stimulation by AngII is the trans-activation of the EGF receptor, thought to be mediated through the activation of the sheddase ADAM17 and the subsequent proteolytic release of the cell surface HB-EGF (*297*). Thus in this study we test the hypothesis that AngII, acting via the AT1R, may likewise stimulate ACE2 shedding, leading to reduced peptide degradation, and thus further enhancing/prolonging the actions of angiotensin.

5.2 Experimental Procedures*(detailed in chapter 2)*

5.2.1 Cell culture and transfection

Huh-7, HEK-NHA-AT1, and CHO-K1 cells were maintained as described in *Chapter 2.1*. Transient expression of the full-length human ACE2 in HEK-NHA-AT1 cells was achieved by the calcium phosphate transfection protocol outlined in *Chapter 2.6.1*. Stable expression of full-length human ACE2 in CHO-K1 and HEK-NHA-AT1 cells were achieved by Lipofectamine™ 2000 transfection protocol outlined in *Chapter 2.6.3*. All cells were maintained at 37°C in 5% CO_2.

5.2.2 Plasmid construction

The expression construct for full-length human recombinant ACE2 containing a C-terminal FLAG sequence was engineered as described in *Chapter 2.3.1* (pcDNA3.1/V5-His-TOPO-ACE2)and *Chapter 2.3.3* (pIRESpuro-ACE2). Restriction digest and DNA extraction from gels were performed according to methods described in *Chapter 2.5*. The purification of plasmid constructs and protein expression were achieved as described in *Chapter 2.6.3.*Purified plasmid constructs were sequenced as described in *Chapter 2.4*.

5.2.3 Cell treatment and protein extraction

Huh-7 cells endogenously expressing human ACE2, CHO-K1 cells expressing ACE2 mutants, and HEK-NHA-AT1 cells were grown to 80% confluence in 100-mm tissue culture dishes and rinsed twice with serum-reduced medium prior to experimentation. Huh-7 cells were incubated with angiotensin I and II peptides *(see Chapter 2.13 for detailed protocols)*, EDTA, or vehicle control. HEK-NHA-AT1 cells were incubated with angiotensin I and II peptides, in the presence of ACE inhibitor, captopril, and specific AT1R and AT2R inhibitors including losartan, and PD123319, PMA, EDTA or vehicle control *(see Chapter 2.13 for detailed protocols)*. HEK-NHA-AT1 cells stably expressing full length human ACE2 and CHO-K1 cells stably expressing full length human ACE2 were both incubated with non-peptide AT1R agonist, L-162,313, as detailed in *Chapter 2.13*. Secreted proteins from the growth medium and proteins expressed on the cell surface were harvested according to methods described in *Chapter 2.7*. Protein concentrations were also determined, as detailed in *Chapter 2.8*.

5.2.4 ACE2 activity assay

The catalytic activity of the shed or soluble form of human ACE2 was measured using a highly specific fluorogenic substrate, as detailed in *Chapter 2.10.* Known amounts of protein were analysed by following the hydrolysis of a specific quenched fluorescent substrate monitored over time. Results were analysed against the appropriate controls and the significance of data sets were determined on normalised data using one-way ANOVA statistical analysis.

5.2.5 SDS-PAGE and immunoblotting

Concentrated medium samples, and harvested cell lysates and extracted protein bound to affinity resins were separated by SDS-PAGE and proteins electroblotted to PVDF, as described in *Chapter 2.17*. The membranes were incubated with antibodies to human ACE2 ectodomain or hemagglutinin antigen, according to the methods outlined in *Chapter 2.18*. Bands (staining intensity and relative retention) were compared to soluble recombinant human ACE2, which served as a positive control.

5.3 Results

5.3.1 Angiotensin peptides stimulate ACE2 shedding in Huh-7 cells

To explore our hypothesis, we initially incubated Huh-7 liver cells that endogenously express ACE2 protein, with angiotensin peptides for 12, 18 and 24 hours. Results showed that there are marked increases in ACE2 activity in the medium of the hepatocytes at all three time points upon incubation with 100 nM AngI and 100 nM AngII peptides, but not in the presence of EDTA. After 12 hours incubation, ACE2 activity in the medium of AngI and AngII

peptide treated cells had increased significantly increased (162.1 ± 6.0% and 153.4 ± 4.5%, respectively; n≥3, one-way ANOVA, $P< 0.05$), when compared to control. After 18 hours of incubation, ACE2 activity in the medium of AngI and AngII peptide treated cells showed increase of 146.9 ± 5.1% and 137.5%, respectively(n≥3, one-way ANOVA, $P< 0.05$), when compared to control. Furthermore, at 24 hours post-incubation, AngI and AngII peptides treated cells also exhibited an increase in ACE2 activity when compared to control (133.2 ± 2.5% and 137.9 ± 1.0%, respectively,n≥3, one-way ANOVA, $P< 0.05$). As a negative control, Huh-7 cells that were treated with 500 µM EDTA for 24 hours showed no ACE2 activity.

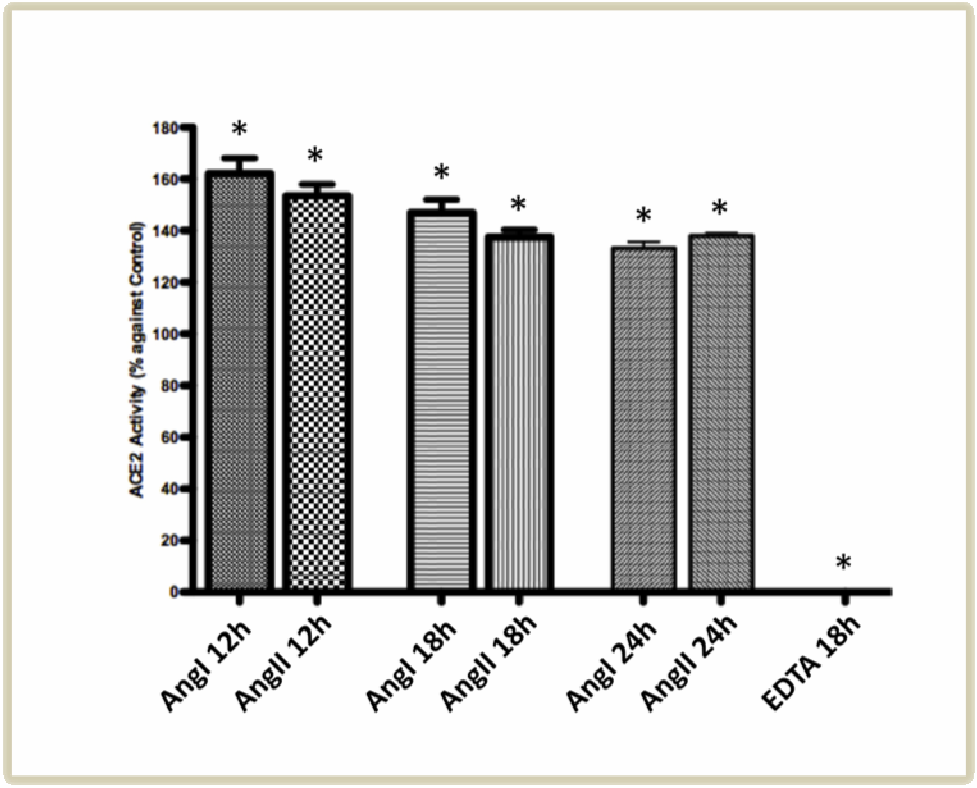


Figure 5-1: ACE2 activity from the concentrated medium of Huh-7 cells. Cells were stimulated with AngI or AngII peptides (100 nM), EDTA (500 µM) or in an equal volume of carrier (Me_2SO) for 12, 18 or 24 hours. Secreted protein (10 µg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from at least three independent experiments(n≥3, one-way ANOVA). Asterisks denote significant differences ($P< 0.05$) compared to all data under the same category.

5.3.2 Angiotensin peptides stimulate ACE2 shedding in HEK-NHA-AT1 cells.

After the initial observation in which both AngI and AngII peptides can both increase ACE2 secretion in Huh-7 cells, we next looked at the ability of these peptides to stimulate to the same level in different cells. This was achieved by transfecting HEK-NHA-AT1 cells, stably expressing AT1R, with full-length ACE2 constructs. Using this model system we have generated both transient and stable ACE2 expression cell lines, for the measuring the differences in shedding profiles observed following angiotensin peptides incubation. In the first set of data, HEK-NHA-AT1 cells, transiently expressing the ACE2 protein showed that AngI peptides (100 nM), AngII peptides (100 nM) or PMA (1 μM), alone, caused significant increase in ACE2 activity in the medium after 4 hours of incubation when compared to control (198.5± 14.7%, 205.0±10.3%, and 226.6± 11.9%, respectively; n≥3, one-way ANOVA, $P< 0.05$) (Fig. 5-2*A*). However these increases in ACE2 shedding is sensitive to the different antagonists used (Fig. 5-2*B*). AngI peptides (100 nM) in the presence of the ACE inhibitor, captopril (100 μM), and AngII peptides (100 nM) in the presence of the AT1R antagonist, losartan (1 μM), showed reduced ACE2 activity in the medium when compared to control (60.9 ± 9.8% and 51.6 ± 6.4%, respectively; n≥3, one-way ANOVA, $P<0.05$) (Fig. 5-2*B*). AngII peptide (100 nM) in the presence of AT2R antagonist, PD123319 (1 μM) showed no significant difference in ACE2 activity compared to control. As a negative control, cells that were incubated in the presence of EDTA (500 μM) showed only background fluorescence activities (Fig. 5-2*A* and 5-2*B*).

In HEK-NHA-AT1 cells that stably express full-length human ACE2, both cell lines (transfected using ACE2 gene in pcDNA3.1/V5-His-TOPO-ACE2 and pIRESpuro3-ACE2 vectors), displayed relatively high levels of ACE2 expression compared to non-and mock-transfected cells, measured by relative fluorescent units per minute per mg of protein

(2002.0 ±186.5 and 1596.0 ± 32.5 RFU/min/mg of protein, respectively; n≥3, one-way ANOVA, *P*< 0.05) after 42-days post-transfection (illustrated in Fig. 5-3*A* and Fig. 5-4*A*). AngI and AngII peptides (100 nM) and PMA (1 μM) treated cells showed a marked increase in ACE2 activity in the medium in both cell lines. To examine the expression of the AT1R receptor and ACE2, HEK-293 cells stably expressing the N-terminally epitope-tagged AT1R (NHA-AT1) and ACE2 were solubilised, and the immunoprecipitated receptor protein and ACE2 were analysed by SDS-PAGE. As shown in Fig. 5-3B and Fig. 5-4B, a broad band was observed that ranged between 70 and 140 kDa. This feature is likely to be associated with the heavy N-glycosylation of the AT1R receptor protein, as previously described by Thomas et al (*298*). When compared to control cells, incubation with AngII (100 nM), and PMA (1 μM), the HEK-NHA-AT1-ACE2 cells (generated by pcDNA3.1/V5-His-TOPO-ACE2 vector) showed a significant increase of 171.4 ± 26.2%, and 181.7 ± 20.0%, respectively (n≥3, one-way ANOVA, *P*< 0.05) ACE2 activity (Fig. 5-3*C*). In the different HEK-NHA-AT1-ACE2 cells (generated by pIRESpuro3-ACE2 vector), similar treatment showed a significant increase in ACE2 activity detected in the medium in the presence of 100 nM AngI peptides (154.5 ± 16.3%), 100 nM AngII peptides (152.5 ± 20.8%), 100 nM AngII peptides in the presence of AT2R antagonist, PD123319 (1 μM) (157.6 ± 13.1%), and 1 μM PMA (184.3 ± 11.0%)(n≥3, one-way ANOVA, *P*< 0.05)(illustrated in Fig. 5-4*C*).

