Characterisation of the Ectodomain Shedding of Angiotensin-Converting Enzyme

A PhD thesis by

Zenda Loren Woodman BSc (Med) (Hons)

Thesis Presented for the Degree of
DOCTOR OF PHILOSOPHY
in the Division of Medical Biochemistry,
Faculty of Health Sciences
University of Cape Town
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Abstract

Angiotensin-converting enzyme (ACE) is a zinc dipeptidyl carboxypeptidase that is involved in blood pressure, electrolyte and fluid homeostasis. It is a Type I ectoprotein comprising an active N and C domain that is shed from the cell surface by cleavage within its juxtamembrane stalk region. The secretase responsible for this cleavage event is a member of the ADAMs family of metalloproteases. In order to identify and isolate the ACE secretase, a membrane assay was developed to detect ACE secretase activity in CHO cell membrane fractions containing recombinant testis ACE. However, low detergent-solubilisation of the ACE secretase and the presence of endogenous ACE secretase activity within the substrate-containing membranes did not permit detection of exogenous ACE secretase activity. Characterization of the shedding of ACE revealed that the juxtamembrane cleavage site of somatic ACE was identical to that of testis ACE although somatic ACE was shed less efficiently. The inefficient shedding of somatic ACE was not due to steric hindrance by the N domain in the native protein, as an ACE mutant comprising two C domains (2C-domACE) was shed as efficiently as testis ACE and was cleaved both within the juxtamembrane stalk and inter-domain bridge regions. This suggested that the C domain comprises an ACE secretase recognition motif, which enables the secretase to bind to each domain, orientate itself and sequentially cleave ACE within both the stalk and bridge regions. In order to identify this C domain recognition motif, regions of testis ACE sequence were replaced with sequences from the corresponding regions of the N domain, generating three mutants: SomNdomBglII, SomNdomNheI and SomNdomBglII-NheI. This enabled the identification of a putative recognition motif within the region Arg191-Val214 of the C domain. Moreover, in order to investigate whether the shedding of ACE was also dependent on an accessible juxtamembrane stalk region adjacent to the transmembrane domain, the juxtamembrane region of testis ACE was replaced with the epidermal growth factor (EGF) domains of the low-density lipoprotein receptor (LDL-R) and factor IX, as well as a synthetic disulfide-linked domain, min23. The introduction of disulfide-linked domains within the juxtamembrane region of testis ACE did not abrogate shedding, but affected the efficiency at which testis ACE was released from the membrane. To further investigate the effect of the three-dimensional structure of a disulfide-linked

juxtamembrane domain on ACE shedding, three amino acid residues were deleted from the disulfide-linked region of the ACE mutant comprising the epidermal growth factor domain of the low-density lipoprotein receptor. This mutational analysis showed that accessibility of the juxtamembrane region is determined by both the tertiary structure of this region, and the identity of its amino acid residues. The orientation of bulky side chains of aromatic amino acid residues affects the ability of the ACE secretase to cleave within the stalk region. Thus, we have shown that shedding of ACE is determined by the presence of a putative recognition motif in the C domain, the tertiary structure of the juxtamembrane stalk region, and the orientation of amino acid side chains within this region.

Chapter 1

The significance of shedding on the systemic and local functions of angiotensin-converting enzyme

1.1 Introduction

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase involved in the hydrolysis of angiotensin I and bradykinin, chief effectors of blood pressure regulation. From as far back as 1954, the activity of ACE has been detected within plasma (Skeggs et al, 1954). Although the level of ACE activity within blood plasma is low, it clearly demonstrated the presence of circulating ACE (Alhenc-Gelas, 1983). Further evidence suggests that specific tissues (i.e. vascular, renal and cardiac) produce all components of the renal-angiotensin system (RAS) locally. This therefore raises the important question of the purpose of circulating ACE. It is likely that although blood pressure regulation is the main physiological function of ACE, it is not its only function. ACE has been proposed to be involved in fertility, inflammation, organ development, and tissue regeneration. This review will look at the significance of shedding in terms of the local and systemic production of ACE and the possible implications this has on its function *in vivo*.

1.2 ACE Structure

There are two ACE isozymes named according to their site of expression: somatic ACE and testis ACE. These isoforms of ACE are transcribed from a single gene (Howard et al, 1990; Kumar et al, 1991) resulting in the generation of the somatic isoform in the lung, kidney, intestine and vasculature and the testis isoform in spermatozoa (Langford et al, 1993; Sibony et al, 1993). Testis ACE is expressed from a spermatozoa-specific promoter in the 12^{th} intron of the *ace* gene (Howard et al, 1990). The translation of the *ace* gene in the endothelium generates a protein of 1306 amino acid residues with a 29-amino-acid-residue signal peptide (Soubrier et al,

1988). The molecular weight of this protein is ~180 kDa, and ~150 kDa after deglycosylation (Hooper and Turner, 1987). The sequence of somatic ACE indicates the presence of 17 potential N-glycosylation sites and treatment with *N*-linked-specific glycanases indicated that most of the ACE-associated carbohydrates were *N*-linked (Soubrier et al, 1988; Hooper & Turner, 1987). Testis ACE comprises 732 amino acid residues with a 31-amino-acid-residue signal peptide (Lattion et al, 1989; Ehlers et al, 1989). The first 36 amino acid residues of testis ACE are unique to this isozyme while the remainder are identical to the C domain of somatic ACE (Figure 1.2.1). In addition to containing 7 of the potential glycosylation sites of somatic ACE, testis ACE also has a Ser/Thr-rich region in the unique 36-amino-acid-residue region at the N-terminal end of the protein that is highly *O*-glycosylated (Ehlers et al, 1989; Ehlers et al, 1992).

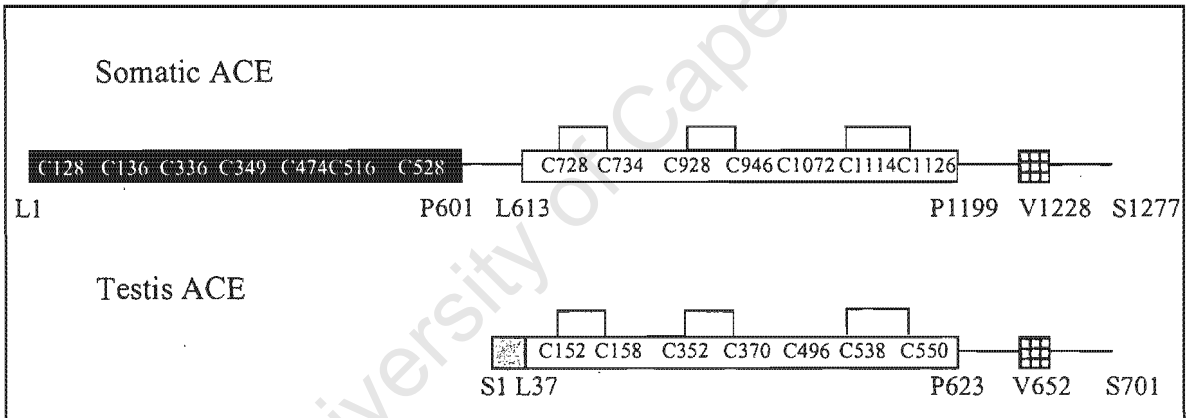


Figure 1.2.1 Schematic comparison of somatic and testis ACE The structure of somatic ACE is indicated at the top of the figure (Soubrier et al, 1988) and testis ACE is shown underneath (Lattion et al, 1989; Ehlers et al, 1989) without the signal peptide. The two domains of somatic ACE, N and C domains, are indicated by black and open bars, respectively, and span the regions L1 to P601 and L613 to P1199, respectively. The TM is shown as a hatched box and starts at V1228 and the ultimate amino acid residue is S1277. Testis ACE ectodomain is indicated by an open bar and its unique N-terminal region is shown by a grey box. The amino acid residues of testis ACE, corresponding to the C domain of somatic ACE are indicated where L37 marks the start of the identical region, P623 indicates the end of the ectodomain, V652 marks the start of the TM and S701 is the final amino acid residue. The boundaries of somatic and testis ACE domains were determined by sequence alignment. The cysteine residues are shown with the disulfide bridges indicated (Sturrock et al, 1996).

The three-dimensional structure of human testis ACE (residues 37-625) indicated that it is an ellipsoidal structure with a central groove that divides the structure into two sub-domains (Natesh et al, 2003). The amino acid residues, D40 and G615, define the N and C-terminal boundaries of the ectodomain of testis ACE and the region from 618-625 (stalk region) is disordered. It has high α-helical content comprising of 20 α-helices and only 6 short β-structures (Figure 1.2.2). The active site occurs within the groove and the first three N–terminal α-helices act as a "lid" structure thought to prevent access of large polypeptides to the active site. The carbohydrate moieties occur on the surface of the structure and binding of the two chloride ions involves residues from both sub domains (Natesh et al, 2003).

Somatic ACE is comprised of two highly homologous domains that are likely to be due to gene duplication. Both the N and C domains have a zinc-binding motif (HEMGH) starting at residues 360 and 959, respectively (Soubrier et al, 1988; Bernstein et al, 1989). The alignment of the 2 domains of somatic ACE and testis ACE (Appendix 1.4) indicates that there is ~60% sequence identity between the C and N domain of somatic ACE (Liu et al, 2001). Each domain has 7 cysteine residues in homologous positions. Six of these form 3 disulfide bridges in recombinant testis ACE, and the *aabbcc* pattern of disulfide bonding is proposed to occur in both the N and C domains (Figure 1.2.1) (Sturrock et al, 1996). There are 14 unique amino acid residues between the two domains of somatic ACE from P602 to T615, which we have called the bridge region. Both isozymes of ACE are Type I membrane proteins that have a 20-amino-acid-residue hydrophobic-rich transmembrane (TM) region and a cytoplasmic tail consisting of 30 amino acid residues (Wei et al, 1991a) (Figure 1.2.1). Sequence alignment of the two domains of somatic ACE indicates that sequence identity ends at P601 and P1199 for the N and C domain, respectively (Appendix 1.4). The region between P1199 and V1128 is termed the stalk region and links the ectodomain of ACE to the TM (Figure 1.2.1). ACE is released extracellularly by cleavage within the stalk region (Wei et al, 1991b; Ehlers et al., 1991b).

Although the two domains have high sequence homology, they exhibit different characteristics. Specific monoclonal antibodies can distinguish between the domains

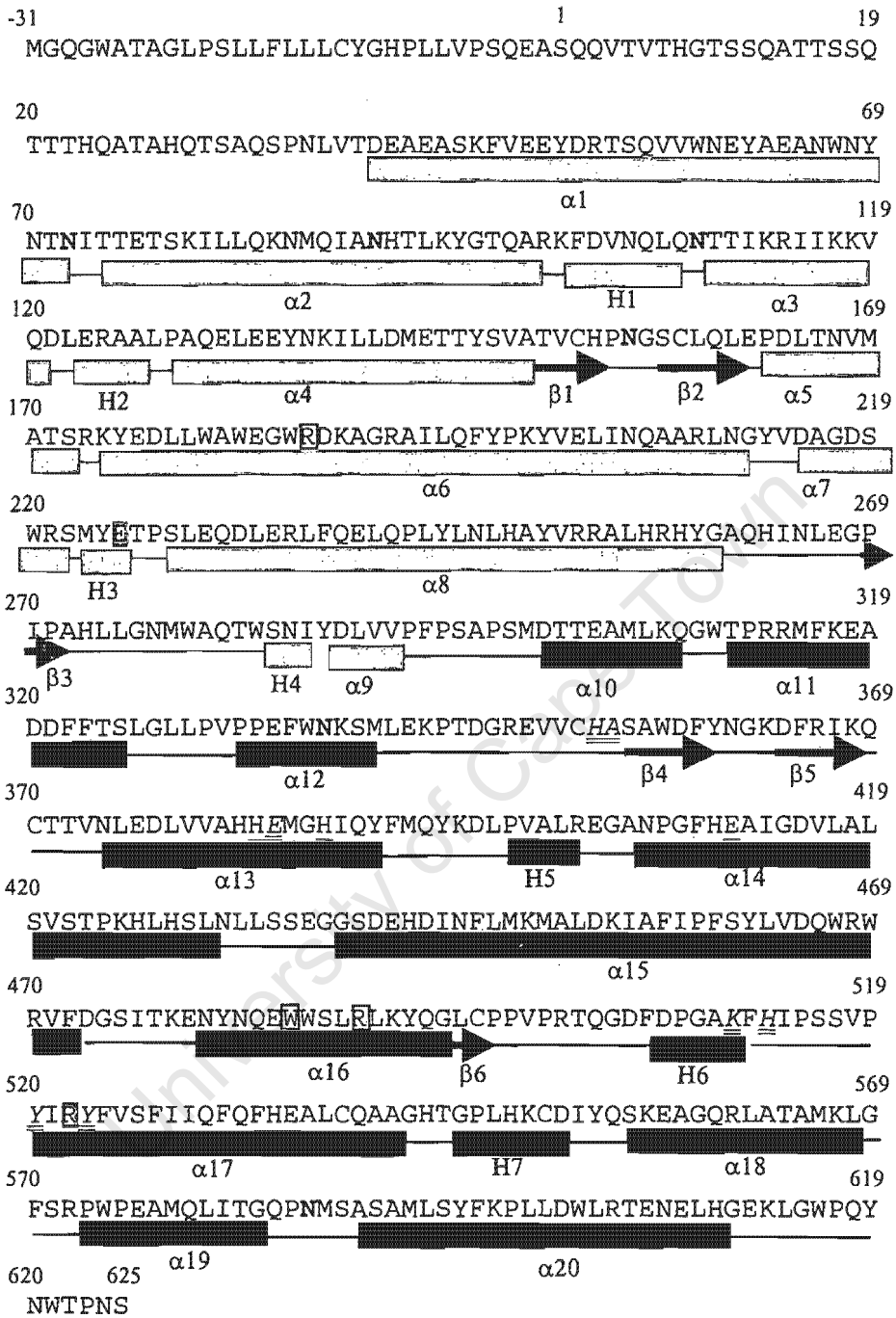


Figure 1.2.2 Secondary structure of human testis ACE Two sub-domains, I and II, are shown in grey and black, respectively, with the α-helices and 3_{10} helices depicted as bars and β structures shown as arrows. Residues involved in zinc binding (underlined), lisinopril binding (italics and double-underlined), and chloride ion co-ordination with chloride ion I (open box) and chloride ion II (grey box) are indicated. N-linked glycosylated residues are in bold (Natesh et al, 2003).

implying that the domains are structurally distinct (Danilov et al, 1994, personal communication). In addition, the N domain seems to determine the immunogenicity of somatic ACE, as monoclonal antibodies raised against somatic ACE, selectively recognise the N domain (Danilov et al, 1994). This suggests that the N domain folds around the C domain. The selective thermo-denaturation of the C domain within the parent somatic ACE molecule while maintaining the activity of the N domain indicates that the domains have different levels of stability and fold independently (Voronov et al, 2002). The proposed conserved *aabbcc* disulfide-bonding pattern also suggests independent folding of the two domains (Sturrock et al, 1996)

1.3 Regulation of ACE

The more distal 5´-region of the human *ace* gene contains response elements for glucocorticoid and cAMP (Shai et al, 1990). Both testis ACE and somatic ACE expression are responsive to cAMP (Krulewitz & Fanburg, 1986; Kessler et al, 1998). Interestingly, cellular somatic ACE and the generation of circulating somatic ACE seem to be regulated differently. Corticosteroids, thyroid hormones, and theophylline, a phosphodiesterase inhibitor, increase both forms, whereas dibutyryl cAMP and isoproterenol, a β-adrenergic agonist, only increase cell-associated somatic ACE (Krulewitz & Fanburg, 1984; Krulewitz & Fanburg, 1986). Activators of protein kinase C (PKC), such as phorbol 12-myristate 13-acetate (PMA) causes dissociation of ACE from PKC isozymes, except PKCλ, and increase ACE shedding (Santhamma & Sen, 2000). Moreover, PMA increases ACE mRNA levels and this induction occurs via transcriptional factors, activating protein 1 (AP-1) and early growth response gene 1 (Egr-1) (Villard et al, 1998) (Eyries et al, 2002).