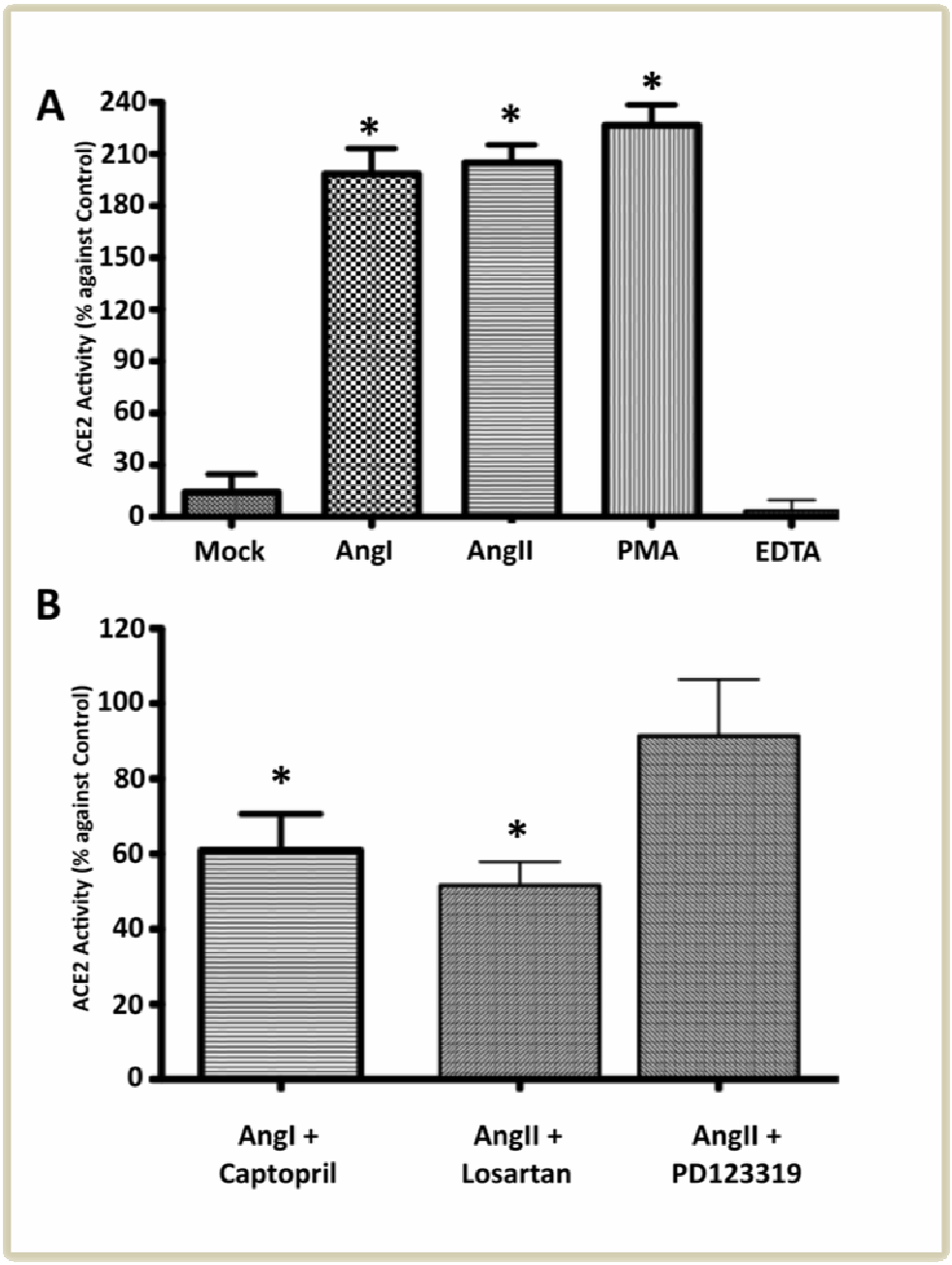


Figure 5-2: ACE2 activity from concentrated media of HEK-NHA-AT1 cells transiently expressing full-length ACE2. 10 µg of plasmid DNA(pcDNA3.1/V5-His-TOPO-ACE2) was used to transfect 60% confluent cells in 100 mm cell culture dishes using the calcium-phosphate transfection method described under 'Materials and Methods'.48 hours post transfection, cells were washed and serum free media added prior to further incubation for additional 16 hours.*A.* Cells were stimulated with AngI or AngII peptides (100 nM), PMA (1 µM), EDTA (500 µM)or in equal volume of carrier (Me_2SO) for 4-hours. B. Cells were stimulated with AngI

peptide (100 nM) + captopril (100 μM); AngII peptide (100 nM) + either losartan (1 μM) or PD123319 (1 μM), or in equal volume of carrier (Me_2SO) for 4-hours. Secreted protein (10 μg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from three independent experiments($n \geq 3$, one-way ANOVA, $P < 0.05$). Asterisks denote significant differences ($P < 0.05$) compared to against control.

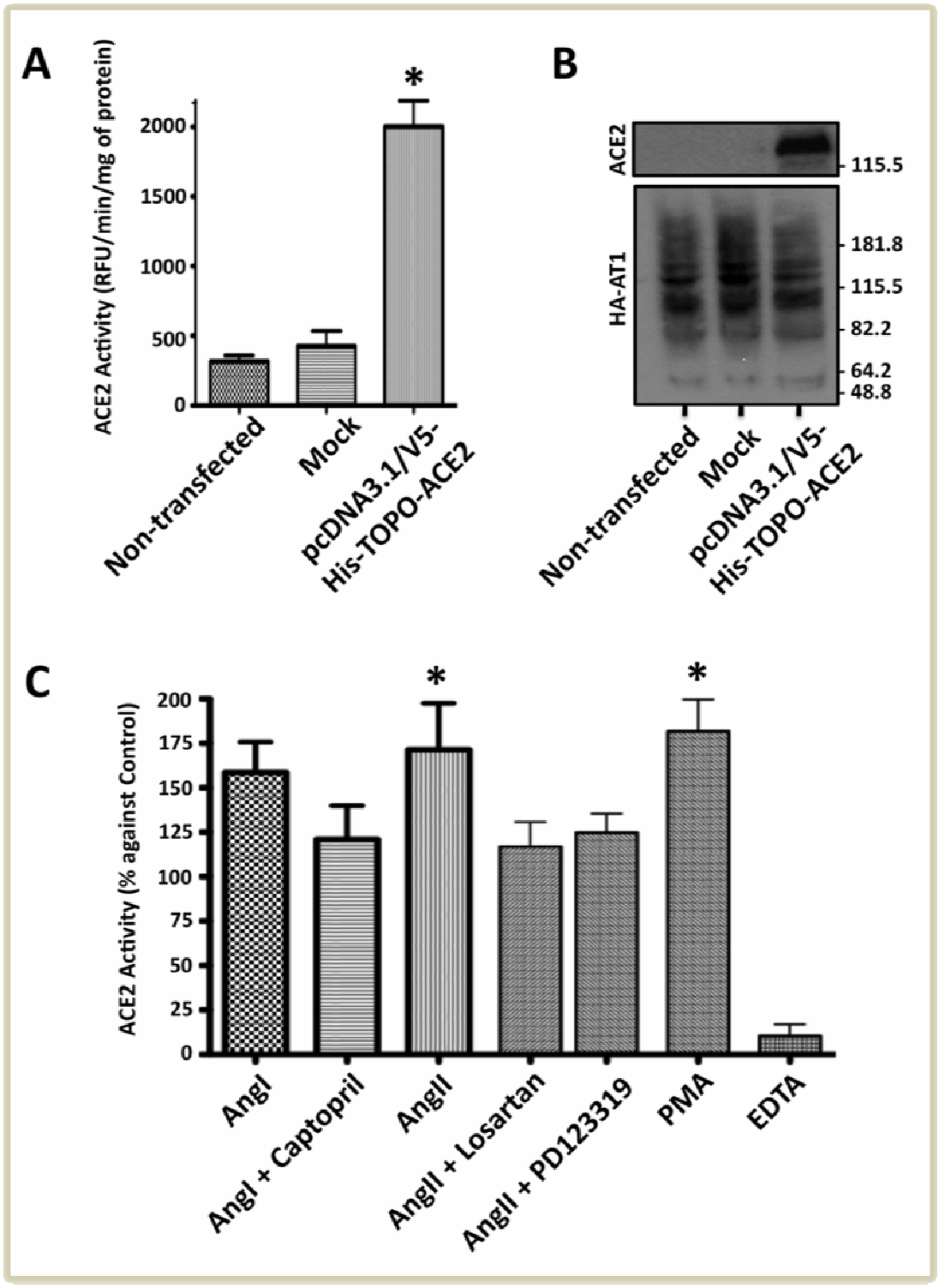


Figure 5-3: ACE2 activity from concentrated media of HEK-NHA-AT1 cells stably expressing full-length ACE2. 10 µg of plasmid DNA(pcDNA3.1/V5-His-TOPO-ACE2) were used to transfect 60% confluent cells in 100 mm cell culture dishes using the Lipofectamine™ 2000 transfection method described under 'Materials and Methods'.Fourty-two days post transfection, cells were screened for ACE2 level of expression.*A.*10 µg (total protein) of cell

lysate from non-transfected cells, mock-tranfected cells and cells tranfected with pcDNA3.1/V5-His-TOPO-ACE2 plasmid were assayed twice for ACE2 activity. *B.*250 µg (total protein) of the cell lysate from stable HEK-NHA-AT1 cells expressing ACE2 were separated by SDS-PAGE and western immunoblotted with a monoclonal anti-ACE2 and anti-HA antibodies. *C.*Cells were stimulated with AngI peptide (100 nM), and together in the presence of captopril (100 µM); AngII peptide (100 nM), and together in the presence of losartan (1 µM) or PD123319 (1 µM); PMA (1 µM), EDTA (500 µM)or in equal volume of carrier (Me_2SO) for 4-hours. Secreted protein (10 µg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from three independent experiments($n \geq 3$, one-way ANOVA). Asterisks denote significant differences ($P < 0.05$) compared against control.

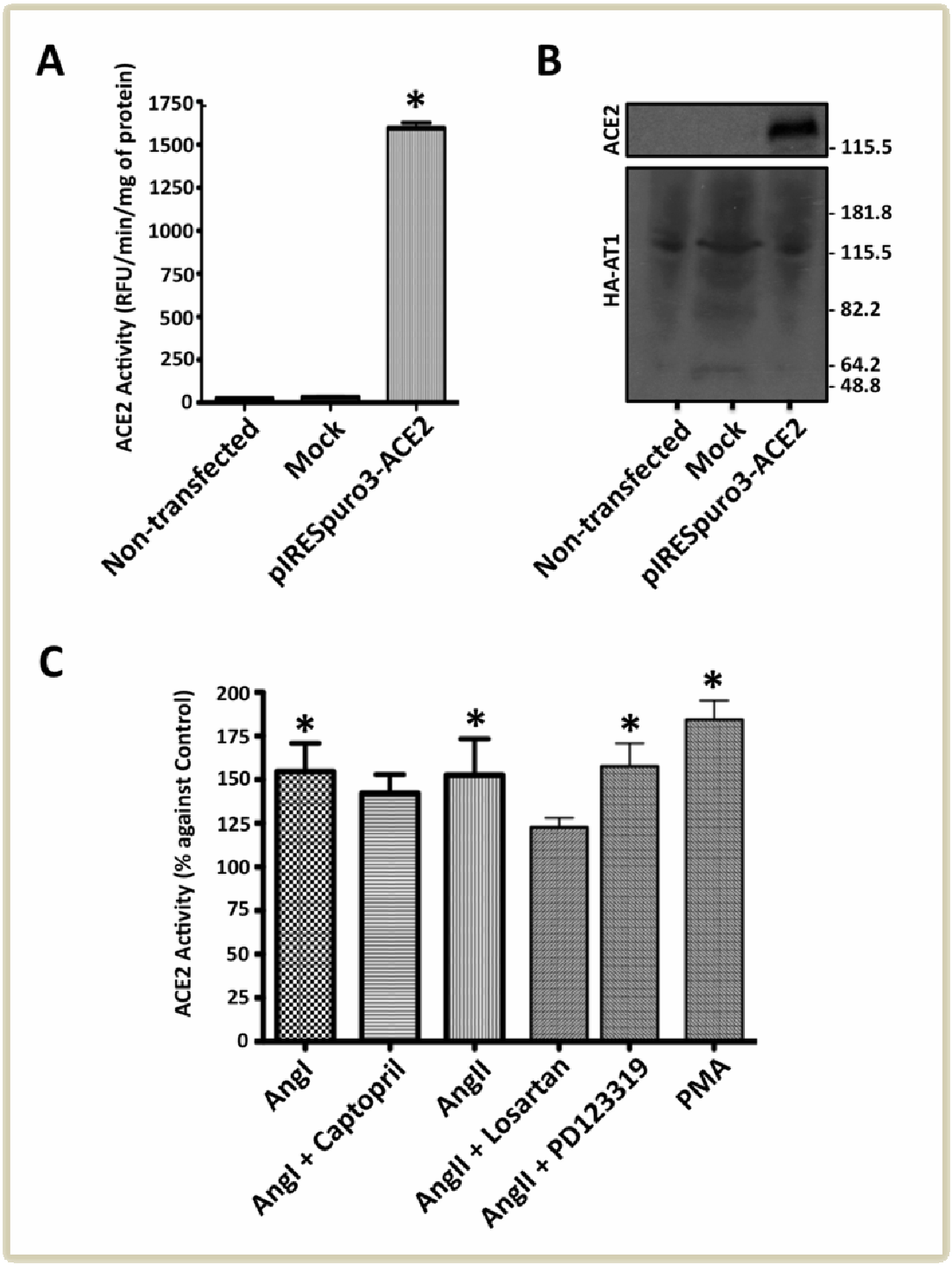


Figure 5-4: ACE2 activity from concentrated media of HEK-NHA-AT1 cells stably expressing full-length ACE2. 10 µg of plasmid DNA(pIRESpuro3-ACE2) were used to transfect 60% confluent cells in 100 mm cell culture dishes using the Lipofectamine™ 2000 transfection method described under 'Materials and Methods'.Fourty-two days post transfection, cells were screened for ACE2 level of expression.*A.*10 µg (total protein) of cell lysate from non-

transfected cells, mock-tranfected cells and cells tranfected with pIRESpuro3-ACE2 plasmid were assayed twice for ACE2 activity.*B.*250 µg (total protein) of the cell lysate from stable HEK-NHA-AT1 cells expressing ACE2 were separated by SDS-PAGE and western immunoblotted with a monoclonal anti-ACE2 and anti-HA antibodies. *C.*Cells were stimulated with AngI peptide (100 nM), and together in the presence of captopril; Angiotensin II peptide (100 nM), and together in the presence of losartan (1 µM) or PD123319 (1 µM); PMA (1 µM), or in equal volume of carrier (Me_2SO) for 4-hours. Secreted protein (10 µg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from at least three independent experiments(n≥3, one-way ANOVA). Asterisks denote significant differences ($P < 0.05$) compared against control.

5.3.3 Effects of AT1R receptor agonist (L-162,313) on ACE2 shedding

In addition to stimulating ACE2 secretion in HEK-NHA-AT1-ACE2 cells, cells were also treated with the metabolically stable non-peptide AT1R receptor agonist L-162,313 (*299*) for 4 hours. Following treatment, ACE2 activity was measured in the media. Results showed that incubation of the cells with 1 µM L-162,313 showed significant decrease in ACE2 activity (72.7 ± 6.2%) when compared to control (n≥4, one-way ANOVA) after 4 hours of incubation. As positive control, 1 µM PMA showed significant increase in ACE2 activity (139.1 ± 6.2%) in the concentrated medium compared to the control (n≥4, one-way ANOVA) (Fig. 5-5*A*). In a parallel experiment, CHO-K1 cells that stably express full-length human ACE2 were also incubated with L-162,313 and PMA. When compared to control cells, the use of 1 µM of L-162,313 however, resulted in no significant changes in ACE2 activity, while 1 µM PMA significantly increased ACE2 activity in the medium (153.0 ± 4.2%) (n≥3, one-way ANOVA) (Fig. 5-5*B*). We also investigated the changes in ACE2 activity when HEK-NHA-AT1-ACE2 cells were incubated with L-162,313 over a time course of 4 hours.

Significant reductions in ACE2 activity in the concentrated medium from treated cells were detected after stimulation with L-162,313 following 2 hours of incubation (T = 2 hours: 78.0 ± 7.6% reduction, T = 3 hours: 76.1 ± 2.2% reduction, T = 4 hours: 65.1 ± 3.3% reduction; n≥3, one-way ANOVA) when compared to controls)((Fig. 5-5*C*). In addition, any potential phenotypic consequences of L-162,313 incubated HEK-NHA-AT1 cells were examined by phase microscopy (Fig. 5-6). HEK-NHA-AT1 cells that were stimulated with L-162,313 displayed an altered morphology typified by smaller, irregularly-shaped cells, which were poorly attached to the tissue culture dishes (Fig. 5-6*B*) compared to mock-stimulated cells (Fig. 5-6*A*).

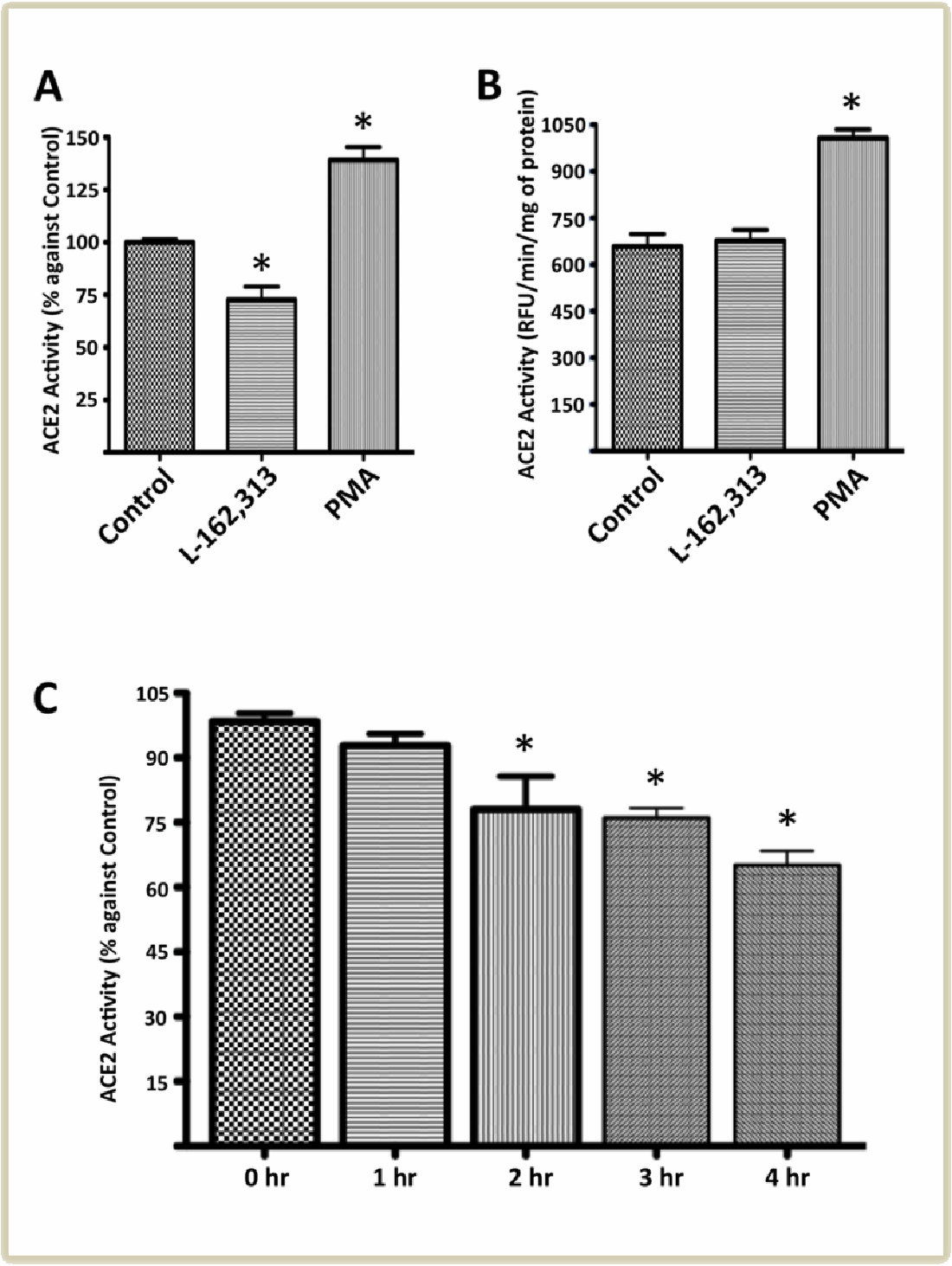


Figure 5-5: ACE2 activity of the concentrated media from cells stimulated with AT1R agonist, L-162,313. *A.* HEK-NHA-AT1 cells stably expressing full-length ACE2 were treated with the non-peptide AT1R agonist, L-162,313 (1 μM), PMA (1 μM) or with an equal volume of carrier (Me_2SO) for 4 hours. *B.* CHO-K1 cells stably expressing full-length ACE2 were stimulated with L-162,313 (1 μM), PMA (1 μM), or with an equal volume of carrier (Me_2SO) for 4 hours. *C.*

Time course stimulation of non-peptide AT1R agonist L-162,313 (1 μM) in HEK-NHA-AT1 cells stably expressing full-length ACE2. Secreted protein (10 μg) was analysed using an ACE2 specific quenched fluorescent substrate assay. Data are normalised against the appropriate controls from at least 4independent experiments(n≥4, one-way ANOVA). Asterisks denote significant differences ($P < 0.05$) compared to T = 0-hour.

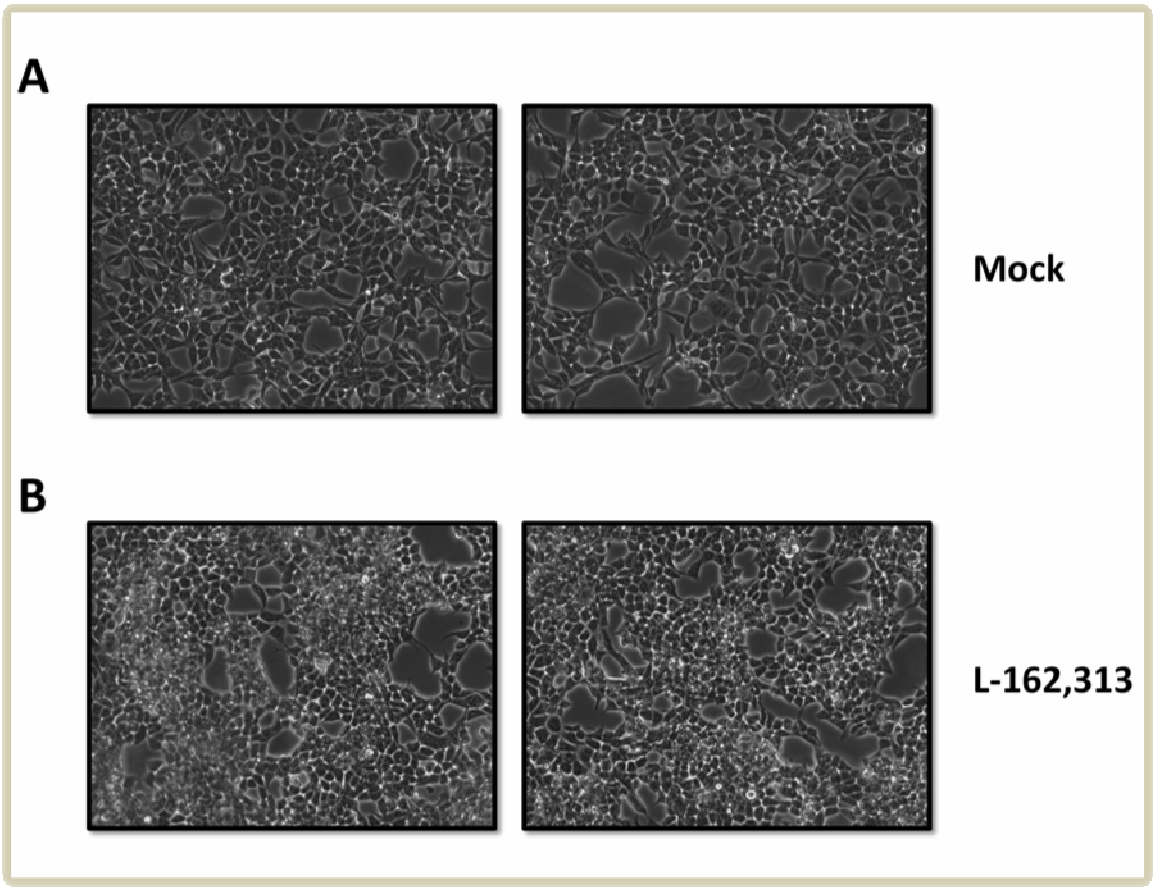


Figure 5-6: Changes in cell size and phenotypic morphology of HEK-NHA-AT1 cells incubated with AT1R agonist, L-162,313. HEK-NHA-AT1 cells stably expressing full-length ACE2 were stimulated with *A.* mock (Me_2SO), or *B.* L-162,313 (1 μM), in Opti-MEM® serum reduced medium for 4 hours prior to visualisation using phase microscopy.