1.4 ACE Enzyme Activity

ACE was discovered by its ability to cleave the prohormone angiotensin I to angiotensin II, by a carboxydipeptidase action that releases the dipeptide, His-Leu (Skeggs et al, 1956). In addition, ACE inactivates bradykinin and Lys-bradykinin (Yang et al, 1967). Angiotensin, bradykinin and Lys-bradykinin (kallidin 10) are important regulators of blood pressure and inflammatory responses (Ryan et al, 1988).

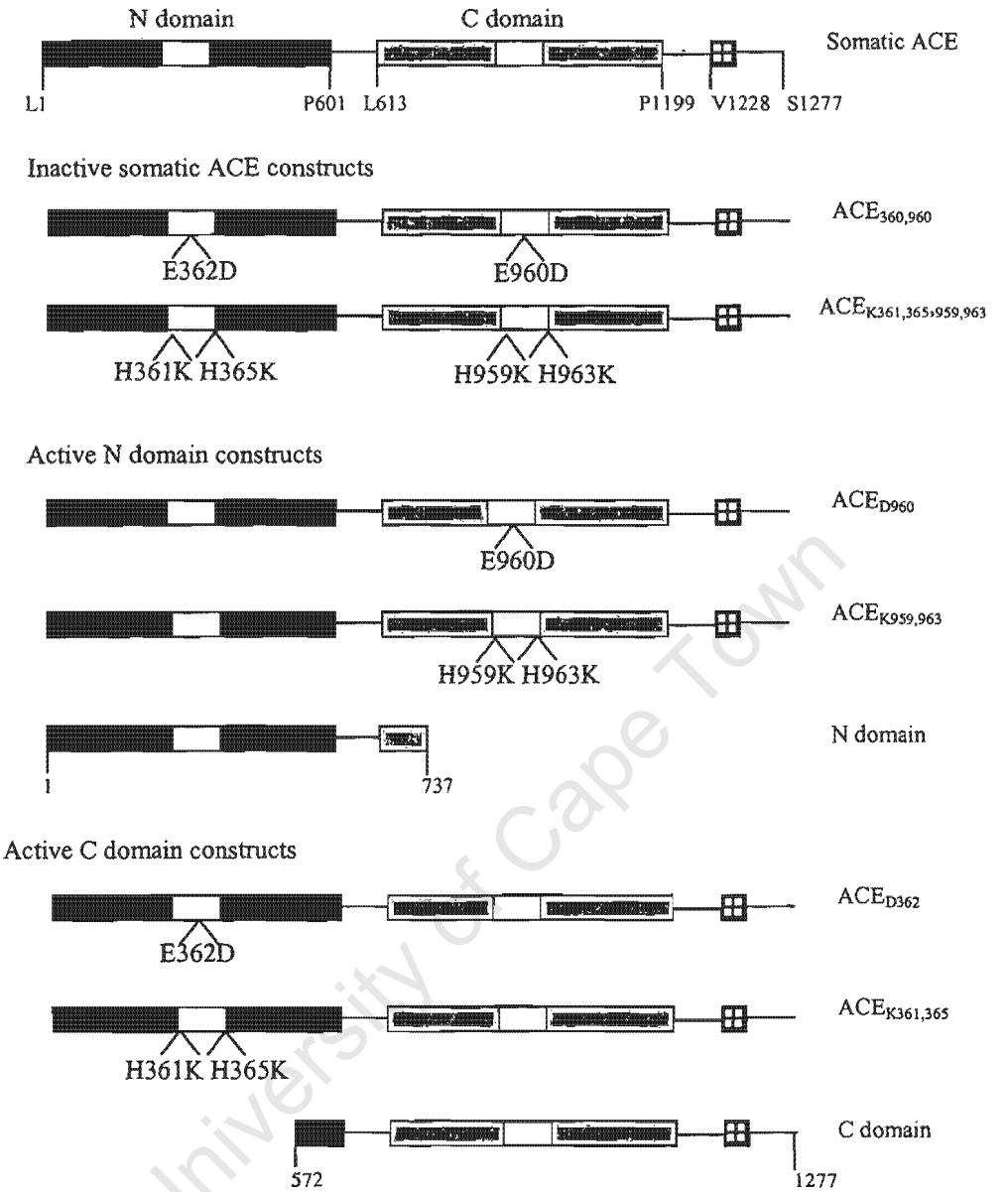


Figure 1.4.1 Constructs of somatic ACE Somatic ACE is shown above with the N and C domains shown as the black and grey bars and the TM as a hatched box. Substitution mutations were introduced at E362, E960, H361, H365, H959 and H963 within the active site (white box) as indicated below the respective domains. The truncated N domain mutant comprised of the signal peptide and residues 1-737 and the truncated C domain constructs comprised of the signal peptide, residues 1 to 4 of mature ACE, followed by two heterologous amino acids (P and W) and residues 572-1277 of ACE (Wei et al, 1991a).

It has also been shown that ACE is able to cleave substance P, luteinising-hormone releasing hormone (LHRH), and the enkephalins *in vitro* (Skidgel et al, 1984; Nau et al, 1985; Skidgel et al 1985). Whether these are natural substrates for ACE is doubtful

doubtful as the specificity constants for these peptides are very low. In fact, the specificity constant for LHRH is 3-fold lower than that for bradykinin (Ryan et al, 1988). As the two homologous domains of somatic ACE are active, various studies have been performed attempting to identify the specific kinetic parameters of the two domains with respect to angiotensin I, hippuryl-histidine-leucine (Hip-His-Leu/ HHL) and z-phenylalanine-histidine-leucine (z-PHL) hydrolysis. Wei et al replaced E362 and E960 with glutamic acid residues in the N and C domains, respectively, inactivating the active sites of both domains and rendering full-length somatic ACE inactive (Figure 1.4.1) (Wei et al 1991a). Similarly, somatic ACE was rendered inactive by the replacement of H361, H365, H959 and H963 with lysine residues in both domains. This indicated that these amino acid residues are vital for enzyme activity. The three-dimensional structure of testis ACE confirmed that residues H959 and H963 (H383 and H387, testis ACE numbering) are involved in binding of the zinc ion (Figure 1.2.2) (Natesh et al, 2003). Full-length somatic ACE with an inactive N domain and full-length somatic ACE with an inactive C domain was produced by substitution mutations of the relevant residues within the domain-specific active sites (Figure 1.4.1) (Wei et al, 1991a). In addition, somatic ACE was truncated to produce the N domain and the C domain (Figure 1.4.1).

The two domains of somatic ACE exhibited different chloride activation profiles: the N domain had higher activity than the C domain in the absence of chloride but the C domain activation resulted in higher maximal activity (Wei et al, 1991a). Thus, the C domain has an absolute requirement for chloride anions and hydrolyses angiotensin I faster than the N domain at high chloride concentrations. This suggests that there are structural and functional differences between the two catalytic sites and that the C domain is more active in the extracellular environment and the N domain is more active intracellularly, where concentrations of chloride are high and low, respectively (Wei et al, 1991a). In a similar experiment, truncated somatic ACE mutants were generated using sequence alignment of the two domains: N domain construct (signal peptide and residues 1-609) and the C domain construct (signal peptide and residues 611-1201) (Liu et al, 2001). Liu et al determined that the amino acid residue, R1098, within the C domain, is critical for Cl^- dependence and confirmed the domain-specific chloride activation profiles determined previously. The three dimensional structure of testis ACE identified R1098 (R522, according to testis ACE numbering) as a chloride

ion ligand (Natesh et al, 2003). This residue is conserved between the two domains. The only chloride-coordinating residue not conserved in the N domain is R186 of testis ACE, which is replaced by H164 in the N domain. This residue is likely responsible for the different activation responses to chloride concentration observed for the two domains.

According to the data of Liu et al, at 20 mM NaCl (saturating Cl^-), the N domain hydrolyses angiotensin I 2.5-fold more efficiently than the C domain (k_{cat}/K_m ratios of 2.7 and 1.1 $s^{-1}\mu M^{-1}$ for the N and C domains, respectively) (Table 1.4.1) (Liu et al, 2001). Wei et al determined that the full-length active C domain construct,

Table 1.4.1 Comparison of the catalytic constants of angiotensin I hydrolysis by the human isoforms of ACE

Authors	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
Wei et al, 1990	sACEwt	16	40	2.5
	[a]ACE $_{K361/K365}$	18	34	1.9
	[b]ACE $_{K959/K963}$	15	**11**	0.73
Liu et al, 2001	C domain	47	52	1.1
	N domain	23	**62**	2.7
Sturrock et al, 1997	C domain	82	36	0.4
	N domain	70	**10**	0.14
Ehlers et al, 1991a	sACE wt	23	32.6	1.4
	tACE wt	32	34.2	1.06

[a]ACE $_{K361/K365}$ Full-length somatic ACE with an inactive N domain
[b]ACE $_{K959/K963}$ Full-length somatic ACE with an inactive C domain

$ACE_{K361/K365}$ hydrolysed angiotensin I, 2.6-fold better than the full-length active N domain construct, $ACE_{K959/K963}$, (0.73 and 1.9 $s^{-1}\mu M^{-1}$ for the N- and C- domains, respectively) at 50 mM NaCl (Table 1.4.1) (Wei et al, 1991a). Moreover, the k_{cat} of the N domain constructs differed 6-fold (in bold). Liu et al attributed this discrepancy to the presence of the inactive domains in the full-length constructs and proposed that inter-domain interactions could affect the efficiency at which the individual domains hydrolyse angiotensin I (Liu et al, 2001). However, the k_{cat}/K_m ratios of truncated N and C domains of somatic ACE indicated that the C domain hydrolysed angiotensin I with 2.8-fold greater efficiency than the N domain, corroborating the results of Wei et al (Sturrock et al, 1997).

The full length, active N domain mutants, hydrolysed angiotensin I, ~25% of wild type somatic ACE and the full-length active C domain construct hydrolysed angiotensin I approximately ~75% to that of wild type (Figure 1.4.1)(Table 1.4.1) (Wei et al, 1991a). The sum of the enzyme activity (k_{cat}) of each domain equals the activity of full-length somatic ACE. These results suggested that somatic ACE comprised two functional and independent catalytic sites (Wei et al, 1991a). Liu et al did not determine the k_{cat} of somatic ACE but the sum of the activity of the separate domains did not equal the activity of wild-type somatic ACE determined by Wei et al (40 s^{-1}), suggesting that in native somatic ACE, the activities of the domains are different compared to when they were separated from one another (Liu et al, 2001). However, the k_{cat} of testis ACE did not differ from that of somatic ACE, and the k_{cat} of the N and C domains generated by limited proteolysis was identical to the full-length constructs of Wei et al (Table 1.4.1) (Ehlers et al, 1991a; Sturrock et al, 1997). This would suggest that the N domain does not contribute to somatic ACE hydrolysis of angiotensin I and that the presence of the N domain sequence does not affect the activity of the C domain, and vice versa, in the native full-length somatic ACE. The catalytic efficiency constants of somatic ACE (underlined) (Table 1.4.2) determined from the data of Wei et al is ~2-fold higher than that determined by Ehlers et al (Wei et al, 1991a; Ehlers et al, 1991a). However, the relative activity of somatic ACE to the C domain and the relative activity of somatic ACE to testis ACE for the two experiments are similar (ratio 1.3). It is difficult to explain the differences observed

between these two experiments as they were performed in identical buffer systems and under saturating chloride conditions (Wei et al, 1991a; Ehlers et al, 1991a).

The differences in the kinetic data makes it difficult to suggest that, 1) the activity of somatic ACE comprises the sum of the activities of the two independent domains, 2) that the C domain is responsible for 75% of the activity of somatic ACE, or 3) that the two domains are functionally independent within native somatic ACE. However, a transgenic mouse model, producing only active soluble N domain, was only able to convert ~20% of plasma angiotensin I to angiotensin II (Esther et al, 1997). Thus, it is likely that the C domain hydrolyses angiotensin I with much higher efficiency than the N domain, whether this is carried out independently of the N domain in full-length somatic ACE is uncertain.

Table 1.4.2 Kinetic parameters of HHL hydrolysis by somatic ACE and the separated domains

	Hip-His-Leu		
Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)
Wild-type	1540	408	0.26
N fragment	1980	ND[a]	
ACE_{D960}	2000	40	0.02
$ACE_{K959/K963}$	2000	40	0.02
C fragment	2000	ND[a]	
ACE_{D362}	1590	359	0.23
$ACE_{K361/K365}$	1590	364	0.23

[a] Not determined
Values derived from Wei et al, 1991a

The substrate specificity of the two domains of somatic ACE was also demonstrated by the hydrolysis of HHL (Table 1.4.2). The full length, active N domain mutants, hydrolysed HHL 10-fold slower than that of the wild-type enzyme. The full-length active C domain fragments hydrolysed HHL 90% to that of wild type. The sum of enzyme activity of each domain equals the activity of full-length somatic ACE. This result suggests that the N domain activity only accounts for 10% of the total activity of somatic ACE (Wei et al, 1991a). The chloride dependence for the enzymatic activation of the domains was similar to that determined for angiotensin I hydrolysis (Wei et al, 1991a).

Table 1.4.3 Kinetic constants and ratios using the substrates, z-Phe-His-Leu and Hip-His-Leu

	k_{cat} z-PHL	k_{cat} HHL	Ratio k_{cat} z-PHL/HHL
Somatic ACE	387.6[d]	408[b]	0.95[c]
N domain	292[a]	40[b]	7.3
C domain	322[a]	364[b]	0.9
Ratio: C domain / N domain	1.1	9.1	

Values are derived from the published data of:
[a] Danilov et al,1994
[b] Wei et al, 1991a
[c] Williams et al,1996
[d] The calculated value from the data of Wei et al, 1991a and Williams et al, 1996

The substrate specificity of the N domain and C domain allows us to differentiate between the activity of the domains. Table 1.4.3 lists the k_{cat} values for the hydrolysis of HHL and z-PHL. The synthetic substrate, z-PHL, is hydrolysed at a similar rate by

both the N domain and the C domain as indicated by the ratio of 1.1 (Danilov et al, 1994). The ratio of C domain/N domain HHL hydrolysis is 9.1 indicating that the C domain hydrolyses HHL 9-fold more efficiently than the N domain (Table 1.4.3)(Wei et al, 1991a). The k_{cat} z-PHL/k_{cat} HHL ratios of hydrolysis of the N domain and C domain are 7.3 and 0.9, respectively. These differed from the published ratios for the N domain and C domain of 4.5 and 0.67 (Williams et al, 1996). However, these ratios still indicate the differences with which the domains hydrolyse HHL and z-PHL, namely that the C domain hydrolyses these two substrates at a similar rate and the N domain is more specific for z-PHL than HHL. These two substrates can thus be used to determine N domain- or C domain-specific activity.