5.4 Discussion

The present study investigates the use of angiotensin peptides (namely AngI and AngII), together with specific AT1R and AT2R inhibitors in stimulating the release of the ectodomain of human ACE2 present in endogenous and tranfected cell lines. In the initial experiment Huh-7 cells, endogenously expressing ACE2, were treated with both AngI and AngII peptides, and the resulting effects of these peptides on ACE2 shedding were measured. The hypothesis to be tested proposes that AngII, acting via the AT1R, may lead to the initiation of ACE2 ectodomain shedding. AngII however, is also the preferred physiological substrate of ACE2. Despite the fact that AngII peptide could be hydrolysed by membrane bound andsoluble form of ACE2 during cell incubation, there was a detectable increase in the amount of ACE2 shed, reflecting the significant increase in ACE2 activity in the medium (Fig. 5-1). In a separate experiment performed by a colleague in our laboratory (Iresha Hanchapola, Monash University) also demonstrated a similar increase in ACE2 activity in the medium of these cells in the presence of AngII peptides, in varying concentration (100 nM, 1 μM, and 10 μM) (unpublished data). More interestingly, the results from these experiments also indicate an increase in ACE2 activity in the media when these cells were incubated with AngI peptide (Fig. 5-1). AngI alone is an inactive peptide and does not play role itself in the RAS pathway. In order to effect cardiovascular and respiratory regulation, AngI must first be converted to AngII. Hence, the above-observations could be possibly due to: (1) the removal of two amino acids in the amino end of the peptide, as a result of peptide degradation or breakdown during the incubation period, (*157*) the presence of ACE on Huh-7 cells, converting AngI into AngII, or perhaps (3) the presence of an unknown receptor that interacts with AngI to stimulate ACE2 ectodomain shedding. Unfortunately, during the course of this preliminary study, we were unable to precisely confirm the relative level of AT1R expression in these cells, as no antibody against AT1R was made available commercially. Although the AT1R is known to be constitutively expressed in hepatocytes *in vivo*, some studies have previously shown that when cells are placed in culture, its expression is

diminished or even lost (*122, 294*). This could potentially be resolved by stably transfecting the Huh-7 liver cells with a recombinant AT1R construct.

As an alternative, transfected HEK-293 cells that stably express AT1R bearing N-terminal HA epitope tag (NHA-AT1) generously provided by Dr. Walter Thomas (University of Queensland) were used in subsequent experiments. As predicted, very low levels of ACE2 activity were detected in non-transfected and mock-transfected cells (Fig. 5-2*A*, Fig.5-3*A* and Fig. 5-4*A*). In a similar experiment, Li et al have also previously demonstrated that mock-transfected HEK-293 cells express very low level of ACE2 (*182*). The HEK-NHA-AT1 were further transfected to express both transient and stable full-length ACE2 constructs. Transient expression of ACE2 protein in these cells showed significant increase in ACE2 activity detected in the medium, when incubated with AngI and II peptides (Fig. 5-2*A* and Fig. 5-2*B*). Some slight variations in percentage increase in shedding profile of these transient ACE2 expressing cells versus Huh-7 cells were observed, this variation may be generated as a result from varying transfection efficiencies between each transfection experiment (each batch of transient ACE2 expressing cells were transfected separately for each incubation period, to a total of three or more separate stimulation experiments being individually performed). In the presence of captopril (ACE inhibitor) and losartan (AT1R antagonist), ACE2 activity in the medium is reduced to a comparable level measured against control. It would not be expected that AngII, in the presence PD123319 (AT2R antagonist), would have an effect on ACE2 shedding. However, a significant reduction in ACE2 activity was observed in the medium compared to AngII treatment alone. This could perhaps also be due to the difference in transfection efficiencies between each of the transfection experiments.

To address this issue, two separate stable cell lines were generated that express ACE2 using different ACE2 constructs (Fig. 5-3*A* and Fig. 5-4*A*). First, the data from stable expression of ACE2 protein in HEK-NHA-AT1 cells showed an increase in ACE2 activity in the

medium of cells incubated with AngI and AngII peptides (Fig. 5-3*B* and Fig. 5-4*B*), following a similar shedding profile to that observed in Huh-7 and HEK-NHA-AT1 cells that transiently expressed ACE2 in the previous experiments. In the first stable cell line (transfection using ACE2 construct expressed in pcDNA3.1/V5-His-TOPO vector), a similar shedding profile was observed compared to the cells expressing transient ACE2. If the hypothesis is true, it would be expected that an improved result compared to transient cell line, such as higher ACE2 activity in the medium from cells that were treated with AngII peptides and AT2R antagonist (PD123319) would be observed. However, a reduced effect in ACE2 shedding was noted when cells were incubated with AngII peptide in the presence of PD123319). The acquired HEK-NHA-AT1 cells, first generated by Thomas et al (*300*), using the NHA-AT1 construct engineered in an adenovirus vector that is resistant to neomycin selective antibiotic. The attempt to create a second generation of stable cell lines; by stably transfecting the HEK-NHA-AT1 cell line expressing ACE2 using the ACE2 construct in pcDNA3.1/V5-His-TOPO vector, (a vector also resistant to neomycin selective antibiotic), was perhaps not the optimum approach due to selectivity issue. Nonetheless, during the colony selection process (Day 07 post-transfection), using fluorescence assisted cell-sorting technology, individually selected cells were collected and maintained separately for a period of 42 days, prior to individually screening for ACE2 expression using the ACE2 QFS assay.

As an alternative, the second approach used was to excise the human ACE2 gene into a different vector that is resistant to a different antibiotic. In this case, the pIRESpuro3 vector that is resistant to puromycin antibiotic was chosen, and a similar screening process was repeated and maintained over the duration of 42 days prior to further experimentation. Finally, when incubated with angiotensin peptides, it was observed that captopril had a minimal effect on AngI peptide stimulated shedding of ACE2 (Fig. 5-4B), suggesting that the increase in ACE2 activity was not due to the conversion of AngI to AngII by ACE, but perhaps due to non specific peptide degradation. To eliminate the issue of AngII potentially being

hydrolysed by ACE2, the cells were also treated with the metabolically stable AT1R receptor agonist L-162,313 (*299*). L-162,313 has been previously shown to mimic the effect of AngII in a variety of cultured cells, such as in human monocyte-macrophages, rat aortic smooth muscle cells, CHO cells, COS-1 and COS-7 cells (*299, 301-306*). However, the use of this AT1R agonist in our experiment showed not only a significant reduction in ACE2 activity in the medium, but also resulted in significant changes in cell morphology, compared to control cells. In a separate experiment, CHO-K1 cells that stably express full-length ACE2, but not the AT1R receptor, were treated with equal amount of L-162,313 for 4 hours. In this experiment, no changes in ACE2 activity from the concentrated medium were observed when compared to ACE2 (Fig 5-5*B*). Nonetheless, we were unable to shed any light on understanding the nature of this effect (HEK-NHA-AT1 cells treated with L-162,313). To date, no study has been performed using HEK-293 (derived/variant) cells for AT1R stimulation by L-162,313. One possible explanation is that perhaps the two-step transfection procedure is responsible; first, the generation of the NHA-AT1 stable cell line, and that the stable ACE2 expression, has a detrimental effect on these cells when stimulated with L-162,313. This issue can be potentially examined by performing a reciprocal experiment, by co-transfecting CHO-K1 cells stably expressing the full-length human ACE2 with the AT1R construct.

Future experiments could perhaps be carried out in the presence of a specific ACE2 inhibitor, such as MLN-4760. This would inhibit the action of ACE2 in hydrolysing AngII peptide, and thus exclude the possibility that the product of ACE2 action on AngII, Ang(1-7), which may itself modulate ACE2 shedding. Currently, MLN-4760 is the only ACE2 inhibitor reported to inhibit ACE2 with high efficacy and potency(*307*). Additionally, a commercially available ACE2 inhibitor, DX600 (Phoenix Pharmaceuticals) is reported to have 100-fold less potency compared to MLN-4760(*308*). Of course the problem with this strategy would be the sensitive detection of ACE2, the fluorescent cleavage assay used in this study would not work in the presence of the ACE2 inhibitor.

6 GENERAL DISCUSSION

6.1 Summary

As detailed in Chapter 1, the renin angiotensin system or RAS is one of major regulators of cardiovascular homeostasis, and its over activity has been implicated in the pathogenesis of cardiovascular disease leading to stroke, myocardial infarction and end-stage renal disease. ACE and ACE2 are two homologous enzymes known to play pivotal roles in hydrolysing and thus generating various angiotensin peptides in the RAS cascade. Since its discovery in 2000, the bulk of the research on ACE2 has focused on the potential physiological roles for this enzyme in the regulation of the cardiovascular, renal and respiratory function. However, to date, there has not been a comprehensive study looking at the local regulation of ACE2, particularly examining the mechanisms by which the ectodomain of ACE2 can undergo proteolysis to release an active soluble form of the carboxypeptidase, into the external milieu. The research described in this thesis has characterised several mechanistic components involved in the regulation of ACE2 ectodomain shedding, specifically examining its association with CaM, the potential cleavage site for the shedding mediated by ADAM17 and the potential activation/regulation of shedding by angiotensin itself through AT1R receptor stimulation.

The first set of experiments described in chapter 3 of this thesis examined the potential interaction of CaM, an intracellular calcium regulatory protein, in associating with, and thus regulating the ectodomain shedding of ACE2. CaM has previously been shown to interact with the ACE2 homologue ACE, which in turn leads to activation of the ectodomain shedding of this metalloprotease. In this context, CaM has is known to be involved in

regulating the ectodomain shedding of many other membrane-bound proteins and this is mediated through CAM binding to a region of hydrophilic charged amino acids in the cytoplasmic tail of interacting proteins (*193*). Despite the fact that ACE2 is the only known mammalian ACE homologue (sharing ~42% sequence similarities), the cytoplasmic region of ACE2 however shares no recognisable homology with ACE. Therefore, the initial aim of these studies was to identify and characterise any potential associations between CaM and ACE2. The studies detailed in Chapter 3 of this thesis reveal that CaM is capable of binding to specific ACE2 peptide mimetics based on potential CAM binding sites in the cytoplasmic tail of ACE2, forming a CaM-ACE2 complex as evidenced by the co-migration of the CaM band in a gel shift assay in the presence of ACE2. Additionally, based on evidence that full length ACE2 is able to co-immunoprecipitate with CaM in a pull-down assay, we concluded that CaM is able to bind to a putative CAM binding domain identified in the cytoplasmic tail of ACE2. To explore the possibility that this binding of CaM to ACE2 plays any role in regulating the ectodomain shedding of ACE2, cell stimulation studies were performed using CaM inhibitors. Also detailed in Chapter 3, are a series of experiments describing the application of two different specific CaM inhibitors, both causing significant increases in ACE2 shedding from Huh-7 cells which endogenously express ACE2. Finally it was also demonstrated that this CaM-induced shedding of ACE2 is independent of the PKC signaling pathway.

The following chapter details a series of studies looking at the possible ADAM17 cleavage site within the ACE2 ectodomain which mediates shedding. In 2005, our laboratory demonstrated that the ACE2 undergo ectodomain undergos shedding *in vitro*, and that the rate/level of shedding is increased upon treatment with phorbol esters (*46*). In the same study, our laboratory also successfully identified that ADAM17 is responsible for the cleavage-release of this enzyme. The work described in Chapter 4 builds on these initial findings, to specifically investigate and characterise the cleavage site(s) within the extracellular domain of ACE2. Although another study has recently suggested that the

cleavage site lies within approximately twenty-five amino acid residues proximal to the transmembrane domain (*239*), our preliminary evidence suggests that the cleavage site is actually localised slightly more distal than the region proposed by the original authors and is located at approximately thirty-three amino acid residues proximal to the transmembrane domain. Furthermore, in similar experiments to those described by the authors, we were not able to reproduce the reported findings. While a consensus sequence Ser-Ala-Arg-Ser-Glu-Gly has been previously identified as the ACE cleavage site, with cleavage occurring between the Arg^{663} – Ser^{664}(*207*), no analogous sequences are present in the membrane proximal region of ACE2. Nonetheless, the data generated using peptide mimetics around the putative ACE2 cleavage site suggests that the ADAM17 cleavage site in ACE2 could reside at Arg^{708} – Ser^{709} bond. Both these P1 and P1' residues are found not only at the ADAM17 cleavage site reported for ACE, but also reported for L-selectin (*248*) and amphiregulin(*249*). The final experiments outlined in Chapter 4 also reveal that despite mutations of either or both the P1 and P1' sites these ACE2 mutants expressed in CHO cells were still able to undergo ectodomain shedding, suggesting the possibility of ADAM17 cleaving ACE2 at either an alternative site or that conformation and/or spatial orientation is critical of ADAM 17 recognition and cleavage. Chapter 4 also presents evidence that ACE2 can undergo increased ectodomain shedding when the positively charged amino acids at P1 and P2' position are reversed.

In the final study described in Chapter 5 of this thesis the potential role of angiotensin peptides in the regulation of ACE2 ectodomain shedding was examined. There is very little in the present literature describing the regulation of ACE2 expression and even less about its shedding mechanism. Previous studies from this laboratory have shown that catalytically active ACE2 is present in human plasma and that the levels are elevated as a consequence of cardiovascular disease, likely reflecting the increased tissue expression of ACE2 and thus its shedding in the heart following myocardial ischemia (*3*). AngII is the key peptide product of

the RAS and elevated levels of AngII results in increased local and systemic blood pressure, hypertrophy and fibrosis. AngII mediates most of its cardiovascular actions action via AT1R receptor in a complex series of interacting signaling pathways (*262*). Given the fact that AT1R stimulation has been shown to shed HB-EGF, we hypothesised that AT1R stimulation could perhaps activate the shedding of ACE2. The experiments detailed in Chapter 5 provide preliminary evidence that the activity of ACE2 in the concentrated medium of Huh-7 and transfected HEK-NHA-AT1 cells were increased following the treatment with AngI and AngII peptides. As AngI is an inactive peptide, we suggest that the increased ACE2 activity from AngI peptide was perhaps due to the peptide degradation/endogenous conversion to AngII during the incubation period, even when these experiments performed in the presence of an ACE inhibitor. Nevertheless the experiments mentioned in Chapter 5 clearly show that AngII leads to elevated ACE2 shedding in Huh-7 and HEK-NHA-AT1 cells that stably express ACE2. The results from Chapter 5 also show the effect of the metabolically stable AT1R receptor agonist L-162,313 in transfected HEK-NHA-AT1 cells, showing a gradual decrease in ACE2 activity media, with an accompanying significant change in cell morphology during treatment period, likely reflecting a toxic effect on these cells. Although beyond the scope of the present work, future experiments looking at the effect of Ang(1-7) and/or *mas* receptor on ACE2 shedding in these cells would determine if the experimental findings observed in Chapter 5 are a consequence of AngII hydrolysis.

6.2 Future Directions

Ectodomain shedding of ACE2 is clearly a crucial event for regulating ACE2 catalytic activity at its sites of expression. The studies outlined in this thesis contribute to our knowledge and understanding of both constitutive and regulated shedding mechanisms for ACE2 as well as the protease(s) involved in mediating the shedding event. Given the likely

very important role of ACE2 in regulating cardiovascular and respiratory functions, as well as its proven role in mediating SARS-CoV infection, further studies are needed to identify the precise mechanisms involved in the physiological regulation of ACE2 expression *in vivo* and also its local activity. Such studies for example, could address the identification of other sheddase(s) and the existence of alternative cleavage sites in ACE2 that liberate the active soluble form of ACE2. Sheddases and ectodomain shedding have been well documented in playing important roles, not only for normal cellular development, but also in the pathology of certain diseases. Many of these diseases, such as inflammatory and autoimmune responses, cancer and cardiovascular diseases show elevated level of expression of these sheddases. For instance, one potential study looking at both ACE2 and ADAM17 distribution and expression, would not only provide a better understanding of the regulation of ACE2 and it's shedding, but it would also perhaps provide an avenue for new drug development that targets this event. For example, following the SARS epidemic 2003, ACE2 has now become a new target for therapeutic and vaccine development as well as being a potential biomarker candidate for cardiovascular diseases (*309*). Alternatively, in the RAS cascade for example, the therapeutic manipulation of ACE2 expression and/or its subsequent shedding, could serve to prevent progressive tissue damage, caused by elevated local blood pressure, hyperthrophy and fibrosis induced by elevated levels of locally produced angiotensin II. Finally, although the studies outlined in this thesis have demonstrated that AngII could potentially initiate and regulate the signaling mechanism that mediates ACE2 ectodomain shedding, we are only just barely scratching the surface in understanding this potential complex feedback loop. Studies looking at the potential of each of the individual angiotensin peptides, including the ones not reviewed in this thesis which arise from the proteolytic activity of rennin, ACE and ACE2, and their interaction with their specific receptors, in regulating both ACE2 expression and the ectodomain shedding event is also clearly worthy of further investigation.

In conclusion, this thesis provides strong evidence suggesting the involvement of calmodulin in regulating the ectodomain shedding of ACE2, an event which seems independent of the protein kinase C signaling pathway. We also propose that ADAM17 is able to cleave ACE2 between Arg^{708} – Ser^{709}, and finally, that AT1R receptor activation may lead to the stimulation of ectodomain shedding in ACE2. Given that ACE2 has recently been shown to be the functional receptor for the severe-acute respiratory syndrome (SARS) coronavirus (SARS-CoV) and also that it has been implicated in cardiovascular, renal and respiratory regulation, the studies described in this thesis adds to our knowledge of how the ectodomain shedding of this important membrane protein may be actively regulated. More importantly, the experiments in this thesis have uncovered many new and novel findings, which provide a prelude to more comprehensive studies to further examine the precise mechanism and regulation of ACE2 ectodomain shedding.

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7 PUBLICATIONS

7.1 Publication 1 *(Endocrinology 2009)*

RENAL-CARDIAC-VASCULAR

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-angiotensin 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-

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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′-GGTACCATGTCAAGCTCTTCCTGGCTCC-3′) and reverse (5′-CGCTCGAGTCACTTGTCATCGTCGTCCTTGTAGTCAAAGGAGGTCTGAACATCATC-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

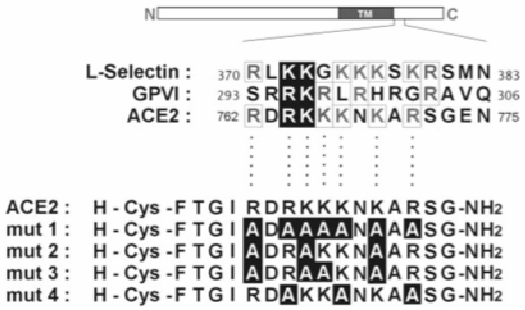

FIG. 1. Sequence comparison of ACE2 to GPVI and L-Selectin. *Top panel*, Cytoplasmic juxtamembrane sequence of human ACE2 compared with functional CaM-binding juxtamembrane sequences that regulate metalloproteinase-mediated ectodomain shedding of platelet GPVI and leukocyte L-selectin (40, 41). Shown 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.

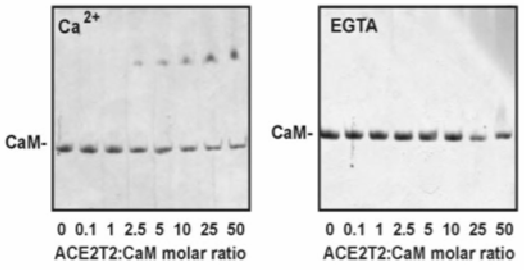

FIG. 2. Calmodulin-peptide complex formation. Purified CaM (300 pmol/tube) was incubated with increasing amounts (0; 30; 300; 750; 1,500; 3,000; 7,500; and 15,000 pmol) of peptide ACE2T2 in the presence of 4 mol/liter urea and either Ca^{2+} or EGTA. Complexes were run on gels containing 4 mol/liter 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*).

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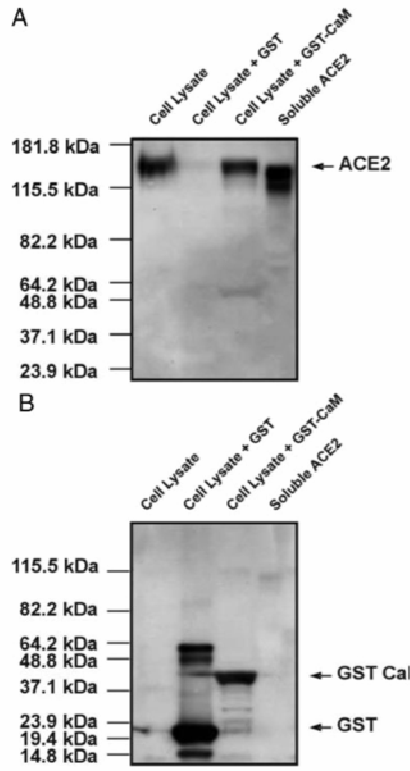

FIG. 3. Binding of ACE2 from cell lysates by GST-CaM. Cell lysates (~2 mg protein) from ACE2-transfected CHOP cells were incubated with glutathione Sepharose beads and GST (20 μg), or GST-CaM (20 μg) bait for 16 h. After incubation, samples were separated by SDS-PAGE and immunoblotted for ACE2 (A) and GST (B) antibodies. Cell lysates (transfected with ACE2) and soluble ACE2 were used as controls for Western blotting.

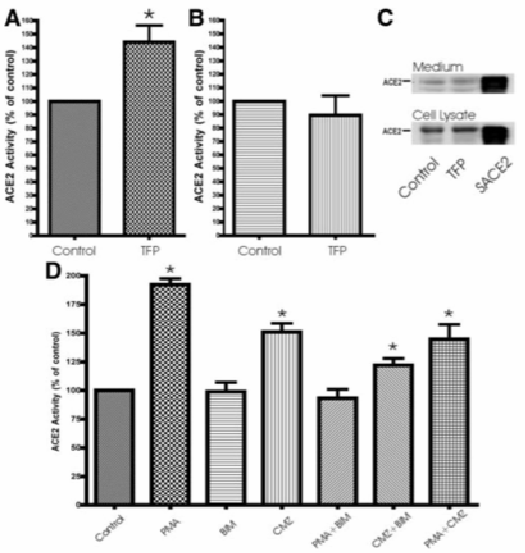

FIG. 4. Shedding of ACE2 is increased by CaM inhibitors. A, Huh-7 cells that endogenously express ACE2 were incubated in OptiMEM containing 25 μmol/liter TFP or in an equal volume of carrier (Me_2SO) for 16 h. The medium was subsequently harvested and concentrated 50-fold, whereas the cells were pelleted 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_2SO) 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 ± 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 ± 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 ± 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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REVIEW

Membrane proteomics: The development of diagnostics based on protein shedding

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Advances in proteomics technologies, in particular the parallel development of highly sensitive mass spectrometers and accurate protein quantitation technologies, have allowed the detection and accurate measurement of low abundance proteins in bodily fluids and tissues. Furthermore, the application of these technologies in biomedical research has led to the identification of proteins and genes with expression patterns that change as a consequence of disease; detection and quantitation of these proteins and genes could provide valuable information for disease diagnosis and prognosis. For example, cell-surface protein expression can change in diseased cells. These proteins may then be secreted or shed from the cell surface; the levels of these proteins in blood or urine could provide valuable information for the diagnosis of disease and disease severity. The focus of this review is the methods by which proteomics-based technologies can be applied to characterize cell-surface proteins and to measure changes to expression levels in diseased states; the review also discusses the soluble counterparts of these surface proteins in the blood; these proteins could be important diagnostic and/or prognostic indicators of disease.

Keywords Biomarker, membrane protein, membrane shaving, protein shedding, proteolysis, proteomics

Introduction

With the increased application of proteomics, momentous advances have occurred in the identification, characterization and quantification of proteins in biological preparations during the first decade of the 21st century. Furthermore, developments in proteomics technologies have led to the design of high-throughput instruments with improved sensitivity and accuracy compared with earlier techniques, providing a significant increase in the number of identified proteins in tissue and/or bodily fluid extracts. Coupled with revolutionary computational software for deconvoluting spectra, and bioinformatics tools that can help to identify and quantify proteins, rapid progression in novel proteomics applications is occurring, including the visualization of proteins and mapping of protein expression in mammalian tissues [1-3]; the identification of potential biomarkers in complex samples, using both label-free and stable isobaric tags [4-6]; and the quantitative analysis of post-translational modifications (PTMs) in proteins [7-9]. Mass spectrometry (MS) is considered the fundamental platform for the majority of proteomics research, acting as a powerful method to profile and quantify many proteins, peptides and amino acids according to their elemental composition. It is not surprising, therefore, that large-scale, MS-based approaches are becoming increasingly important in proteomics [10], particularly for biomarker identification and validation.