The ratio of k_{cat} HHL/k_{cat} z-PHL for somatic ACE was determined to be 0.95, indicating that somatic ACE hydrolyses both substrates equally (Williams et al, 1996). Using the k_{cat} of HHL of 408 s^{-1}, the k_{cat} of z-PHL hydrolysis by somatic ACE was calculated to be 387.6 s^{-1} (Wei et al, 1991a, Williams et al, 1996). The sum of the k_{cat} values determined for the N domain and the C domain for z-PHL hydrolysis does not equal the calculated k_{cat} of somatic ACE (Danilov et al, 1994). In fact the separate domains hydrolyse z-PHL at a similar rate as full length somatic ACE. This would suggest that either, only one domain hydrolyses z-PHL within native ACE or interactions between the two domains affect the ability of the domains to hydrolyse the substrate at maximum catalytic efficiency. In contrast, the hydrolysis of HHL by the individual domains does add up to the total activity of native somatic ACE.

In conclusion, these results suggest that although the two domains have 60% sequence identity, they differ in substrate specificity and therefore structure. Moreover, the catalytic efficiency of the domains seems affected by the presence of the other domain in native somatic ACE. This effect is substrate-dependent and most likely depends on the three-dimensional structure of somatic ACE and the proximity of the two active sites.

1.5 Renin-angiotensin system

The activation of angiotensin I by ACE establishes ACE as an important player in the arena of blood pressure regulation. In addition it has also been hypothesised that ACE plays a role in tissue regeneration and/or apoptosis after injury especially cardiovascular and renal injury.

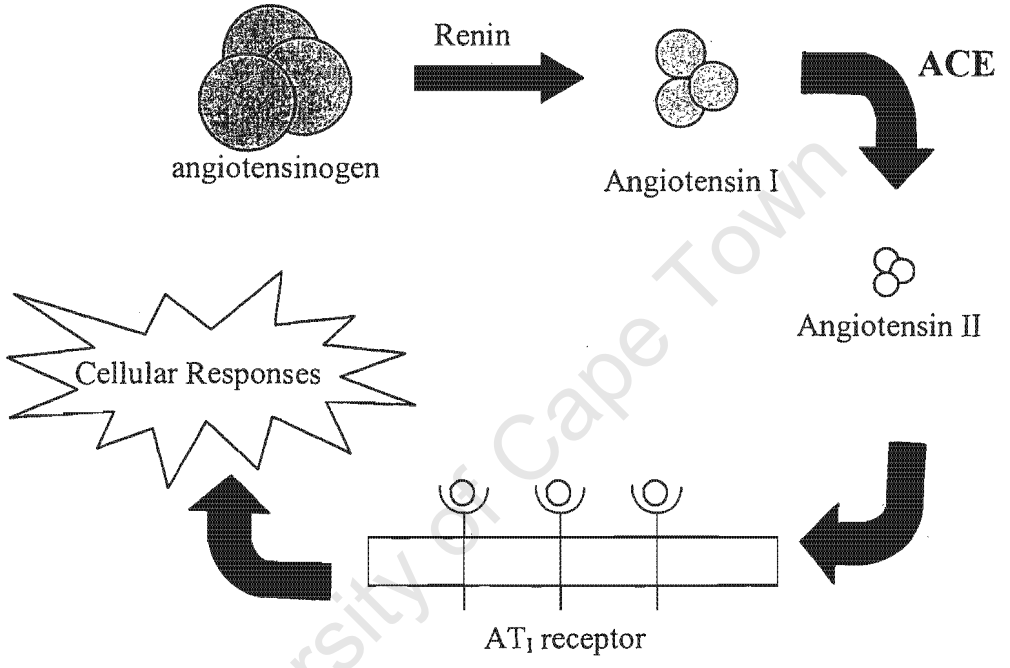


Figure 1.5.1 Renin-angiotensin system The schematic representation of the conversion of angiotensinogen to angiotensin I by renin, the carboxypeptidase processing of angiotensin I to angiotensin II by ACE and the binding of the octapeptide to AT1 receptors of vascular cells eliciting cellular responses.

1.5.1 Components of RAS

The renin-angiotensin system comprises angiotensinogen, renin, angiotensin I, ACE, angiotensin II and angiotensin II receptors in a cascade of proteolytic events that culminate in vasoconstriction; aldosterone, and catecholamine release; secretion of prolactin, and adrenocorticotrophic hormone; and glycogenolysis (Peach, 1977) (Figure 1.5.1).

1.5.1.1 Renin

Renin is an aspartyl protease of ~30-40 kDa and is highly specific for its substrate angiotensinogen (Dzau et al, 1988). It is mainly produced in the juxtaglomerular cells of the afferent arterioles of the kidney, but it was also found to be highly expressed in the adrenal gland, ovary, testis, lung and adipose tissue (Gomez et al, 1990; Sigmund et al, 1992). Moreover, low levels of expression have been determined in the heart and submandibular gland (Sigmund et al, 1992). Renin is produced as prorenin, which is processed to its active form by trypsinlike proteases found in immature granules in kidney, endothelial cells and neutrophils. The low levels of prorenin mRNA in blood vessel tissue has led to the proposal that prorenin is rapidly absorbed by these cells, thus allowing local accumulation (Dzau et al, 1988). The regulation of renin mRNA differs between tissue types. Expression of renal renin is stimulated 5- to 10-fold upon depletion of angiotensin II by the ACE inhibitor, captopril, whereas genital and submandibular-gland renin are regulated by androgens (Table 1.5.1) (Sigmund et al, 1992; Dzau et al, 1986). In addition, sodium depletion and the activation of β-andrenergic receptors increases the expression of renin in kidney, heart and adrenal glands (Dzau et al, 1986)

1.5.1.2 Angiotensinogen

Angiotensinogen (Ao), the substrate of renin, belongs to the family of serine protease inhibitors (serpins) (Doolittle et al, 1983). Due to its large size in comparison to angiotensin I and its increased expression during inflammation, it has been proposed that it could have an alternative function other than a precursor of angiotensin I (Hoj-Nielsen et al, 1987). High levels exist within the plasma and the primary source is the liver (Figure 1.6.2) (Morris et al, 1979). The expression of angiotensinogen is stimulated in rat hepatocytes by glucocorticoid, estrogen and thyroid hormones (Chang & Perlman, 1987). Regulation seems to be dependent on tissue type, as organs rich in neural tissue do not respond as well to thyroid hormone, estradiol and dexamethasone stimulation as organs rich in vascular tissue (Campbell et al, 1986). In addition, angiotensin II has a positive feedback effect on angiotensinogen expression (Table 1.5.1) (Tewksbury et al, 1990).

Table 1.5.1 Regulators of RAS components

	Regulators
renin	Na^+ depletion β-adrenergic receptor activation Androgens Angiotensin II
angiotensinogen	Estrogen, Thyroid hormone Sodium Angiotensin II
ACE	Corticosteroids PKC activators, cAMP Testis ACE: hormonal regulation- FSH/LH testosterone, human chorionic gonadotropin
AT_1	Angiotensin II, cAMP

1.5.1.3 ACE

ACE is a carboxypeptidase involved in the activation of angiotensin I. ACE has been identified in the kidney, ileum, duodenum, uterus, lung, prostrate, jejunum, testis and adrenal gland (Lieberman et al, 1983). In the lung, ACE is found within the vascular endothelium (Erdos & Skidgel, 1985). However, 5- to 6-fold higher concentrations have been identified within the kidney (Erdos, 1967). In addition, it has also been found in serum, seminal fluid, cerebrospinal fluid (CSF) and plasma (Erdos et al, 1986; Lantz & Terenius, 1985) (Figure 1.6.2). It appears to be regulated by PKC activators and cAMP (Iwai et al, 1987) (Table 1.5.1). ACE is an integral Type I membrane protein and the circulating form is generated not by secretion, but by a proteolytic process termed shedding.

1.5.1.4 Angiotensin II

The production of angiotensin II, the product of ACE action on angiotensin I, is correlated with the location of the RAS and is thus found within the circulation as

well as the heart, lung, brain and kidney. Its functions are diverse and include: vasoconstriction; thirst stimulation; release of pituitary hormones; facilitates adrenergic neurotransmission; stimulation of catecholamine secretion; causes glucocorticoid and aldosterone synthesis; stimulates glycogenesis and glucogenesis in the liver; and in the kidney modulates glomerular filtration; arteriole contraction; increases sodium-hydrogen antiporter activity; ammoniagenesis; tubular gluconeogenesis; and tubular and growth-related effects (Tufro-McReddie & Gomez, 1993; Peach, 1986; Navar et al, 1986; Ichikawa & Harris, 1991; reviewed in Gomez & Norwood, 1995).

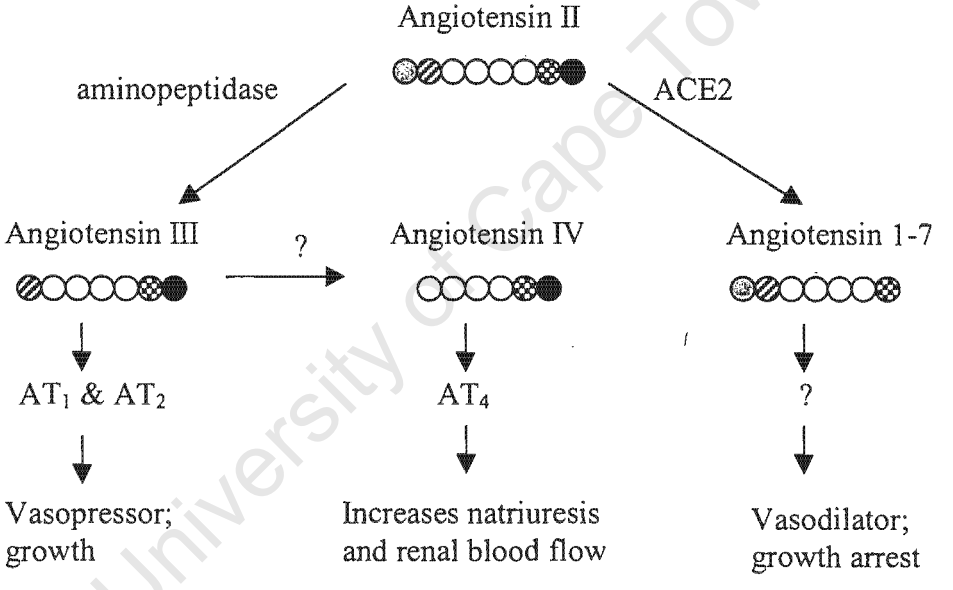


Figure 1.5.2 Degradation products of angiotensin II and their functions Angiotensin II is shown as eight circles, representing each amino acid residue in the octapeptide. Subsequently each degradation product is depicted similarly with the circles appropriately shaded to indicate the amino acid residues that have been removed. AT_4 is a novel receptor (Albiston et al, 2001). Question marks indicate pathways that are unknown.

Angiotensin II action can be regulated by peptidases, which hydrolyse the octapeptide into smaller peptides. Angiotensin II degradation products play a role in blood pressure regulation and renal function (Figure 1.5.2). They bind to angiotensin receptors, and angiotensin III has a similar function to angiotensin II, whereas

angiotensin I-7 acts in opposition to angiotensin I (Wright & Harding, 1997; Brosnihan et al, 1998; Iyer et al, 2000). Angiotensin IV activates multiple signalling pathways during lung endothelium proliferation and stimulates tyrosine kinase activity more effectively than angiotensin II (Li et al, 2002; Ochedalska et al, 2002). In addition, angiotensin IV inhibits ACE activity (Fruitier-Anaudin et al, 2002).

1.5.1.5 Angiotensin II Type I and Type 2 receptors

Angiotensin II receptors are comprised of subtypes dependent on their response to different angiotensin II antagonists. These are classified AT_1 and AT_2 subtypes due to their respective selective inhibition by biphenylimidazoles and tetrahydroimidazopyridines (Bumpus et al, 1991). In addition, AT_1 receptors bind angiotensin II better than angiotensin III, whereas AT_2 receptors respond similarly to these two agonists. The human AT_1 is expressed by a single gene, but there is evidence that it could be post-transcriptionally processed to generate the two sub-types whereas in the rat, two genes on separate chromosomes have been identified (Furato et al, 1992; Elton et al, 1992). Rodent AT_1 receptors have further been sub-divided into AT_{1A} and AT_{1B} sub-types based on their location within the cardiac vasculature or pituitary and adrenal glands, respectively. These have 94% identity in the mouse (Sassamura et al, 1992; Kitami et al 1992; Burson et al, 1994; Gasc et al, 1994).

AT_1 is the final effector of the RAS system and is a member of the family of 7 transmembrane domain (7 TMR) G-protein coupled receptors (Murphy et al, 1991; Sasaki et al, 1991). Its tissue expression is indicated in Figure 1.5.3 and the differential expression of the two subtypes, AT_{1A} and AT_{1B} is most significant in the heart, brain and spleen (Murphy et al, 1991, Kakar et al, 1992a; Kakar et al, 1992b, Iwai et al, 1992a). The signalling pathway of AT_1 is generally well understood. It involves the activation of protein kinase C (PKC) and phosphoinositol generation via phospholipase C (PLC) (Phillips & Sumner, 1998; Blume et al, 1999). In addition, it also activates other signalling pathways implicated in cell growth (Figure 1.6.3) (Blume et al, 1999; Marrero et al, 1995; Lu et al, 1998). The regulation of AT_1 seems to be mediated by angiotensin II in a tissue-specific manner: AT_{1A} mRNA expression was down-regulated by 50% in cultured smooth muscle cells and mesangial cells,