The analysis of peptides and proteins with proteomics methodologies provides comprehensive and unbiased information regarding the regulation and functions of a particular protein or collection of proteins, in both macro- and microorganisms. For example, proteomics has been designated an important tool in biodefense research because of the ability to identify and verify potential agents rapidly for use in biological weapons, such as harmful microorganisms and biotoxins [11]. In plant biology, proteomics approaches have helped to identify and elucidate the differential expression of proteins under various stress factors, and to explain the regulation of trace metal homeostasis and related toxicities in plants [12,13]. In pharmaceutical development research, analytical proteomics provides data to assist in drug metabolite identification in human tissues and cells, and to help predict drug effects, both intended and unintended [14].

The versatility of proteomics technologies, combined with advances in techniques for protein expression, extraction and separation, has prompted a significant increase in the number of comprehensive, publicly available protein catalogs. Moreover, the improved resolution and throughput of proteomics, coupled with enhanced bioinformatics techniques, have resulted in the more effective detection and identification of proteins that are present at low concentrations [9,10]. The field of proteomics is expansive, and novel research in technology and applications is

reported frequently. This review provides an overview of some of the advances in applications being developed to examine cell-surface proteins as potential diagnostic and/or prognostic markers of disease, and discusses analytical techniques and sample preparation; moreover, the article reviews some of the primary papers that demonstrate relevant biological applications. In addition, the proteins that are proteolytically released from the outer cell membrane, and 'sheddases', proteases that mediate these protein shedding events are highlighted.

Surface proteins as potential biomarkers

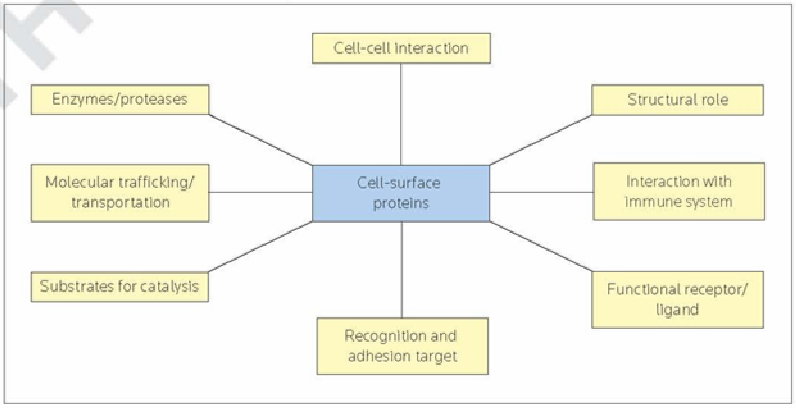
The cell membrane is home to a wide variety of proteins. The proteins expressed on the outer surface of the membrane provide an important means of communication for the host cell and its environment, allowing interactions with surrounding cells and foreign pathogens/compounds, as well as mediating the effects of autocrine and endocrine messengers (Figure 1). For example, surface proteins may serve as functional receptors or may facilitate the trafficking of various molecules across the plasma membrane. Moreover, the surface proteins also may have a role in the regulation of the immune system [15]. Considering this range of functions, membrane proteins appear to be promising drug targets, as well as potential biomarkers for molecular diagnostics and therapeutics. In bacterial cells, surface proteins are used as both recognition and adhesion targets to initiate the invasion of the host cell [16] (Figure 1). In mammalian cells, membrane surface protein expression may be significantly different in diseased cells than in healthy cells, as observed in many types of cancer and cardiovascular diseases [17,18]. The extent of the changes in expression or proteolytic cleavage (shedding) of specific proteins may reflect the severity of disease; thus, the identification and quantification of these shed proteins in blood or urine may provide insights into disease severity and/or may represent early markers of disease [17].

Proteomics techniques are widely used for screening tissues, as well as bodily fluids such as plasma, serum and urine, to measure changes in proteins that occur as a consequence of disease. If proteins are secreted or shed from the cell or tissue, their measurement in bodily fluids may aid disease diagnosis and/or prognosis. Depending on the properties of the proteins under investigation, different separation protocols and mass analyzer instruments can be used, each with its own advantages and limitations. For example, liquid chromatography (LC) coupled to tandem MS (LC-MS/MS) has been used to identify differential glycosylation patterns and sites of membrane proteins in breast cancer cells [19]. In a similar study, electrospray ionization [ESI]-MS/MS has been used to identify a panel of surface proteins as biomarker candidates for thyroid cancer cells [20]. Proteomics methodologies have also been used to investigate surface proteins as potential biomarkers of stem cell fate and self-renewal processes [21], and a combination of gel-based and MS techniques has been used to identify novel macrophage membrane proteins [22].

Separating surface proteins for proteomics analysis

Protein characterization presents many distinct challenges, particularly in the field of life sciences, in which the complexity of proteins present in mammalian tissues in terms of both the variety of proteins and the dynamic range of protein concentrations expressed (eg, in plasma this range is > 10^{10}) is vast. Each protein is constructed from a combination of amino acids, and often incorporates a variety of PTMs that are critical for protein function. After translation, a protein may undergo several modifications, such as proteolytic cleavage, the addition of modifying groups such as sugars and fats, and phosphorylation. Given that protein translation levels can differ both spatially and temporally, the use of genomic analysis to predict protein expression levels has proven to be an unreliable method in a given tissue; the method

Figure 1. The various roles of shed-surface-membrane proteins.



is also inapplicable for measuring PTMs. MS-based quantitative proteomics is a more suitable technology for studying PTMs, particularly given that modern instruments are able to detect and distinguish even the smallest (< 2 Da) differences in molecular weight [8,10]. Identifying proteins in complex mixtures (eg, serum, plasma and tissue extracts) is complicated and presents unique concerns in the identification of potential diagnostics, including the wide concentration range and complexity of the proteins involved [23]. Surface proteins are particularly difficult to measure because of inherent biochemical properties such as poor solubility and extensive glycosylation [24]. These hydrophobic proteins are generally anchored to the lipid bilayer and often span the cell membrane, making isolation and purification of the proteins from tissue extracts difficult. In addition, surface proteins are often in low abundance, and their presence may easily be masked by high levels of intracellular proteins. Several strategies have been developed to address the extraction of surface proteins from the plasma membrane to facilitate proteomics analysis (for reviews, see references [25,26]).

Preparing samples for proteomics analysis

Many well-established tools and techniques are available to assist in the separation and partitioning of peptides and proteins for MS-based proteomics analysis [27-29]. In conventional approaches, the first step in partitioning proteins is often isoelectric focusing (IEF), a method that allows the fractionation of proteins or peptides according to differences in their state of electric charge. A combination of IEF and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), termed 2D gel electrophoresis (2D-PAGE), is commonly used to separate proteins. 2D-PAGE is a powerful tool that can separate hundreds of different proteins and peptides in one run by iso-electric focusing (IEF) and according to their mass. Some membrane proteins have an alkali isoelectric point (pI,) and often require large pH gradients for separation by IEF. In this case, the first dimension of separation (IEF) can be replaced by an anion exchange column, which is followed by classic SDS-PAGE separation. A study pioneering this methodology demonstrated that a superior yield in terms of amount and number of proteins recovered was obtained compared with classical 2D-PAGE separation [30].

One interesting development that has gained considerable research momentum is multidimensional protein identification technology (MudPIT) [31,32]. This HPLC-based technique combines capillary or nano-reversed phase and ion exchange LC, followed by tandem MS. In this process, a membrane protein mixture is digested with trypsin, and the resulting digest is subject to fractionation on a cation exchanger. Reversed-phase HPLC is then used to resolve the resultant peptide digest peaks, and each peak is subsequently subjected to MS/MS, allowing for precise protein identification in terms of both fragment mass and peptide sequence. This technology is amenable to relatively accurate protein quantification when combined with isotope-coded tagging technology [33], which involves the selective labeling of specific reactive groups in a protein (eg, thiols or amines) with isotope-coded tags. In this process, one linker fragments to yield one specific mass pattern, and the second linker has a different pattern. As a result, the peptide digest fragments can be measured concurrently by MS (eg, control versus treated sample) to determine the ratio between the two protein-derived peptide fragments, each distinguished by a unique mass pattern signature. The different isotope tags are easily distinguishable, and the resulting MS/MS spectrum allows for the identification of the peptide fragments.

Alternatively, HPLC separation of surface proteins can be achieved using affinity purification, whereby molecules may be separated using a known, highly specific biological interaction involving the addition of an 'affinity label' or 'tag'. Many different purification columns and affinity labels, including biotin, FLAG and hexahistidine, are commercially available. Affinity labels must be incorporated into the plasmid construct of the surface protein under investigation, should have a minimal effect on the expression and processing of the protein, and should not affect the activity and function of the protein [34,35]. Once expressed, the labeled proteins can be isolated using the appropriate purification column. As a result of the highly specific and relatively strong binding between the tags and the column, proteins of low abundance and those derived from complex mixtures can be purified easily [35]. Given that membrane proteins are often highly glycosylated, the incorporated sugars can be used as affinity tags and the glycosylated proteins can be separated on specific lectin columns [36].

Mass spectrometry analysis

Matrix-assisted laser desorption/ionization (MALDI) and ESI are the two most commonly used techniques for ionizing molecules such as peptides and proteins into a gaseous state for MS analysis. MALDI ionizes molecules by firing a pulsed laser beam onto the surface of a mixture of the dried sample and a crystalline matrix. In contrast, ESI can ionize samples directly from the liquid state following the induction of an aerosol phase in a nebulizer. Both of these ionization technologies are robust and have relatively simple requirements and protocols for sample preparation and proteomic analysis. Once ionized, particles are accelerated into mass analyzers to determine the precise molecular mass of the ions. There are four basic types of mass analyzer: time-of-flight (TOF), ion trap, quadrupole and Fourier transform ion cyclotron (FT-ICR). Each of these mass analyzers exhibits distinct differences in terms of design and performance, and each modality tends to produce complementary data. For example, in terms of analyzing a complete set of peptides in a digest, some peptides ionize more efficiently in one modality type (eg, MALDI versus electrospray), or the increased sensitivity (eg, FT-ICR) allows greater peptide identification in cases of subtle post-translational modifications [37-41].

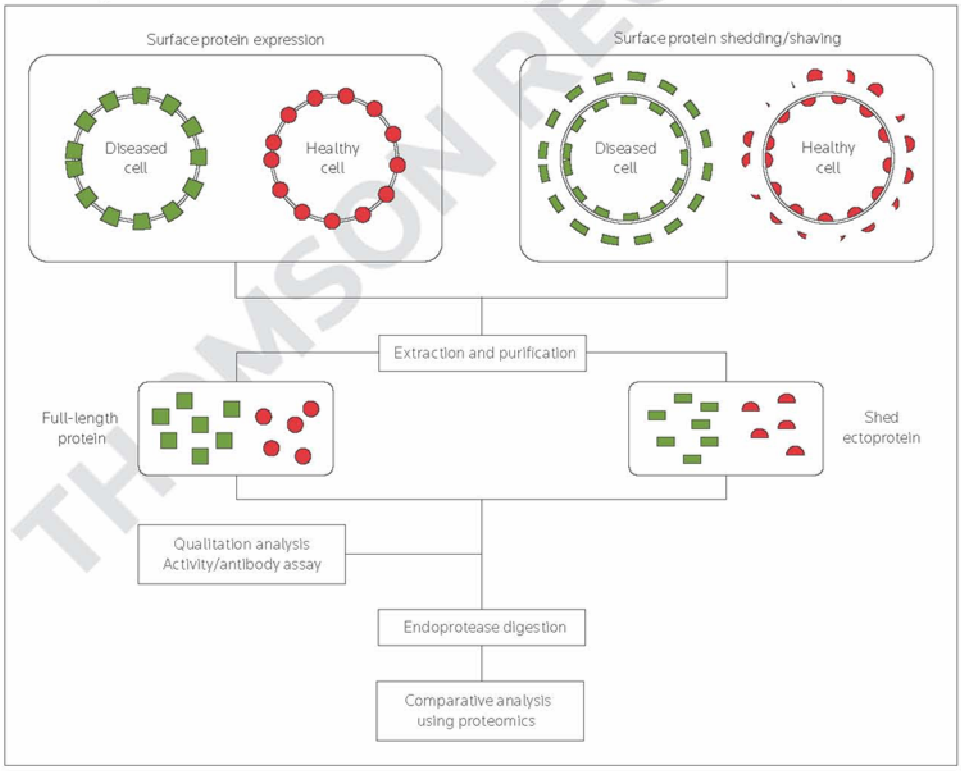
Membrane proteomics analysis

Analyzing cell-surface proteins therefore involves three components: extraction, identification and quantification. As with all protein analyses, it is important to isolate the surface protein under investigation from other contaminating proteins to ensure accurate identification, and to allow for a confident match with a predicted protein in an appropriate annotated gene database. It is necessary to ensure that purified samples are free from contaminants to avoid or limit the potential for false-positive matches or a misinterpretation of results. The overall process is outlined in Figure 2.