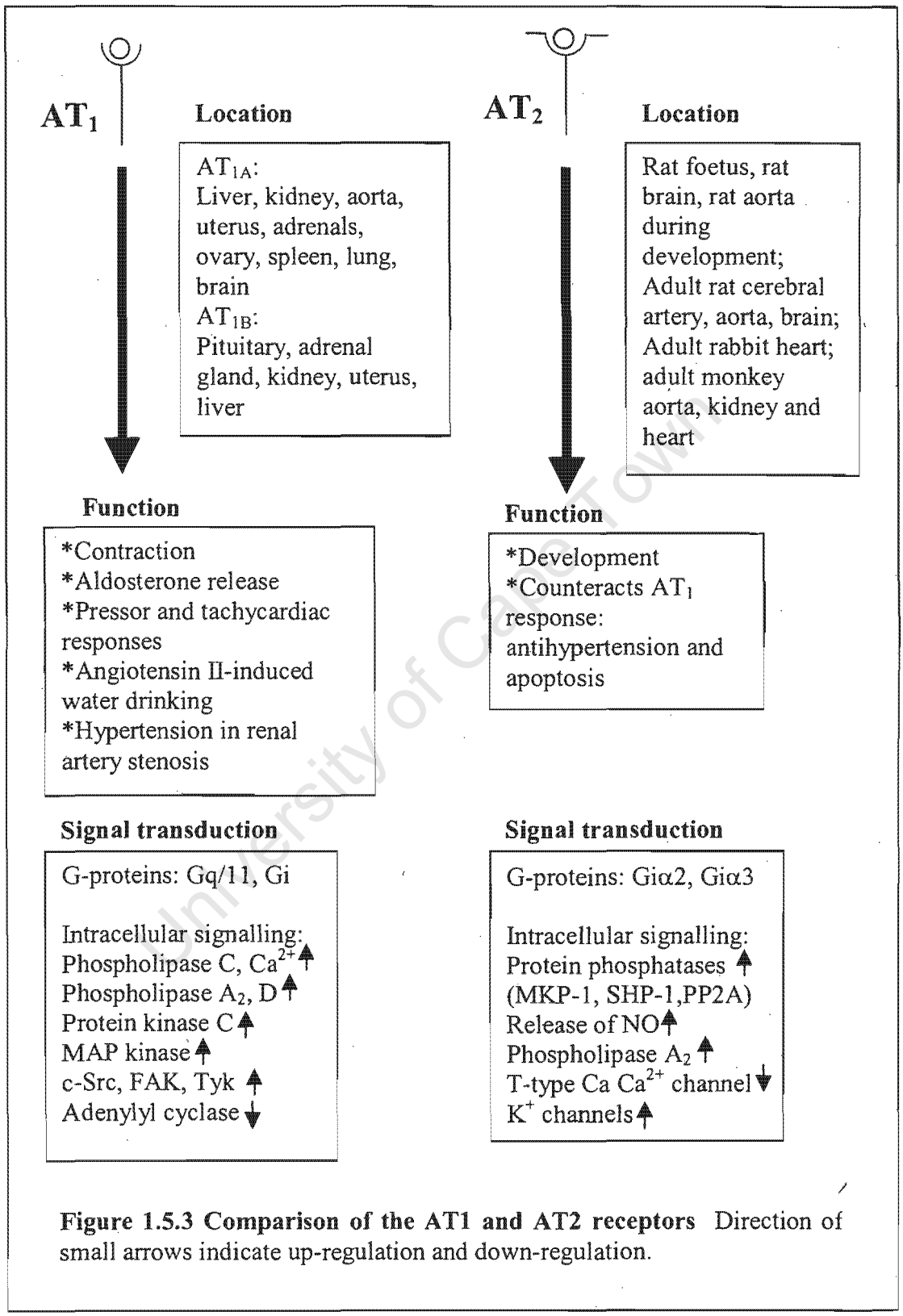


Figure 1.5.3 Comparison of the AT1 and AT2 receptors Direction of small arrows indicate up-regulation and down-regulation.

whereas angiotensin II increased AT_1 expression within the adrenal gland (Makita et al, 1992; Iwai et al, 1992b). The increase of intracellular cAMP also decreased cellular expression of AT_1 (Lassegue et al, 1992; Makita et al, 1992). Thus, cAMP which increases ACE expression in endothelial cells, and likely also increases angiotensin II, would result in a down-regulation of AT_1 receptor in smooth muscle tissue.

AT_2 receptors exhibit 34% identity to AT_1 and are more highly expressed in embryonic and neonatal tissue than adult tissue (Kambayashi et al, 1993b; Grady et al, 1991; Millan et al, 1991; Viswanathan et al, 1991; Tstsumi et al, 1991). However, as indicated in Figure 1.5.3, AT_2 receptors have also been identified in various adult tissues, but this expression seems to be species specific (Tsutsumi et al, 1991; Chang et al, 1991). In primary cultures of neonate and adult rat myocytes, both AT_1 and AT_2 are found, whereas cardiac fibroblasts only produce AT_1 (Booz et al, 1996; Rogers et al, 1986). However, compared to the rat, human cardiac fibroblasts have a lowered number of angiotensin II receptors (Gallagher et al, 1998; Neuss et al, 1996; Matsubara et al; 1994). Although adult tissues show low expression of AT_2, there is an increase of expression during heart failure, renal failure, vascular injury, myocardial infarction and lesions of the nervous system (Ohkubo et al, 1997; Chung et al, 1998; Nakajima et al, 1995; Zhu et al, 1996; Makino et al, 1996). The expression of AT_2 during foetal development and after tissue injury suggests that angiotensin II acts via the AT_2 receptor in development and cell differentiation. AT_2 expression is down-regulated in the presence of growth factors such as phorbol esters (Kambayashi et al, 1993a; Ichiki, 1995). Although the exact signalling pathway(s) activated by AT_2 remains to be elucidated, the action of AT_2 seems to be mediated by the activation of phosphorylases to inhibit phosphorylation steps in growth signalling (Figure 1.6.3) (Bottari et al, 1992). The anti-proliferative function of AT_2 was elucidated when AT_2 receptors inhibited the AT_1-induced growth of cultured microvascular cells and over-expression of this receptor in rat smooth muscle cells inhibited DNA synthesis, MAP-kinase activity and proliferation (Stoll et al, 1995; Nakajima et al, 1995). Not only is AT_2 involved in the inhibition of cell growth, it is also implicated in apoptosis of vascular smooth muscle (Yamada et al, 1996). Thus, the activation of AT_2 by angiotensin II acts in opposition to the function of cell growth mediated by the activation of AT_1.

1.6 ACE Function

1.6.1 Systemic blood pressure regulation

The historic endocrine perception of the regulation of blood pressure begins with the production and secretion of renin by the kidney (Figure 1.6.1), which converts angiotensinogen to angiotensin I. Angiotensinogen, produced by the liver, occurs in high concentrations in blood and thus renin can carry out its function during transit through the circulatory system. The circulating angiotensin I is then converted to angiotensin II by ACE found on the luminal endothelium of the lung (Ryan et al, 1975; Caldwell et al, 1976; Wigger & Stalcup, 1978). It has been shown that total plasma angiotensin I is converted to its active counterpart during a single transit through the lung (Ng & Vane, 1967). Angiotensin II then binds to AT_1 receptors in the endothelium of the vasculature and initiates the sequence of events that lead to vasoconstriction and an increase in blood pressure. These include activation of phospholipase C and modulation of intracellular Ca^{2+} and induction of the release of the vasoconstrictor, endothelin-1 (Griendling et al, 1997; Imai et al, 1992). In addition to causing vasoconstriction, the binding of circulating angiotensin II to AT_1 in the adrenal gland results in aldosterone release (Sancho et al, 1976). Aldosterone causes the kidney to retain Na^+ and this results in water retention leading to increased blood pressure within the kidney. This localised increase in blood pressure stimulates the release of renin and the cycle begins again. In addition to the production of the vasoconstrictor, angiotensin II, ACE also inactivates bradykinin, which effectively inhibits the kallikrein-kinin pathway. ACE inhibitors are thus effective means of curbing hypertension.

In terms of this systemic regulation of blood pressure, the role of circulating ACE is of significant interest because it raises the question as to what role shedding of this molecule has on ACE function. As angiotensin I occurs within the plasma, circulation can transport this substrate through ACE-producing organs. Thus, lung ACE is sufficient to produce plasma angiotensin II (Ng & Vane, 1967) and overproduction of ACE by the liver in $ACE^{-/-}$ mice can maintain blood pressure regulation (Cole et al, 2002). This suggests that the production of circulating ACE is not necessary for the functioning of the RAS. One can speculate that it does serve as a secondary

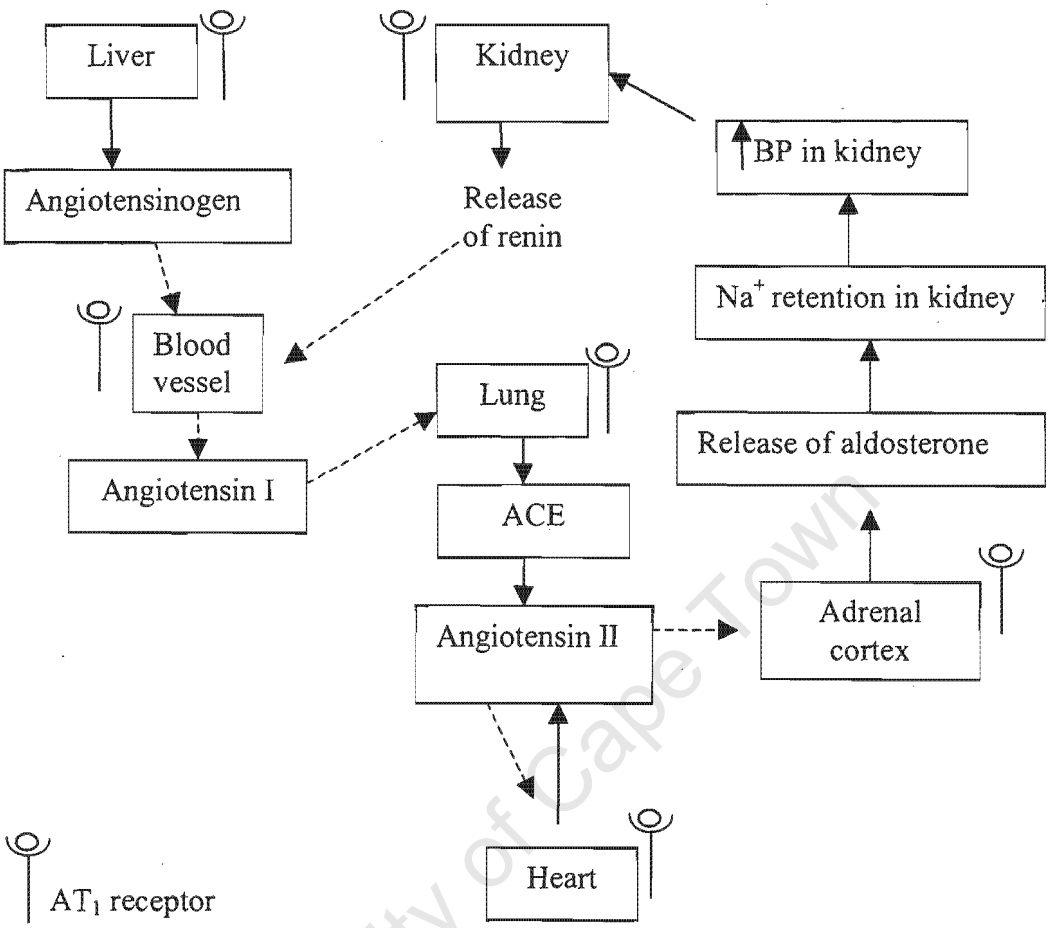


Figure 1.6.1 Endocrine pathway of RAS Angiotensinogen is produced by the liver and is found in blood plasma where renin produced by the kidney, converts it to angiotensin I. Angiotensin I is circulated to the lung and converted to angiotensin II, which is then transported to the adrenal cortex where it stimulates aldosterone release. Aldosterone stimulates renin release in the kidney by increasing renal blood pressure (BP). Solid arrows indicate production and release, broken arrows indicate circulation within blood plasma and the AT_1 receptor indicates effector systems in the organs.

mechanism for providing ACE activity locally. However, the local production of ACE within a number of organs, including that of the vasculature, makes circulating ACE redundant. It has been hypothesised that shedding could act as a down-regulatory mechanism for local removal of concentrated ACE activity. Cell lines expressing recombinant ACE can have different levels of ACE expression depending on transfection efficiency and the level of soluble ACE is a reflection of the level of

cell-associated ACE. Thus, if the level of plasma ACE is indicative of the level of its tissue-bound form, shedding could act as a means of maintaining the level of ACE attached to the membrane. Whether these two forms have different functions has yet to be elucidated.

1.6.2 Localised RAS systems

The concept of local RAS within the various systems, i.e. renal, cardiac and vasculature, is an important point to consider. The level of therapeutic effect of ACE inhibition on hypertension could not be correlated with the level of plasma ACE inhibition (Waeber et al, 1989). This suggests that tissue-ACE is of physiological

	Components				
Source of RAS	Renin	Angiotensinogen	ACE	AT_1	AII
Heart	+	++	+	+	+
Kidney	+++	+	++	++	+
Liver	++	++	+	++	+
Lung		+	+	++	
Intestine			++		
Vascular endothelium	+?	+?	++	+	+
Choroid plexus			+++		
Submandibular gland	+				
Prostate			+		
Adrenal gland	++	+	+	+	+
Brain		++	+	+	+
Ovary	++		+	++	
Uterus			+	++	
Testis, epididymis	++		++		
Blood			+		+
Urine			+		
CSF			+		
Seminal plasma			+		

Figure 1.6.2 Localisation of RAS components Positive signs indicate production of a RAS component, with increasing number of signs indicating increased expression levels. Question marks indicate controversial evidence regarding its location (Lieberman et al, 1983; Erdos & Skidgel, 1985; Erdos, 1967; Erdos et al, 1986; Lantz & Terenius, 1985; Igic et al, 1977) .

significance to the production of local angiotensin II within localised RAS. The production of local angiotensin II peptides able to bind to local AT_1 receptors makes the production of circulating components of RAS obsolete. Evidence to support the important role of tissue ACE in hypertension was derived from the differential inhibition of tissue and plasma ACE by two specific ACE inhibitors. The more effective inhibitor of hypertension (Hoe498) was shown to inhibit tissue ACE preferentially (Unger et al, 1984). Therefore, tissue ACE and the local production of angiotensin II within these tissues is responsible for blood pressure regulation. The conversion of tissue ACE into plasma ACE by proteolytic shedding could thus be an effective means of regulating this function. This review will now look at the localised RAS's within specific organs and attempt to link the action of shedding to the functioning of ACE in these tissues. To do this we will discuss ACE function within the vasculature, renal and cardiac tissues under normal and pathological conditions. These tissues express all the components of the RAS, although in varying concentrations (Figure 1.6.2).

1.6.2.1 Cardiac RAS

All components of the RAS have been localised in the myocardium (Dostal & Baker, 1999). In addition to locally produced renin, circulating renin has been shown to contribute to cardiac-specific synthesis of angiotensin II (Prescott et al, 2000). The presence of these components elicited great interest after it was noted that ACE inhibitors and AT_1 antagonists played a preventative role in the pathophysiology of cardiac hypertrophy and heart failure (Garg et al, 1995; Lijnen & Petrov, 1999). Cardiac hypertrophy is a compensatory reaction to increased blood pressure. In order to maintain contractile function while reducing the stress per unit area of the heart, hypertrophy of cardiac tissue occurs. However, with prolonged hypertension, the contractile function of the heart is reduced leading to cardiac dilation and diminished cardiac output, eventually leading to heart failure. As cardiac hypertrophy is linked to hypertension, the indirect role the RAS has to play in heart function is obvious. However, the RAS is directly implicated in cardiac hypertrophy: 1) myocardium produces angiotensin II; 2) RAS components increase during hypertrophy and heart dysfunction; 3) angiotensin II is able to stimulate the hypertrophy of cardiac myocytes

and fibroblast proliferation *in vitro*; and 4) ACE inhibitor and AT_1 receptor antagonist treatment are effective for cardiac failure (reviewed in Dostal, 2000).