Surface proteins can exhibit a variety of PTMs, including alkylation, amidation, phosphorylation, formylation, oxidation, sulfation, acetylation and glycosylation, all of which can impact peptide mass. Given that MS instrumentation is highly sensitive and uses highly specific mass measurements, a small change to the mass of the parent peptide can affect the ability of MS to correctly identify proteins by mass comparison to proteins in annotated databases. Therefore, the possible occurrence of any type of PTM must be considered when studying these proteins. For example, glycosylation is one of the most abundant PTMs in membrane-associated proteins, and can affect peptide mass fingerprint identification. To simplify analysis, proteins can be deglycosylated prior to proteomic analysis. Other potential PTMs can be accounted for prior to bioinformatic analysis by including the likely masses of these modified proteins when programming database search algorithms. In addition, affinity chromatography using lectin affinity columns is a useful tool for concentrating glycosylated proteins such as membrane proteins prior to analysis [42].

Some tryptic peptides may fail to ionize or may not fragment to yield sufficient quantities of daughter ion to allow identification by MS. To address this issue, a powerful proteomics technique, known as Edman sequencing, can be used to sequence proteins efficiently.

Figure 2. Protein expression on the cell surface and the differential shedding profiles of diseased cells versus healthy cells.



Full-length membrane proteins are extracted from the plasma membrane using techniques such as detergent solubilization. Shed ectoproteins are harvested and can be purified using techniques such as affinity chromatography, size exclusion columns and gel-based purification. Purified proteins can then be identified and quantified using isotope-coded tags, or by functional activity- or antibody-based assays for comparative analyses with a proteomics approach.

This process involves the sequential chemical cleavage of individual amino acid residues from the N-terminal of the protein. The cleaved amino residues are identified according to their absorption spectra following a series of chemical reactions, with the cycle repeated for each subsequent residue as it becomes exposed [43]. An advantage of this technique compared with MS-based protein sequencing is the ability to differentiate isomeric residues such as leucine and isoleucine, or isobaric residues such as lysine and glutamine. However, protein sequencing using the Edman degradation technique is not as sensitive as MS-based techniques and requires the protein or peptide sample to be completely pure and free from contamination. The Edman technique is also not suitable for sequencing peptides or proteins with blocked N-terminal ends [44,45].

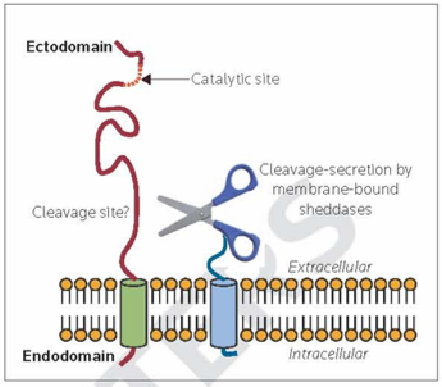
Proteolytic shaving for membrane protein profiling

One technique that has been developed to selectively catalog cell-surface proteins is 'membrane shaving'. This approach involves the treatment of the whole cell with a specific endoprotease, directly liberating small peptide fragments to allow for subsequent proteomic identification of the parent protein. The major advantage of this technique is the ability to selectively shave proteins on the outer surface of the cell for rapid profiling, without the need to isolate and fractionate the proteins from soluble cytosolic proteins. Another advantage is the ability to assess the topology of surface proteins, as proteolytic cleavage is limited to external sites [46]. Furthermore, this technique is useful for evaluating proteins that are traditionally difficult to measure as a result of their poor solubility because only the soluble components of the protein are liberated. The generation of peptide fragments from surface proteins without the use of detergents is ideal for direct analysis by MS, as detergents are able to suppress MS signals. Shaving technology has potential use for highlighting the differences in protein expression between healthy and diseased cells. Several studies have demonstrated the use of membrane shaving techniques in the characterization of surface proteins; for example, studies on group A streptococcus have been used to develop potential vaccine candidates [47,48]. A similar technique was conducted in *Bacillus subtilis* [49] and *Helicobacter pylori* [50] to identify surface proteins for protein sorting. Moreover, these techniques are equally applicable for eukaryotic cells, including HeLa cells [51,52].

Protein shedding from the membrane surface

Membrane-associated proteins may be released into the extracellular space by either regulated or constitutive proteolytic cleavage from the cell surface, a process known as protein or ectodomain shedding. Proteins are generally cleaved in the extracellular domain close to the transmembrane domain, resulting in the liberation of the 'ectodomains', also known as ectoproteins, which are often physiologically active. Figure 3 illustrates how a

Figure 3. The method by which proteins expressed on the cell surface undergo ectodomain shedding.



Surface proteins are usually cleaved at the membrane-proximal region by proteases or sheddases to release a soluble form of ectoprotein that can be detected by ligand binding assays, immunodetection or mass spectrometry-based proteomics methodologies if sufficiently abundant. Quantitative analysis of catalytically active ectoproteins can be measured using activity-based assays.

catalytically active protein expressed on the cell surface can undergo cleavage at the juxtamembrane region by a sheddase. Protein shedding is crucial for regulating the activity of many surface proteins, including growth factors, adhesion molecules and cytokine receptors [53-55]. The release of a soluble and sometimes active form (in terms of its ability to bind or to cause catalysis) of a protein may reflect changes in the biological roles and actions of the protein, allowing it to function systemically. Moreover, shedding may also provide a means of preventing pathogens from binding host cells. The true physiological consequences of protein shedding from the cell surface are largely unknown; however, there is potential value in examining these proteins as biomarkers.

Proteomics approaches in studying ectodomain shedding

Various proteomic approaches have been undertaken to characterize the ectodomain shedding of membrane proteins; this section reviews some examples. One such study proposed that the cleavage site for the ectodomain shedding of ACE2, the receptor for the SARS coronavirus, is regulated by ADAM17 (a disintegrin and metalloprotease 17) [56]. Jia and colleagues digested the cleaved ectoprotein with trypsin prior to sequence analysis by MS/MS. The data allowed for the identification of the key residues present in the ectodomain that are responsible for directing the ectodomain shedding of ACE2 [56]. ADAM17 has a broad range of biological substrates and, along with ADAM10, appears to be one of the major proteases mediating ectodomain shedding [57]. Other than ACE2, ADAM17 is involved in the shedding of many membrane proteins,

including TNFα [58,59], TGFα [60-64], heparin-binding-EGF (HB-EGF) [64,65], amphiregulin [64,66], epiregulin [64] and amyloid precursor protein (APP) [67,68]. A proteomics study profiling the expression of surface proteins and protein shedding in three ovarian cancer cell lines, OVCAR3, CaOV3 and ES2, was completed [69]. In the study, surface proteins were labeled with a lysine isotope prior to avidin-mediated affinity capture of biotinylated proteins, while proteins secreted from the cell media were concentrated [69]. Both samples were fractionated and analyzed using LC-MS/MS. Comparative proteomic analyses were conducted to determine the identity of shed proteins; marked differences were observed in the quantities of proteins shed from these cells [69]. Another method that has been used to characterize ectodomain shedding is the generation of overlapping synthetic peptides mimicking the membrane-proximal region. For *in vitro* cleavage experiments, these peptide mimetics can act as substrates for sheddases responsible for discarding the surface protein. Proteomic analysis of the cleaved peptide products can reveal the potential cleavage sites in the parent protein, and can be confirmed subsequently by site-directed mutagenesis. One limitation of this technique is that the sheddase must have already been identified, usually with specific inhibitors of candidate sheddases. This approach was demonstrated in the study of platelet glycoprotein VI (GPVI) ectodomain shedding [70]. Mimetic peptides corresponding to GPVI juxtamembrane region were successfully cleaved by human recombinant ADAM10 for MALDI-TOF-MS analysis [70]. The cleavage site was confirmed using cells that were transfected with a GPVI plasmid construct containing mutation at the cleavage site, which failed to cause shedding. In summary, the information gathered from these types of experiments allows for the prediction of potential ectodomain cleavage sites in related proteins.

Protein shedding in disease states: Biomarker potential

Accurate disease diagnosis is particularly important for many potentially chronic conditions, such as cardiovascular and neurological diseases and many cancers, which, in early stages, can often be present without any obvious symptoms. Early diagnosis of disease can allow for the initation of effective treatments before the development of more advanced diseases.

In diseased cells, cell-surface proteins often undergo a change in expression, which can lead to a change in protein shedding profile. As such, shed proteins may have a role as markers of disease status or progression. Information is accumulating regarding the physiological mechanisms and consequences of protein shedding in disease. One classic example in human brain disease is the accumulation of β-amyloid (Aβ) in plaques as a consequence of aberrant secretase activities (for reviews, see references [71,72]); this accumulation is believed to contribute to the pathology of Alzheimer's disease [73-76]. Furthermore, measurements of Aβ in plasma and CSF may have predictive potential for the diagnosis and assessment of severity of Alzheimer's disease [77,78]. Studies in both rats and humans have demonstrated that ACE2 undergoes an increased level of proteolytic cleavage to release its active soluble form as a result of myocardial infarction [17]; therefore, ACE2 may have diagnostic and/or prognostic potential. In 2003, ACE2 was reported to be the functional receptor for the binding SARS coronavirus [79]. Studies have focused on the shedding of ACE2 as a potential target for controlling the infectivity of the virus [80]. Another surface protein to be considered as a biomarker is GPVI, a member of the immunoglobulin receptor family discussed in the *Proteomics approaches in studying ectodomain shedding* section, that binds collagen to regulate homeostasis and thrombosis [81,82]. Patients with acute coronary diseases usually exhibit a significant increase in the expression level of platelet GPVI [83], which subsequently leads to increased shedding of the receptor [70]. The regulation of ectodomain shedding is tightly controlled, both in healthy and diseased cells, as observed in a study of ectodomain shedding of membrane anchored HB-EGF. Mice that expressed non-cleavable HB-EGF mutants exhibited heart failure, whereas mice that expressed only the soluble form of HB-EGF had hyperplasia in the skin and heart [84]. Finally, many cancers exhibit a change in membrane protein profiles as a consequence of malignancy, and many of these proteins appear to be selectively shed from the cell surface. An increasing number of reports in the literature suggest that shed proteins could be specific biomarkers for many cancers, such as ovarian cancer [69] and thyroid cancer [20]; alternatively, some proteins may be suitable as more general markers of tumors [85]. In summary, proteins shed from the cell surface not only contribute to disease pathology, but may have a role as biological markers of disease and disease progression.

Conclusion

Membrane proteins have potential as diagnostic and/or prognostic markers of disease, particularly those that can be shed from the cell surface and exhibit increased expression as a consequence of disease. Moreover, many membrane proteins exhibit a defined physiological/biological function, which is sometimes not revealed until ectodomain shedding occurs. Membrane-specific proteomics can provide valuable information on the post-translational processing and regulated shedding of proteins. Early studies of membrane proteins have had limited success. The classic methods of protein identification using a gel-based approach and/or protein detection by LC are often hampered by a low abundance of membrane proteins, which often have poor solubility. Enzymatic activity or antibody-based assays can improve the sensitivity of such protocols by using the inherent biological activity of the membrane proteins. The large-scale application of membrane proteomics is slowly gaining research momentum as protocols are becoming more established and robust [86]. As with most proteomics applications, the most significant

limiting factor for the study of proteins by peptide mass fingerprinting and MS/MS sequencing is the availability of a fully annotated genome for the species being studied. However, the proteomics sequencing ability of MS-based technology and Edman degradation are unequivocal compared with all of the other theoretical data that proteomics analyses provide. Sequences obtained can be used as primers in a molecular approach to aid in the identification of the parent protein. Despite its limitations, there is an expectation that proteomic analysis of membrane proteins (either shed or embedded in the cell membrane) may be widely used in the analysis of potential target cells, such as those expressed in tumors or in injured and/or diseased cells, including the sensitive detection of 'shed' proteins in complex mixtures (eg, blood, serum, plasma and urine). All of these exciting prospects emphasize the need to investigate new diagnostic and/or prognostic markers of disease.