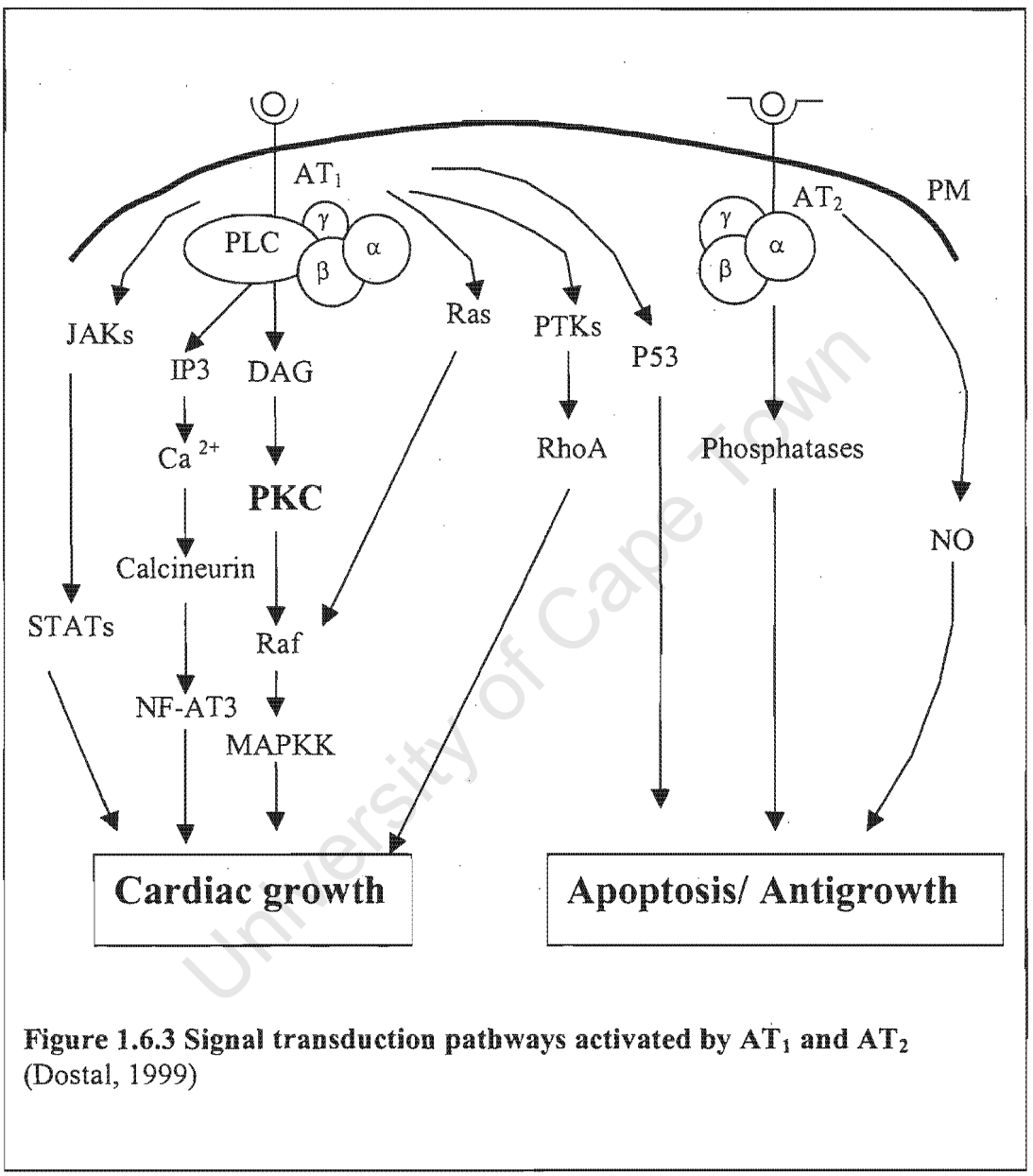


Figure 1.6.3 Signal transduction pathways activated by AT_1 and AT_2 (Dostal, 1999)

The presence of angiotensin II receptors in the atria, ventricles, cardiac myocytes and cardiac fibroblasts suggests the presence of a functional localised RAS in cardiac tissue (Sechi et al, 1992; Urata et al, 1989; Nozawa et al, 1994; Bastien et al, 1996; Matsubara et al, 1994; Gallagher et al, 1998, Neus et al, 1996). Additional evidence to support this is that the RAS is stimulated during cardiac hypertrophy (Dostal & Baker, 1999). Mechanical stretch results in angiotensin II release from rat cardiac myocytes

and prolonged mechanical stress culminates in AT_1, AT_2, angiotensinogen, and renin mRNA production (Sadoshima et al, 1999; Tamura et al, 1998; Kijima et al, 1996). Thus, once hypertension induces mechanical stretch within cardiac tissues, components of the RAS are produced locally, and angiotensin II is then able to bind to AT_1 receptors, induce vasoconstriction of the coronary vasculature, and activate a number of signalling pathways, which could culminate in cardiac hypertrophy (Berk, 1999; Blume et al, 1999). The putative signal transduction pathways induced by AT_1 and AT_2 are indicated in Figure 1.6.3 (Dostal, 1999). In addition to cardiohypertrophy, myocardial infarction (MI) also increases the level of the RAS components at the wound site and ACE inhibitors are effective therapy following MI (Zhu et al, 1997; Waring, 2000). Following myocyte loss, MI results in inflammatory cell and fibroblast invasion, collagen deposition by the fibroblasts, and myocyte hypertrophy to compensate for the initial loss of myocytes. However, the hypertrophy finally culminates in heart failure (Pfeffer & Braunwald, 1990). The cardiac remodelling observed following MI could be a function of ACE, as the blocking of AT_1 and the bradykinin (B_2) receptor produced at the infarction site alters the process of tissue remodelling (Carvallo Frimm de et al, 1997; Carvallo Frimm de et al, 1996).

In Figure 1.6.4 the effects of angiotensin II on the two angiotensin II receptors and their antagonists are indicated. AT_1 antagonists block cardiac hypertrophy in response to cardiac overload in mice and rats (Lijnen & Petrov, 1999). This is accomplished in 2 ways: 1) inhibition of cell growth by AT_1 blockage, and 2) increased level of available angiotensin II. The expression of AT_2 receptors is increased after myocardial infarction and the increased level of angiotensin II would promote inhibition of cell growth. Moreover, AT_2 has been shown to stimulate nitric oxide (NO) production via a bradykinin-dependent mechanism, thus enhancing the tissue-protective effects of AT_1 antagonists (Gohlke et al, 1998). When antagonists to AT_2 receptors were administered, the improved cardiac function achieved through AT_1 antagonists was completely reversed (Nio et al, 1995; Liu et al, 1997).

This mechanism explains the beneficial role that AT_1 antagonists play in the treatment of cardiac hypertrophy. Similarly, ACE inhibitors may also result in reduced hypertrophy as a result of lowered angiotensin II production and increased bradykinin-mediated NO protection (Gohlke et al, 1993; Golhke et al, 1994).

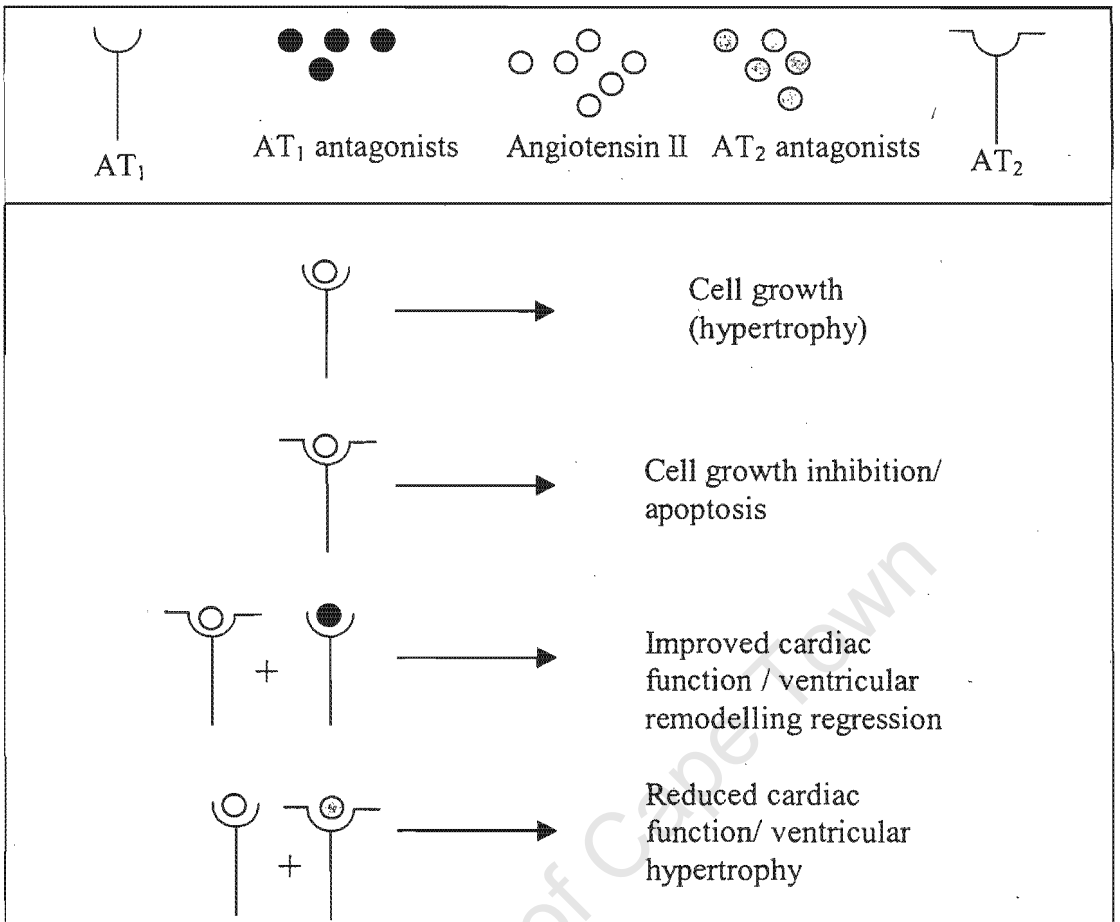


Figure 1.6.4 Effect of AT_1 and AT_2 antagonists on cardiac hypertrophy Binding of angiotensin II to AT_1 promotes cell growth whereas binding to AT_2, inhibits cell growth and stimulates apoptosis. After MI, AT_2 number increases. Treatment with AT_1 antagonists improves cardiac function but a follow-up treatment with AT_2 antagonists reverses the effect.

However, the therapeutic effect of ACE inhibitors after MI was similar when comparing wild-type to AT_2 receptor$^{-/-}$ knockout mice, whereas AT_1 antagonists indicated reduced therapeutic effect in the AT_2 receptor null mice (Xu et al, 2002). This would suggest that the mechanism behind the therapeutic action of ACE inhibitors, which reduce the level of available angiotensin II, differs from that of AT_1 antagonists. Table 1.6.1 lists the transgenic experiments and the cardiac-related phenotypes generated by the gene disruption or over-expression of components of the RAS. AT_1 receptors and AT_2 receptors clearly play a role in the hypertrophic effects of increased pressure load. However, ACE $^{-/-}$ and tissue-ACE$^{-/-}$ (lacking membrane-

bound ACE) mice do not exhibit structural cardiac abnormalities, unlike AT_{1A} and AT_{1B} null mutants where there were structural defects in the ventricular septum and high mortality rates. Compared to wild-type mice, tissue-$ACE^{-/-}$ mice had reduced maximal stroke volume and cardiac output, but there were no structural differences

Table 1.6.1 Phenotype of transgenic mice

Genotype	Phenotype	Reference
Over-expression of AT_1 in myocytes	Bradycardia, & heart block, myocytes hyperplasia	Hein et al, 1997
Over-expression of AT_1 in ventricles	Increased hypertrophy after increased pressure load	Hoffmann et al, 2001
Over-expression of angiotensinogen (Ao) in mice heart	Hypertrophy & fibrosis	Mazzolai et al, 1998
$Ao^{-/-}$ in mice	High mortality	Kim et al, 1995
$AT_1^{-/-}$ & $AT_{1A}^{-/-}$	Normal cardiac development; hypertrophy in response to pressure load	Ito et al, 1995; Hamawaki et al, 1998
$AT_{1A}^{-/-}$ + $AT_{1B}^{-/-}$	Mortality; severe ventricular septum defects	Tsuchida et al, 1998
$AT_2^{-/-}$	Impaired drinking responses; reduced body temperature; increased vassopressor responses to angiotensin II; renal tissue remodelling enhanced; reduced apoptosis; exagerated growth phenotype	Hein et al, 1995; Ichiki et al, 1995; Ma et al, 1998
$ACE^{-/-}$	Histologically and functionally normal heart	Krege et al, 1995; Crackower et al, 2002
Tissue-$ACE^{-/-}$	Diminished functional adaptation to MI	Aartsen et al, 2002

after MI. However, although no tissue ACE was present within the heart, there was no difference in the level of cardiac angiotensin II compared to wild-type mice (Aartsen et al, 2002). Thus, local cardiac ACE is not vital for cardiac re-modelling after MI. An attempt was made to correlate angiotensin II levels in the heart, kidney, lung and plasma to the genotypes of $ACE^{-/-}$, $ACE^{-/+}$ and $ACE^{+/+}$ mice. $ACE^{-/-}$ mice angiotensin levels in the heart, lung and kidney were not different compared to wild-type, but plasma angiotensin II was very low (<2 fmol/ml) and angiotensin I was very high (765 fmol/ml). Thus, ACE is needed for plasma angiotensin I conversion but is not needed in the tissue (Wei et al, 2002).

Angiotensin II can be generated by alternative proteinases, such as cathepsins and chymase (Figure 1.6.5) (Klickstein et al 1982; Perry et al, 2001). Chymase, a serine protease, is produced by mast cells, of which there is an influx in cardiac tissues upon hypertrophy and cardiac dysfunction (Hara et al, 2002). Chymase is present in higher quantities than ACE in most organs and human heart chymase is responsible for ~80% of angiotensin II formation in vitro and ex *in vivo* (Akasu et al, 1998; Urata et al, 1990; Wolney et al, 1998). In $ACE^{-/-}$ mice chymase activity was increased 1.5-fold (Wei et al, 2002). Thus, within cardiac tissues the lack of ACE could be compensated by the action of chymase to produce sufficient quantities of angiotensin II. However, this would not explain the effective therapeutic effects of ACE inhibitors on cardiohypertrophy and myocardial infarction (Figure 1.6.5) (Zhu et al, 1997; Waring, 2000).

The discussion above suggests that the production of local angiotensin II might not be the sole effector of the RAS within cardiac tissue. An ACE homologue, ACE2, has been identified in vascular endothelial cells of the heart and kidney. It acts as a carboxypeptidase and converts angiotensin I to angiotensin 1-9, and angiotenisn II to angiotensin 1-7, by the removal of the C terminal residue (Tipnis et al, 2000). It has a higher specificity constant for angiotensin II than for angiotensin I (Figure 1.6.5) (Vickers et al, 2002). Homologous recombination was used to delete the *ace2* gene and the phenotype of this gene disruption included lower contractile function of the heart, which eventually culminated in lowered blood pressure. This progressive phenotype suggested that the lowered blood pressure was due to severe cardiac dysfunction and not a direct effect of ACE2. The $ACE2^{-/-}$ phenotype included

increased angiotensin II levels in the plasma, heart and kidney. Thus, it is possible that ACE2 either competes with ACE for angiotensin I, and/or degrades angiotensin II. The double gene-deletion mutant, $ACE^{-/-}ACE2^{-/-}$, which lacked ACE and ACE2

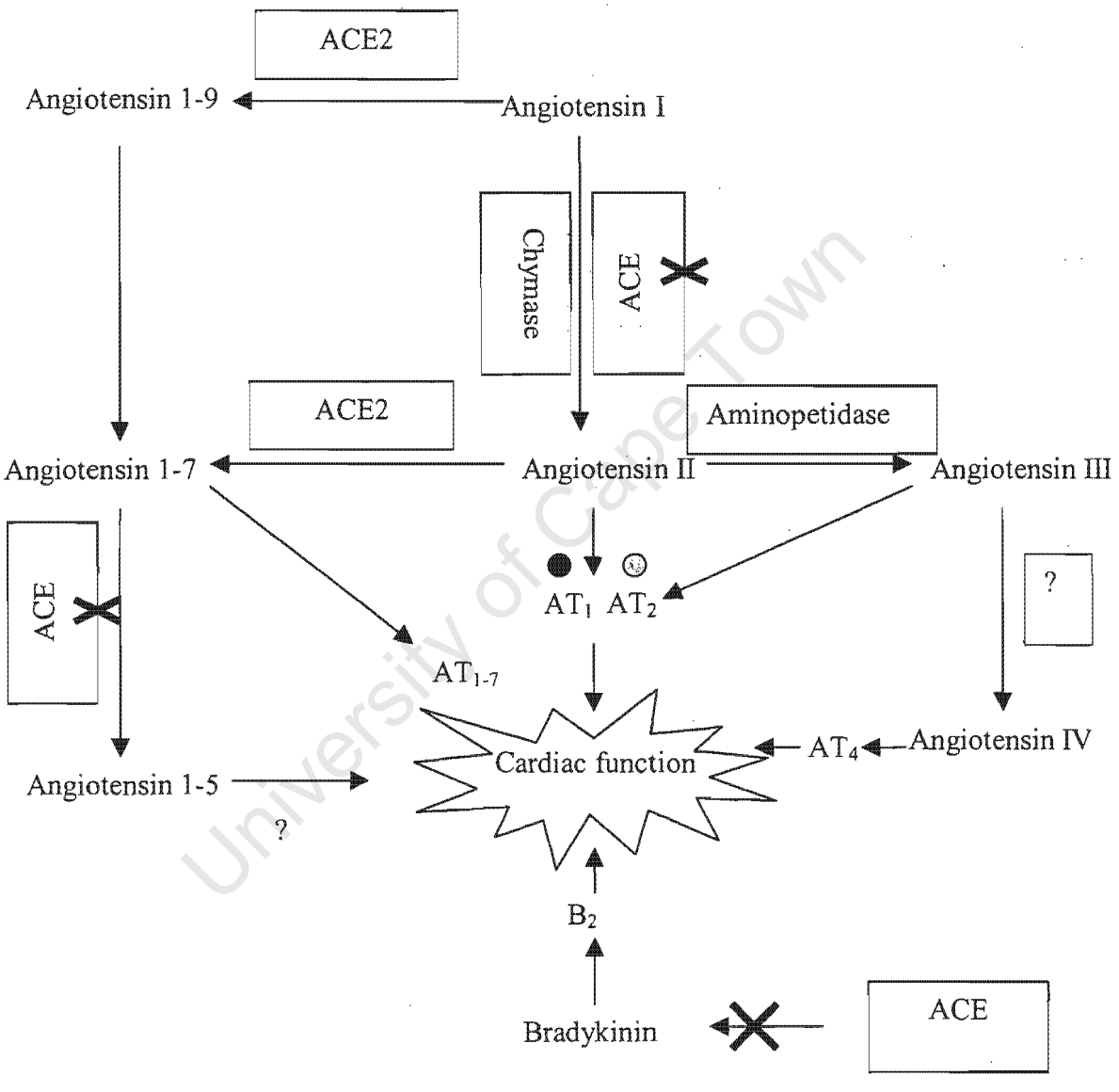


Figure 1.6.5 Putative effectors of the RAS and the non-RAS angiotensin I degradation products involved in cardiac function Both ACE inhibitors (black cross) and AT_1 antagonists (black circle) are therapeutic agents for cardiohypertrophy and MI. AT_2 antagonists are indicated by the grey circle. The exact mechanism of action of ACE inhibitors is complicated by alternative pathways involved in angiotensin II production and other angiotensin I degradation products that could play a role in cardiac function.

expression, rescued the cardiac dysfunction observed in $ACE2^{-/-}$ mice, indicating that the pathology of this phenotype was due to the effect of ACE on cardiac function (Crackower et al, 2002). One can speculate that ACE2 is needed to decrease angiotensin II levels to prevent increased levels from resulting in cardiac dysfunction.

The concentration of angiotensin 1-7 is higher than angiotensin I in a number of tissues and its functions oppose that of angiotensin II. Some of its functions include: 1) inhibition of vascular smooth muscle growth, 2) release of NO, 3) prostaglandin release, and 4) vasodilation (Freeman et al, 1996; Tallant et al, 1999; Jaiswal et al, 1992; Porsti et al, 1994; Ferrario et al, 1997; Brosnihan et al, 1998). Moreover, angiotensin 1-7 is involved in the differential AT_1 mRNA expression in smooth muscle tissue in a strain–specific manner (Neves et al, 2000). This vasodilator has also been shown to rescue cardiac function after MI (Loot et al, 2002). The therapeutic effects of this peptide could be due to it being a substrate for ACE. ACE converts angiotensin 1-7 to angiotensin 1-5 and a His-Pro dipeptide, mainly through the action of the N domain while inhibiting the C domain (Deddish et al, 1998). It could thus out-compete angiotensin I and bradykinin for the ACE active site and lead to decreased levels of angiotensin II and increased levels of bradykinin, resulting in release of NO (Li et al, 1997). The vasodilation caused by angiotensin 1-7 was abolished by the addition of B_2 receptor agonists, indicating that the hypotensive effect of angiotensin 1-7 is mediated by bradykinin (Abbas et al, 1997). The potentiation of bradykinin on its B_2 receptor by angiotensin 1-7 is not mediated by direct action on the bradykinin receptor, or activation of its own receptor, as potentiation was only achieved in the presence of ACE (Deddish et al, 1998; Tallant et al, 1997). However, inhibition of ACE-mediated kinin break-down by angiotensin 1-7 was not responsible for this response, as ACE-resistant B_2 receptor agonists were still potentiated (Deddish et al, 1998). Crackower et al did not determine the level of angiotensin 1-7 in $ACE2^{-/-}$ mice but the generation of the vasodilator can be carried out by prolyl endopeptidase, prolyl carboxypeptidase, and neutral endopeptidase (Welches et al, 1996; Welches et al, 1993; Greene et al, 1982). Thus, the pathology observed for $ACE2^{-/-}$ mice may have no bearing on angiotensin 1-7 as a vital modulator of the RAS within cardiac tissue (Figure 1.6.5).

ACE links the RAS to the kallikrein-kinin pathway and thus the effect of ACE inhibitors could extend beyond that of angiotensin II. Bradykinin receptors, B_1 and B_2 have different ligands and are the final effectors of the kallikrein-kinin system (Erdos et al, 1999). B_2 is the chief mediator (Bhoola et al, 1992). As bradykinin receptor antagonists abolished the beneficial effect of ACE inhibition on cardiac protection, the important role of kinins on cardiac tissue protection cannot be disregarded (Erdos et al, 1999; Ito et al, 1997; Linz et al, 1995). Bradykinin increase has been shown to influence the short-term effects of ACE inhibitors on blood pressure regulation, but in congestive heart failure patients, bradykinin does not seem to play a significant role in the long-term effects of the ACE drug (Gainer et al, 1998, Davie et al, 1999). However, the accumulation of bradykinin and inhibition of AT_2 is involved in protection against ischaemia/reperfusion induced by MI (Wang et al, 2001). ACE inhibitors act similarly to angiotensin 1-7, and do not only result in increased bradykinin levels, but also potentiate bradykinin action through B_2 when ACE is expressed at the cell surface, and directly activate B_1 receptors resulting in elevated calcium levels and NO release from cells (Ignjatovic et al, 2002). It has been suggested that ACE inhibitors induce the formation of ACE/B_2 heterodimers and this crosstalk either results in resensitisation of the receptor, and/or affected the sequestration of B_2 receptors within caveolin-rich membrane microdomains (Marcic et al 1999; Marcic et al, 2000a; Benzing et al, 1999). The potentiating effect of ACE inhibitors of bradykinin on B_2 of isolated tissues is thus not due to the inhibition of kinin hydrolysis, but the direct action of the inhibitor (Erdos et al, 1999). This direct action of ACE inhibitors could account for its effectiveness in cardiac protection, but the inhibition of ACE is likely to affect a range of interacting peptides such as angiotensin II, kinins such as bradykinin, and the RAS modulator, angiotensin 1-7 (Figure 1.6.6).

Although $ACE^{-/-}$ mice exhibited no structural cardiac abnormalities, the fact that this genotype rescued the cardiac dysfunction of $ACE2^{-/-}$ mice, implies that ACE plays a role in cardiac function (Crackower et al, 2002). Furthermore, tissue-$ACE^{-/-}$ mice had altered cardiac function compared to tissue-$ACE^{+/+}$ mice even though angiotensin II was present in significant amounts. The role that ACE plays in cardiac protection and/ or function has been further demonstrated by gene-linkage analysis. The insertion/deletion (I/D) polymorphisms of ACE have been linked to altered plasma

activity of the enzyme and are associated with renal and cardiovascular function (Wuyts et al, 1997). The homozygous D/D polymorphism is associated with increased plasma ACE activity and it has been suggested that this genotype is linked to ventricular hypertrophy in normotensive individuals and MI (Holmer & Riegger,

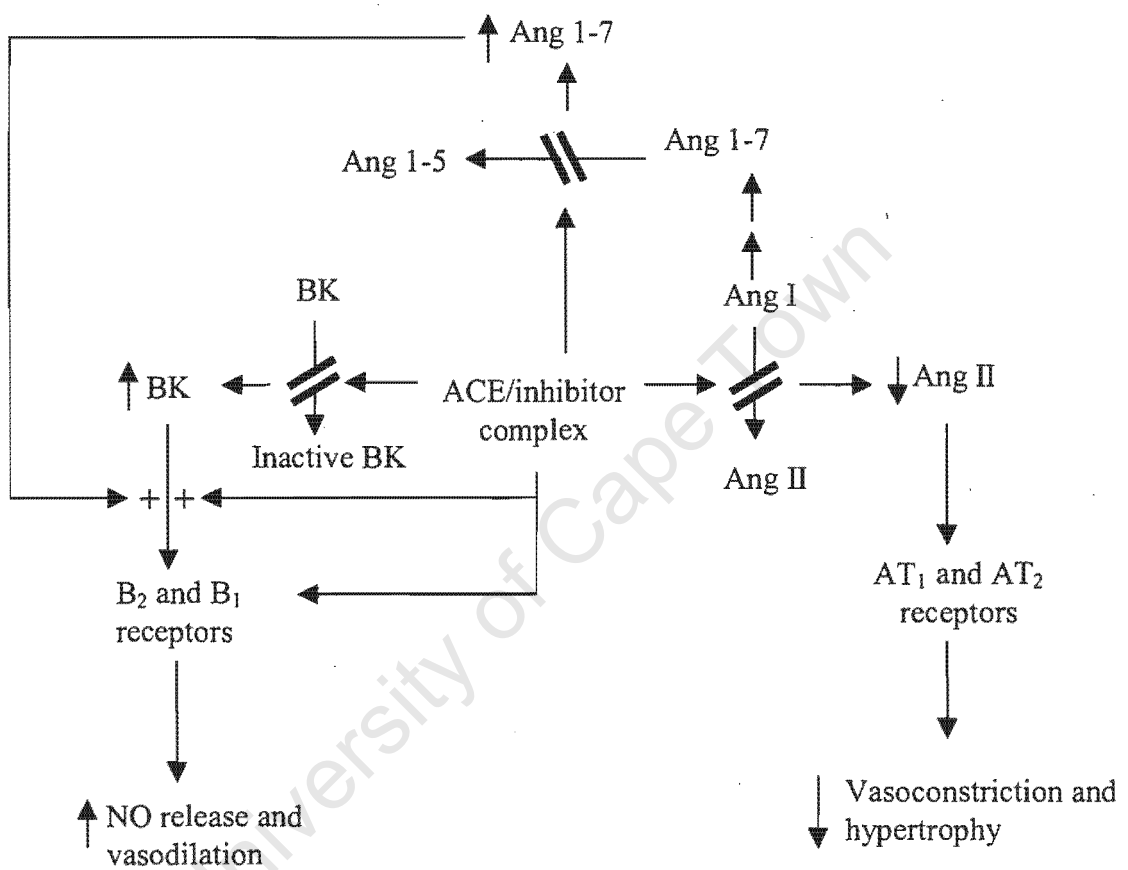


Figure 1.6.6 ACE inhibitors affect multiple effector systems ACE/Inhibitor complexes abrogate angiotensin II (Ang II) production, bradykinin (BK) degradation and angiotensin 1-5 (Ang1-5) generation, while enhancing BK action on B_2 receptors. The direction of the arrows next to the text indicates increase (upwards) and decrease (downwards) and the plus sign represents potentiation of BK action.

1995; Cambien et al, 1992). The increase in plasma levels associated with this genotype has been suggested to be due to increased gene transcription and would thus be a reflection of increased tissue-ACE and not increased shedding of this mutant ACE molecule (Cambien et al 1994). Thus, extrapolation to the significance of

increased plasma levels (and perhaps decreased tissue levels) on cardiac protection and function cannot be made. It has been shown that cellular and circulating ACE is differentially regulated and that hypoxia increases cellular ACE without a concomitant increase in the soluble form (Krulewitz & Fanburg, 1984). This suggests that during oxygen shortage, the upregulation of cellular ACE might be of functional significance. The potentiation of bradykinin action of B_2 receptors by ACE inhibitors and angiotensin 1-7 requires the co-localisation of ACE and B_2 in the membrane (Erdos et al, 1999). The cross-talk between ACE and the receptor is dependent on close proximity of the ectodomain of ACE with the B_2 receptor, and not dependent on the TM or cytoplasmic tail of ACE (Marcic et al, 2000a). Whether soluble ACE was able to elicit the same response was not investigated. Thus, we can speculate that the shedding of membrane-associated ACE from cardiac tissue may affect the role ACE plays when bound to angiotensin 1-7 as a potentiator of bradykinin activity. If tissue ACE were important for MI-induced cardiac tissue remodelling, then shedding would affect this function. However, the phenotype of tissue ACE deficient transgenic mice after MI did not differ from their wild-type counterparts, expressing membrane-anchored ACE even though the overall pump function of the heart was altered (Aartsen et al, 2002). Thus, shedding of ACE from cardiac tissue is unlikely to affect the role that ACE plays in the structural remodelling of cardiac tissue after MI, but may play a role in regulating the function of ACE in the overall pump action of the heart.