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•• of outstanding interest
• of special interest

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7.3 Publication 3 *(Chemistry Today 2008)*

Peptides

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Quenched fluorescent peptide substrates

Tools for the discovery of novel biomarkers

ABSTRACT

The use of quenched fluorogenic labelled peptide substrates is growing and they have proved to be a valuable and effective tool for the rapid and sensitive screening for enzyme (particularly proteolytic) activity. Short synthetic peptides containing a quencher and the fluorophore provides high specificity and sensitivity to monitor enzyme cleavage and they thus have the potential to rapidly identify novel protease biomarkers for disease.

INTRODUCTION

Often patients are diagnosed with a life-threatening disease only as the symptoms become apparent, it can then be too late for medical intervention and long term survival. The World Health Organization (WHO) has recently projected that by 2030, cardiovascular diseases and cancer will become two of the major causes of global death (1), thus it is vital to have the necessary therapeutic tools for the early intervention on the affected patients or perhaps prevention of these diseases. As our knowledge in the field of medical research continues to grow, one of the potential solutions is the identification of biomarkers, which can aid in assessing the different biological parameters leading to disease progression (2). Identification of new biomarkers can provide us with better understanding on the critical checkpoints in a biological pathway, and also provide potential therapeutic or diagnostic targets for pharmaceutical intervention.

QUENCHED FLUORESCENT SUBSTRATE

Fluorescence technology has been widely used in the field of medical research in the past decade. One of its many popular applications is the use of fluorogenic assays in analysing the presence and amount of genetic markers, peptides or proteins. Fluorogenic assays using quenched fluorescent substrates (QFS) are a powerful technology based on the principle of fluorescence resonance energy transfer, also known as FRET, an energy transfer mechanism in which the light emitted from a donor fluorophore is absorbed by an acceptor fluorophore in close proximity of less than 60 Å (3). In designing a QFS, the acceptor fluorophore is replaced by a quencher moiety that shields the light emitting from the intramolecular donor fluorophore. Both the quencher and fluorophore pair generally flank a peptide sequence that is recognised and can be cleaved only by the specific protease of interest. Some of the common fluorophore and quencher pairs used are o-Aminobenzoic acid (fluorophore) and 2,4-dinitrophenyl (quencher), EDANS (fluorophore) and DABCYL (quencher), but many other combinations are possible. The ability of an enzyme to cleave a quenched fluorescent substrate lies in the peptide sequence. The QFS compound should be designed in a way that allows for efficient quenching of the fluorophore while mimicking the conformation and/or sequence of the existing natural substrate to achieve high specificity for enzyme cleavage. Once the enzyme of interest recognises and cleaves the peptide, the fragment containing the quencher diffuses away, thus causing an increase in fluorescence emanating from the fluorophore. This fluorescence is then easily detected using a fluorometer set to the appropriate excitation and emission wavelengths.

ASSAYS USING QUENCHED FLUORESCENT SUBSTRATE

There are currently many different strategies used in identification of biomarkers. Although classical proteomic techniques seem to be the current trend for screening biomarkers, reflecting their capacity for rapid screening, their lack of sensitivity remains a major issue that needs to be addressed. For example, the presence of the low abundance (yet clinically important) proteins in crude biological samples like blood plasma and tissue extracts, which are complex matrices, often cannot be readily detected using a proteomics strategy alone. Bioassays using quenched fluorescent substrate combined with other techniques can provide a very effective strategy that allows high sensitivity, straight-forward and relatively fast screening of biomarkers without the need of a complex sample purification step (4). The application of QFS has been shown to be highly effective in direct identification of proteases in biological fluids, and in addition, fluorescently quenched nucleic acids have been used as molecular probes for detecting genetic biomarkers (5, 6). QFS also work well when substrates are not readily available for enzymatic assays. For example, in the case of matrix metalloproteases where the substrates are generally insoluble proteins, chemically modified and engineered quenched fluorescent substrates have proved to be a good substitute. Hence, there are currently at least four different QFS type assays using different fluorescent and quencher pairs, that have been developed for assaying five specific human matrix metalloproteases whose expression has been implicated in a number of cancers (7).

Peptides

POTENTIAL BIOMARKER FOR CARDIOVASCULAR DISEASE

Some of our studies using QFS have focused on a zinc-metalloprotease called angiotensin-converting enzyme 2 (ACE2). ACE2 is the only known mammalian homologue of angiotensin converting enzyme (ACE), a well-characterised enzyme that is involved in the regulation of cardiovascular function (8, 9). Unlike ACE, ACE2 was discovered only relatively recently, and thus its precise physiological role is yet to be resolved.

Studies have demonstrated that ACE2 is able to inactivate angiotensin II, the catalytic product of ACE, which is also a potent vasoconstrictor peptide (10). The product of this hydrolysis is angiotensin 1-7, a peptide thought to be a vasodilator that could thus mediate the counter-regulatory response of ACE (11, 12).

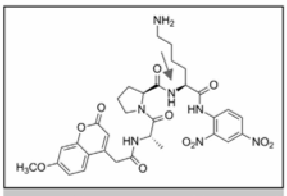

Figure 1. Schematic structure of the quenched fluorescent substrate (QFS) for ACE2. Arrow indicates ACE2 cleavage site.

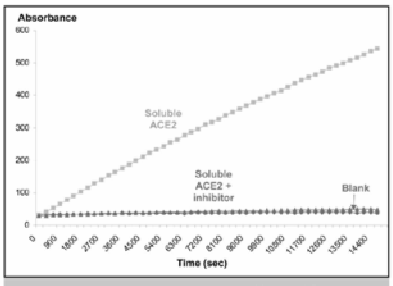

Figure 2. ACE2 activity assay measuring liberated fluorescence over time. An increase in relative fluorescence units indicates hydrolysis of the 50 µM quenched fluorescent peptide substrate by soluble ACE2 (25 µg of protein) in the absence (orange), and presence (green) of 1 µM ACE2 inhibitor, MLN4760. ACE2 buffer containing 100 mM Tris, pH 6.5, 500 mM NaCl were added to final volume of 100 µL. The data presented are the mean of duplicate wells in a 96-well microplate format using filter set 320 nm excitation and 405 nm emission at 37°C.

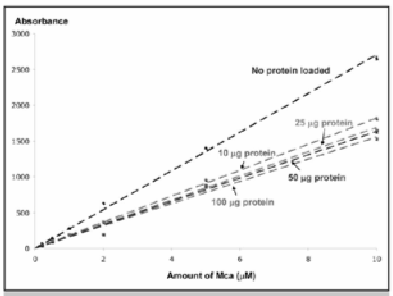

Figure 3. Mca calibration curve for quantitation of ACE2 in the QFS assay. Relative fluorescence units were plotted against known amounts of Mca in the absence (blue), and presence of 10 µg (green), 25 µg (purple), 50 µg (brown), and 100 µg (red) of bovine serum albumin. The data presented are the mean of duplicate wells in a 96-well microplate format using filter set 320 nm excitation and 405 nm emission at 37°C.

Other in vivo studies have also suggested that ACE2 could potentially be an essential regulator of the heart function (13). Since the first discovery of ACE2, many expression and localisation studies have been based on immuno-staining methods, using either in-house raised or commercially available antibodies, which are quite laborious, time-consuming and lack specificity for ACE2.

Synthesising QFS for ACE2 and assay optimisation

Given the increasing evidence that ACE2 may play a significant functional role in cardiovascular regulation, The QFS specific for ACE2 was synthetised commercially (Auspep Pty Ltd, Australia) following the initial strategy described by Vickers et al. (10). This QFS contains a fluorophore, (7-methoxycoumarin-4-yl)acetyl (Mca) on the N-terminal end, and a quencher, dinitrophenyl (Dnp) on the C-terminal end, flanking a tripeptide backbone (Ala-Pro-Lys) that is specific for ACE2 cleavage (Figure 1). To avoid any non-specific cleavage of QFS by other peptidases, it is necessary to optimise the conditions in which only the enzyme of interest (in this case, ACE2) is the active enzyme in the assay under study.

While establishing the protocol to measure ACE2 activity, we have tested the ability of the enzyme to cleave this QFS in a range of different pH's, salt concentrations, excitation and emission wavelengths, and have also tested the QFS for its robustness in different buffer condition, all in order to optimise for both ACE2 and QFS activity in conditions that mimic the physiological environment. To allow us to determine the absolute specificity of cleavage of the QFS by ACE2, we have also incorporated a highly specific ACE2 inhibitor (MLN-4760, a gift from Dr. Natalie Dales, Millennium Pharmaceuticals Inc. Cambridge, MA, USA) in all of our assays (Figure 2.)

We are able to determine the actual amount of the substrate hydrolysed using a calibration curve plotted using the commercially available fluorophore (Mca), as illustrated in Figure 3.

Depending on the actual amount of protein assayed, we can use the different curves to calculate the actual amount of QFS hydrolysed per min per unit of protein. An example of ACE2 analysis is illustrated in Figure 4, showing significant ACE2 activity in the membrane lysate of Huh-7 cells. This cleavage of QFS is significantly inhibited in the presence of ACE2 specific inhibitor.

Characterisation of ACE2 Using QFS

QFS has proved to be an important tool for our and other investigators work on ACE2 (10, 14-18). Assays using QFS have allowed us to demonstrate for the first time that this membrane bound metalloprotease undergoes a proteolytic cleavage in the membrane proximal region, thus liberating the catalytically active ectodomain. This is evidenced by the significant increase in the amount fluorescent detected in the media of ACE2 transfected Hek293 cells (15). The use of QFS has also been instrumental in our expression studies where we have shown both in rat and human that ACE2 expression is increased following myocardial infarction (17), and also how ACE2 is upregulated in the diseased the liver (14, 18) Our recent work using QFS assay has very recently shown that ACE2 is present in human plasma (accepted for publication in Experimental Physiology). Further studies are underway to measure ACE2 activity in plasma taken from patients with heart disease to assess the value of plasma ACE2 measurement as a marker for cardiovascular disease.

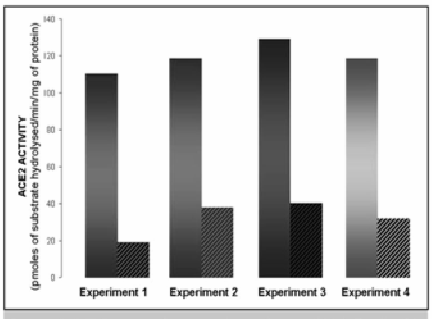

Figure 4. ACE2 activity from membrane lysate of Huh-7 cells. 10 µg of protein were assayed using 50 µM quenched fluorescent peptide substrate in the absence (clear bar), and presence (patterned bar) of 1 µM ACE2 inhibitor, MLN4760. ACE2 buffer containing 100 mM Tris, pH 6.5, 500 mM NaCl were added to final volume of 100 µL. The data presented are the mean of duplicate wells of four independent experiments in a 96-well microplate format using filter set 320 nm excitation and 405 nm emission at 37°C.

OTHER APPLICATIONS USING QFS AND ITS LIMITATIONS

The use of QFS is convenient and versatile. Apart from establishing the presence of the enzyme of interest, the sensitivity of QFS allows for determination and evaluation of enzyme kinetics. This is particularly useful in designing enzyme inhibitors or agonists. For example, the recent use of QFS in g-glutamyl hydrolase studies provides the first continuous activity assay and kinetic analysis where it was previously not achievable using a high performance liquid chromatography strategy (19). In another example, QFS use was implemented in quality control of vaccine production, both to identify and to characterise the presence of protease activity (20).

Like all substrates, the effectiveness and sensitivity of QFS in assays are governed by a number of different factors. Poor quenching of the fluorophore or hydrolysis of QFS by existing endopeptidases or other contaminants, especially in biological samples like blood plasma or tissue extracts, can often cause high fluorescent background and contribute to false positive results. One possible solution is changing to different fluorophore and quencher pairs to achieve better quenching effect, or to incorporate a specific inhibitor to inhibit cleavage activity by other enzymes. On the other hand, low fluorescent readings can sometimes be the result of intermolecular quenching either within sample proteins or by the use of high concentration of substrate. Another potential problem is some QFS demonstrate poor solubility in aqueous solution. Using an organic solvent or incorporating hydrophilic residues in the peptide chain can often assist in solubilizing the QFS. Although many of these potential problems can be easily addressed, it is important that all the different parameters are considered and should be thoroughly tested for when designing QFS, particularly for quantitative biological applications.

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