1.6.2.2 Vascular RAS

If the idea of a local RAS is dependent on all the components being present then the occurrence of renin within this tissue remains controversial. Renin is expressed in cultured smooth muscle cells and endothelium, but in other tissues little or no renin mRNA was detected (Lilly et al, 1985; Dzau et al; 1988; Ekker et al, 1989). Similarly, the expression of angiotensinogen by smooth muscle or endothelium, and not adipose tissue, has not been shown conclusively (Cassis et al, 1988). However, the rapid absorption of prorenin by the vasculature suggests that a functional RAS system can exist within this tissue. ACE is produced by the endothelium of the vasculature, therefore the presence of circulating ACE is not necessary for vascular constriction from the local production of angiotensin II within this tissue. However, when the

expression of endothelial ACE was eliminated in mice by placing the expression of the somatic ACE gene under the albumin promoter, the 87% increase in liver expression of this isozyme compensated for the activity of endothelial ACE and normal blood pressure was maintained (Cole et al, 2002). This would indicate that although the local RAS could exist in the vascular endothelium, the circulating RAS could fulfil its physiological function of blood pressure maintenance. This is

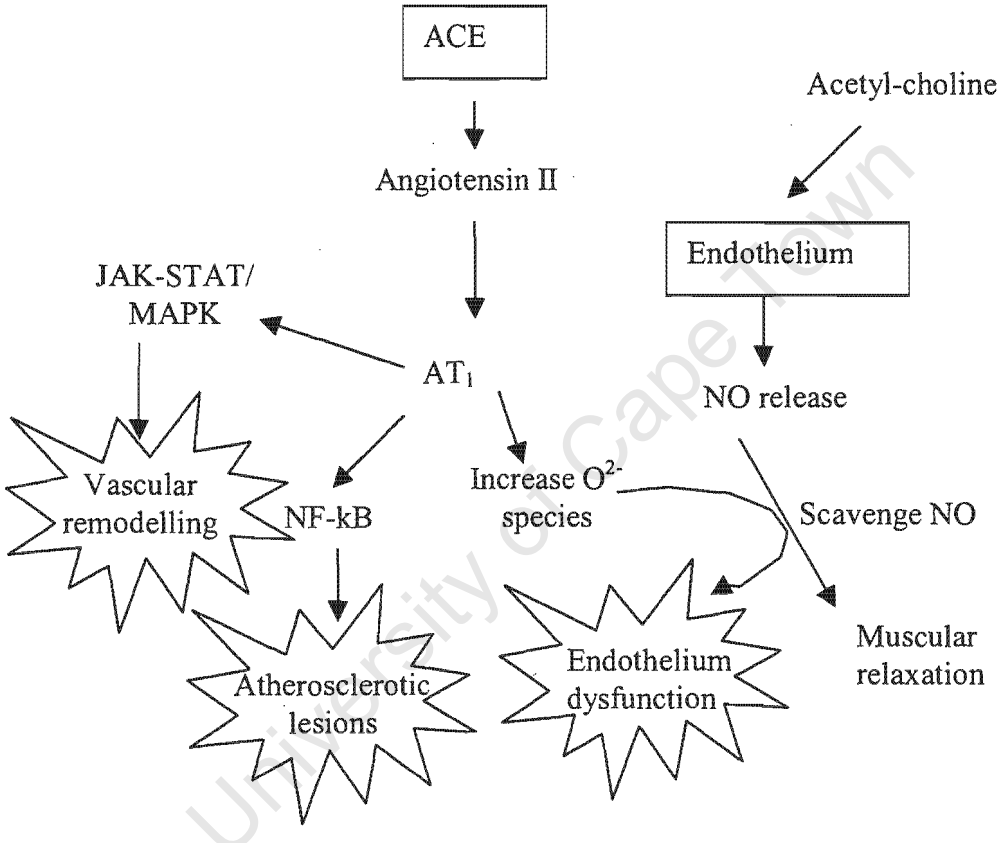


Figure 1.6.7 Role of ACE in atherosclerosis Effects of angiotensin II via the AT_1 receptor in the formation of atherosclerotic plaques and endothelium dysfunction. Under normal conditions, acetyl-choline stimulates the release of NO which facilitates muscle relaxation. Activation of AT_1 increases superoxide species that scavenge the NO and contribute to endothelium dysfunction. In addition AT_1 activates signal transduction pathways involved in vascular remodelling.

corroborated by earlier evidence that total plasma angiotensin I is converted to angiotensin II by circulation through the lung (Ng & Vane, 1967). Thus, if the production of ACE by a single organ is able to provide sufficient circulating angiotensin II, then it's likely that the existence of local RAS is not necessary for the

physiological functioning of blood pressure regulation. However, membrane-bound ACE must be important, as it was shown that tissue-bound ACE is essential for blood pressure regulation and kidney function (Esther et al, 1997). The transgenic mice tested in this experiment expressed ACE with only one active domain, the N domain. This construct was able to convert ~20% available angiotensin I, which, although a significant amount, might affect the conclusion that tissue-bound ACE, is vital for normal blood pressure maintenance and renal function.

The diseased state of the vasculature can give insights into the alternative role of the local RAS within the endothelium of blood vessels (Figure 1.6.7). Atherosclerosis is characterised by remodelling of the blood vessels resulting in reduction of the lumen. Causes for this disease are smoking, hypercholesterolemia, diabetes, hypertension and genetic factors. Atherosclerosis can result in stroke and MI. As a local RAS is located within the vessel wall, the cellular effects of angiotensin II on cell growth could mediate the structural changes seen in atherosclerosis. In fact, angiotensinogen expression is increased during neointima formation, renin expression is induced in medial smooth muscle by balloon injury and AT_1 and not AT_2 is upregulated during atheroscelorosis (Rakugi et al, 1993; Iwai et al, 1997; Yang et al, 1994; Wang et al, 1998). Moreover, angiotensin II seems to colocalize with increased levels of ACE, and not chymase, during atherosclerosis in endothelial cells and neointimal macrophages (Diet et al, 1996; Fukuhara et al, 2000; Ohishi et al, 1999). This suggests that angiotensin II, produced within the vessel wall through the hydrolysis of local angiotensinogen by the RAS, could play a role in vascular restructuring.

The onset of atherosclerosis is characterised by endothelium dysfunction, deposition and oxidative modification of low-density lipoprotein (LDL), and an inflammatory response (Figure 1.6.7). This is associated in part with an increase in reactive oxygen species (ROS). Angiotensin II infusion increases superoxide formation by AT_1 activation independent of its hypertensive function and AT_1 antagonists have been shown to inhibit vascular NAD(P)H oxidase activity and O^{2-} production in hypercholesterolemic animals (Rajagopalan et al, 1996; Warnholtz et al , 1999). The inability of the endothelium to respond to an acetyl-choline vasorelaxation stimulus characterises endothelium dysfunction. This relaxation is dependent on the production of the vasodilator and anti-atherogenic substance, NO, by the endothelium (Palmer et

al, 1987). Angiotensin II effects counteract this NO-induced relaxation by vasoconstriction and an increase of O^{2-} species, which act as scavengers of NO (Beckman & Koppenol, 1996). Angiotensin II is thus directly linked to the initial events of blood vessel remodelling (Figure 1.6.7).

The progression of atherosclerosis is defined as a chronic inflammatory response in the vascular wall (Ross, 1999). Angiotensin II is implicated in the activation of the transcriptional factor nuclear-factor κB (NF-κB), which is involved in the inflammatory response of atherosclerosis (Kranzhofer et al, 1999; Ruiz-Ortega et al, 2000) (Figure 1.6.7). The activation of this transcriptional factor has multiple effects on different cell types in the blood vessel wall and these changes are linked to atherosclerotic lesions (Hernandez-Presa et al, 1997; Hernandez-Presa et al, 1998; Han et al, 1999). In Figure 1.6.3 the signal transduction pathway activated by angiotensin II for cardiac function is indicated. The JAK-STAT and MAPK pathways are involved in vascular remodelling (Marrero et al, 1997; Seki et al, 2000; Xi et al, 1999). Thus, angiotensin II is known to activate a number of signalling pathways leading to extracellular matrix deposition, release of cytokines, expression of adhesion molecules, migration of smooth muscle cells, activation of growth factors, apoptosis, and thrombosis (Tummala et al, 1999; Griffin et al, 1991; Itoh et al, 1993; Diep et al, 1999; reviewed in Schmidt et al 2000). These have all been linked to the progression of atherosclerosis.

Although the local vascular RAS plays a role in atherosclerosis and indicates the significance of local angiotensin II-producing systems, it gives no indication as to the role that shedding of ACE may play.

1.6.2.3 Renal RAS

The RAS components, angiotensinogen and renin, are found in the kidney. The juxtaglomerular cells produce AT_1 and AT_2 receptors but AT_1 seems to be the main mediator of angiotensin II function (Richoux et al, 1983; Gomez et al, 1986; Gibson

et al; 1991; Gasc et al, 1993). ACE is found in the endothelium of the arterioles and glomerular capillaries, as well as the epithelia of the proximal tubules and descending vasa recta (Mounier et al, 1987; Wallace et al, 1978). The localisation of ACE expression changes during ontogeny and this could have implications for foetal renal development (Jung et al, 1993). Localised renal RAS has been suggested to play a significant role in renal function, such as renal blood flow, glomerular filtration, and tubular sodium readsorption due the local production of angiotensin II by ACE of the juxtaglomerular cells (Erdos, 1990). In addition to the functional advantage offered by a localised RAS, angiotensin II is known to act as a growth modulator and could act during the development of the kidney. Adult mesangial cells exposed to angiotensin II undergo hypertrophy, whereas foetal cells undergo hyperplasia (Ray et al, 1991). This indicates that angiotensin II elicits differing responses depending on the differentiation state of the cell. Additional evidence, for a role for angiotensin II in kidney development, was derived from treating newborn rats with AT_1 antagonists. This resulted in decreased renal to body ratios, modulation of cell proliferation and structural abnormalities such as aberrant vascular morphology, glomerular immaturity and tubular dilation (Tufro-McReddie & Gomez, 1993). Moreover, it has been demonstrated that the AT_1 receptor functions in elongation and branching of arterioles, glomerular capillary development, and late stages of nephrogenesis (Gomez, 1994). Thus, in addition to modulating kidney function, angiotensin II is a major determinant of kidney morphology and development (reviewed in Gomez and Norwood, 1995).

Mice, which have their *ace* gene deleted, have significant structural and functional kidney abnormalities. These include atrophy of the renal cortex, cortical thinning, and renal vascular changes (Krege et al, 1995). When the transmembrane domain and C-terminal domain were deleted so that the transgenic mice produced no tissue-ACE, they exhibited a similar phenotype to ACE null mutants, except that the renal pathology was not as severe (Esther et al, 1997). Tissue-$ACE^{-/-}$ mice also had an additional defective urine concentrating mechanism. This defect was associated with the down-regulation of important urea, salt, and water transport proteins (Klein et al, 2002). This suggests that renal development and function is a function of tissue-bound ACE. In addition, ACE secretase activity is not present in membranes isolated from human kidney, which suggests that the loss of tissue ACE by shedding does not occur

(Oppong & Hooper, 1993). Thus, for kidney development, tissue ACE might play a vital role. However, endothelial ACE null mutants that over-expressed ACE in the liver and only 12.5% of wild-type ACE in the kidney had normal renal function and blood pressure (Cole et al, 2002). The normal plasma angiotensin II levels generated in these transgenic mice were able to maintain normal kidney function, indicating that local renal angiotensin II production was not necessary. It was possible that the decreased level of ACE expression in the kidney was sufficient to maintain renal function under normal conditions or that other compensatory mechanisms came into play, which compensated for the 87.5% loss of tissue ACE within the kidney.

Rat mesangial cells have been shown to express and release full-length somatic ACE and N domain (Andrade et al, 1998). Naturally occurring N domain has been shown to occur in the ileal fluid of surgery patients and in urine (Deddish et al, 1994; Casarini et al, 2001). The naturally occurring N domain in the ileal fluid was thought to be due to the limited proteolysis of somatic ACE (Deddish et al, 1994). Thus far, an N domain mRNA species has not been detected, and Pang et al has shown that recombinant N domain was not readily shed from CHO cells (Pang et al, 2001). However, the isolation of the N domain from intact mesangial cells and the culture medium indicated that either limited proteolysis occurs intracellularly or the N domain was produced due to posttranscriptional modification. Alternative splicing of the ACE mRNA has been shown to generate kidney and lung ACE cDNA of 4.9 and 4.1 kb, respectively (Bernstein et al, 1988). Similarly, alternative splicing generated two forms of umbilical vein endothelial ACE, one lacking the TM domain of the protein (3.5 kDa) (Sugimura et al, 1998). These results raise the possibility of alternative splicing mechanisms of the ACE mRNA.

The tissue-ACE null mutants only have a functional N domain, as the C domain was deleted during the construction of the transgene (Esther et al, 1997). Thus, the differences observed between these two genotypes, i.e. soluble N domain with renal abnormalities compared to lowered expression (12.5%) of tissue N domain (and somatic ACE) and normal renal function in tissue-$ACE^{-/-}$ mice, might be of significance. However, the level of plasma angiotensin II was different for these two transgenic models, ~20% and 100% compared to wild type, respectively, and this could contribute to the differences observed. Angiotensin 1-7-producing enzymes

have been identified in the kidney and urine (Erdos & Skidgel, 1990; Handa et al, 1996). This vasodilator had a positive effect on glomerular filtration rate and increased water and electrolyte secretion (Chappel et al, 1998; DelliPizzi et al, 1994). The N domain hydrolyses angiotensin 1-7 efficiently, whereas the C domain is inhibited (Deddish et al, 1998). However, there is insufficient evidence to speculate on the alternate functions of the N domain and angiotensin 1-7 and the role they play in renal physiology.

1.7 Alternative function of ACE

1.7.1 Fertility

The smaller isoform of ACE (80-100 kDa), termed testis ACE, is only produced by developing sperm cells (Sibony et al, 1994; Williams et al, 1995). It has been localised to the periacrosomal plasma membrane (Kohn et al, 1998) and is released from sperm during capacitation (Kohn et al, 1995). Knock-out experiments in male mice have indicated that fertility in mice is dependent on the expression of testis ACE within spermatozoa. $ACE^{-/-}$ mice had a decreased number of spermatozoa in the oviduct and had reduced zona pellucida binding (Hagaman et al, 1998). It was further demonstrated that the specific expression of testis ACE in sperm of $ACE^{-/-}$ mice was able to rescue the infertility of the knock-out mice (Ramaraj et al, 1998). The role of ACE in male fertility cannot be attributed to the RAS, even though all components are present within the testis, because when the expression of angiotensinogen was eliminated in mice, the male mice retained fertility. This does not eliminate the possibility that the peptidase activity of ACE plays a role in mice fertility, as other physiological substrates could exist. However this substrate would have to be specific for testis ACE, as somatic ACE is present within the female reproductive tract. Alternatively, the role testis ACE plays in male fertility could be due to its structure and ability to be efficiently and rapidly shed from the membrane.

Spermatozoa bind to oviductal epithelium as a mechanism of maintaining viability (Smith & Nothnick; 1997). The binding has been postulated to occur via lectin-like interactions as sperm binding can be inhibited by fetuin, fucose, asialofetuin and galactose in a species-specific manner (DeMott et al, 1995; Lefebvre et al, 1995; Dobrinski et al, 1996). The structural difference between the two isoforms of ACE is

the presence of the unique 36 amino acid residues at the N-terminus of testis ACE (Soubrier et al, 1988; Ehlers et al, 1989). This unique region of testis ACE is highly *O*-glycosylated (Ehlers et al, 1992). Thus, the lowered number of $ACE^{-/-}$ mice sperm in the oviductal epithelium could be due to the inability of the sperm to bind to the oviductal epithelium via the *O*-linked sugar moieties of testis ACE. Furthermore, the release of sperm could be mediated via the release of testis ACE during capacitation, which is likely to occur via proteolysis within the stalk region. This mechanism of binding and release could very well resemble that of the rolling mechanism observed for inflammatory cells and could allow the transport of sperm through the oviductal regions in a regulated fashion (Ehlers & Riordan, 1991b). In fact, the binding and shedding of testis ACE could very well be intrinsically linked to the fertilizing capacity of sperm.

1.8 ACE Shedding

ACE was first discovered in blood plasma, indicating that the phenomenon of circulating, soluble ACE is not a new one (Skeggs et al, 1954). However, at the time of discovery, the actual generation of this form was unknown. ACE is an integral membrane protein and is localised on the plasma membrane and caveolae of vascular endothelial cells (Ryan et al, 1987). This review of ACE shedding will attempt to look at the process involved in converting the membrane-associated ACE to its circulating, soluble form.

1.8.1 Shedding

Endothelial ACE is solubilised by EDTA-sensitive proteolysis proximal to the TM domain (Hooper et al, 1987) (Wei et al, 1991a). Similarly, recombinant testis ACE is proteolytically released into the medium (Ehlers et al, 1991b; Sen et al, 1991; Sen et al, 1993). The cleavage secretion of rabbit testis ACE was stimulated by phorbol esters (growth stimulators) and the cleavage site was identified as the R663-S664 bond (R627-S628 bond in human testis ACE) proximal to the TM domain (Ramchandran et al, 1994; Ehlers et al, 1995; Ehlers et al, 1996).

The release of pig kidney ACE was further characterised using isolated membranes. It was discovered that the ACE-releasing proteolytic activity associated with the

membranes was: 1) resistant to high NaCl washes indicating, that it was an integral membrane protein, 2) Mg2+ dependent, 3) optimally active at 37°C and pH 8.4, and 4) present in porcine kidney, lung, testis and human lung and placenta (Oppong & Hooper, 1993). The metalloprotease responsible for ACE shedding was also shown to require ACE to be membrane bound, as it was unable to cleave detergent-solubilised amphipathic ACE (Ehlers et al, 1996; Parvathy et al, 1997). Biotinylation of cell surface proteins localised ACE proteolysis to the plasma membrane (Beldent et al, 1995).

Hydroxamic-acid-based zinc metalloprotease inhibitors such as batimastat, marimastat and BB2116 were synthesised to inhibit matrix metalloproteases (Davies et al, 1993; Gearing et al, 1994). Hydroxamates bind to the active site of the metalloprotease and coordinate the zinc ion (Botos et al, 1996; Zhang et al, 1994). Tumour necrosis factor α convertase (TACE) is inhibited by batimastat, TAPI and BB2116 (Parvathy et al, 1998c). TACE belongs to the ADAMs (A Disintegrin And Metalloprotease) family of zinc metalloproteases (Black et al, 1997). The proteolytic processing of ACE was shown to be sensitive to Compound 3, batimastat (IC50 of 0.47μM) and TAPI-2 (IC50 of 18 μM) and not non-hydroxamate collagenase inhibitors (Parvathy et al, 1998b; Ramchandran & Sen, 1995). It is thus likely that the metalloprotease responsible for the proteolytic processing of ACE, termed the ACE secretase, is not a matrix metalloprotease but a member of the ADAMs family of proteases (Parvathy et al, 1998b). The shedding of ACE and other membrane proteins exhibit different levels of sensitivity to hydroxamic acid-based inhibitors (Hooper et al, 1997). This indicates that more than one secretase is involved in the shedding of membrane proteins and that inhibition profiles can be used to distinguish these proteases (Parvathy et al, 1998c).

1.8.2 Regulation

As discussed before, phorbol esters stimulate ACE release, and this stimulation was inhibited by staurosporin, indicating that the shedding of ACE is regulated by PKC (Beldent et al, 1995; Ehlers et al, 1995). ACE has been shown to interact specifically with isozymes of PKC and the addition of phorbol ester causes the dissociation of these isozymes except for PKCλ (Figure 1.8.1). It was suggested that this dissociation

plays a role in the activation of the ACE secretase (Santhamma & Sen, 2000). In addition, PMA increased *ace* gene expression but whether this plays a role in the increased level of soluble ACE is unlikely, as the response to phorbol esters occurs within minutes and does not involve *de novo* protein synthesis (Eyries et al, 2002; Mullberg et al, 1992). TACE surface expression was down regulated in response to phorbol esters. The link between decreased cell-surface expression of the secretase and increased shedding is unknown (Doedens & Black, 2000). Ionophores did not affect ACE shedding, thus shedding occurs independent of Ca^{2+} (Beldent et al, 1995).

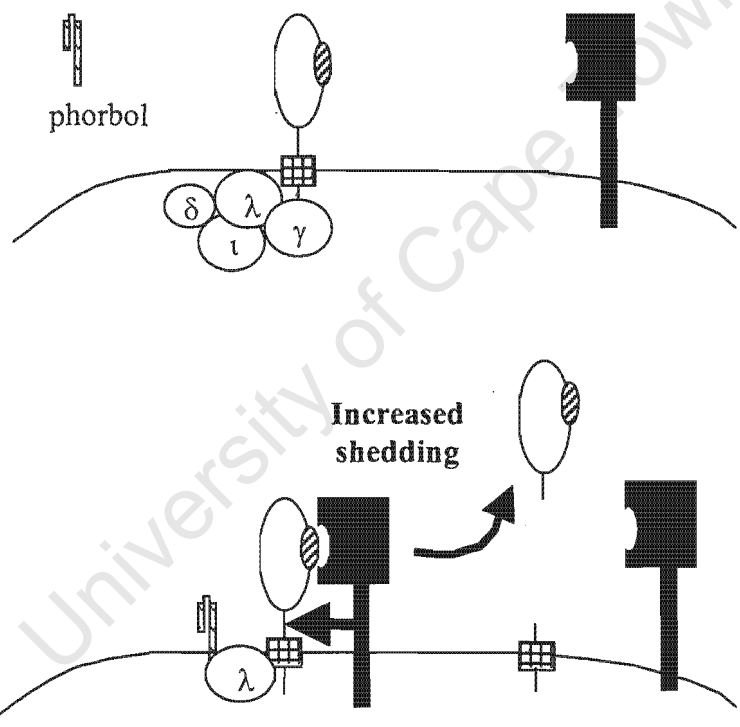


Figure 1.8.1 Effect of phorbol esters on PKC dissociation and stimulation of the ACE secretase Addition of phorbol esters results in the dissociation of PKC isozymes and increased shedding of ACE (Santhamma & Sen, 2000).

Casein kinase 2 (CK2) associates with, and phosphorylates, the cytoplasmic tail of ACE at S1270 (Kohlstedt et al, 2002). Phosphorylation regulates the retention of ACE within the PM, as the unphosphorylated S1270A substitution ACE mutant exhibited

lower association with the PM than wild-type ACE and enhanced constitutive shedding (Figure 1.8.2) (Kohlstedt et al, 2002). PMA was able to stimulate the shedding of S1270A indicating that the regulated shedding of ACE is not affected by the phosphorylation of ACE (Kohlstedt et al, 2002).

Inhibitors of serine proteases like aprotinin, benzamidine and phenylmethanesulfonylflouride (PMSF), and inhibition of aspartic proteases such as

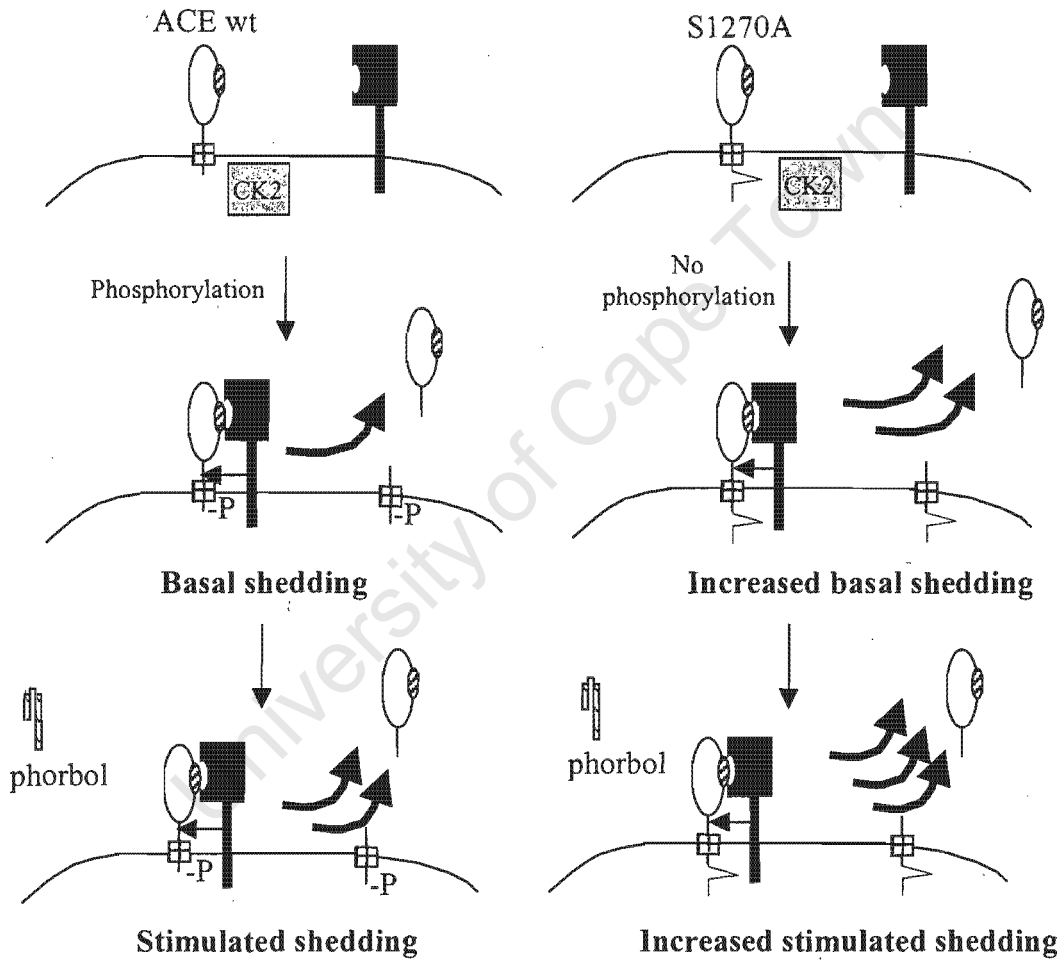


Figure 1.8.2 Effect of phosphorylation on basal shedding of ACE Phosphorylation of wild-type ACE by CK2 results in increased membrane-bound ACE and lowered basal shedding, but does not affect phorbol-induced shedding. The point mutation, S1270A, in the cytoplasmic tail of ACE abrogated phosphorylation and this resulted in increased shedding in the absence and presence of phorbol esters (Kohlstedt et al, 2002).

antipain and pepstatin and of endopeptidases, did not inhibit shedding (Oppong & Hooper, 1997). However, hydroxamate inhibitors, such as TAPI, Compound 3 and batimastat abolished shedding (Parvathy et al, 1997). In contrast, DCI, a serine protease inhibitor, was shown to up-regulate shedding (Alfalah et al, 2001, Schwager et al, 1999). This upregulation of soluble ACE was transient, as metabolic labelling and pulse-chase experiments showed that DCI inhibited the maturation of ACE to its fully glycosylated form and after the initial burst of ACE release, no ACE was solubilised. This was likely due to activation by DCI of the ACE secretase to shed all available PM ACE and once these molecules were shed, the lack of mature ACE resulted in no further ACE solubilisation (Schwager et al, 1999). The regulation of shedding of TGF-α in the absence and presence of phorbol esters was different. The constitutive and phorbol-induced shedding of TGF-α involved different MAP kinase signalling cascades, indicating that these two processes are differentially regulated (Fan & Derynck, 1999). Similarly, the different effect of phosphorylation on basal and regulated shedding of ACE suggests that ACE shedding is differently regulated.

1.8.3 Structural determinants

TACE, responsible for the shedding of TNF-α has been isolated and the three-dimensional structure determined (Black et al, 1997; Moss et al, 1997). This secretase is not responsible for the shedding of ACE *in vivo* as indicated by the transient transfection of ACE cDNA into $TACE^{-/-}$ cell line (Sadhukhan et al, 1999). However, co-expression of TACE cDNA with ACE in the $TACE^{-/-}$ cells increased ACE solubilisation by ~17% and TACE was able to cleave a synthetic peptide spanning the cleavage site of ACE (Sadhukhan et al, 1999). Thus, although TACE is not responsible for ACE solubilisation, it does play a role in the shedding process. The exact role is unknown.

TACE is able to cleave TGF-α, L-selectin, p75 TNF receptor and β-APP (Peschon et al, 1999; Buxbaum et al, 1998; Merlos-Saurez et al, 1998). At this juncture, it was proposed that TACE and the α-secretase were the same metalloprotease, as $TACE^{-/-}$ fibroblast cells were unable to shed β-APP. The authors did not exclude the possibility that TACE might not directly shed β-APP, but act on a separate protein involved in the proteolysis (Bauxbaum et al, 1998). However, although able to cleave

β-APP, the inhibition profile of TACE using batimastat, Compound 1 and Compound 4 was different to that of the α-secretase responsible for normal, physiological shedding of β-APP (Parvathy et al, 1998c). In fact, the inhibition profiles of the α-secretase and the ACE secretase were very similar for a range of hyroxamate inhibitors (Parvathy et al, 1998a). The specific TACE inhibitor, Compound 15, which does not affect α-secretase proteolysis, did not inhibit the release of carbachol-stimulated release of ACE or APP from neuronal cells. Moreover, DCI stimulated APP shedding similarly to ACE (Parkin et al, 2002). This suggests that the secretase responsible for β-APP and ACE cleavage were one and the same or very similar. However, by investigating the structural requirements for the shedding of these two ectoproteins, it was shown that the juxtamembrane region of β-APP, but not that of ACE can direct the shedding of a protein not shed under normal physiological conditions (Arribas et al, 1997, Sadhukhan et al, 1998). These data suggest that the ACE secretase is distinct from the α-secretase as it has different determinants for shedding. The investigation into the determinants for shedding is thus a vital tool for distinguishing the various hydroxamate-inhibited, phorbol-stimulated secretases involved in the shedding of ectoproteins. The stalk and the ectodomain of ectoproteins have been thoroughly investigated to determine whether they contain the sequence or structural determinants for the release of membrane proteins.

1.8.3.1 Stalk

The cleavage site of human testis ACE has been identified as the scissile bond between R627 and S628 (Ehlers et al, 1996; Ramchandran et al, 1994). This cleavage site occurs within the stalk region, 24 amino acid residues from the membrane. As the stalk region is the ultimate target for the ACE secretase this region has received a large amount of interest as a structural determinant for shedding. There is no sequence identity between the stalk regions of ACE and those of other shed proteins (reviewed in Ehlers et al, 1996). When the stalk sequence was replaced with the stalk region of the low-density lipoprotein receptor (LDL-R), ACE was still shed from the cell surface (Ehlers et al, 1996). In addition, the fusion of the ACE stalk sequence to the ectodomain of the unshed protein, CD4, did not result in the shedding of this protein (Sadhukhan et al, 1998). Thus, sequence identity of the stalk is unlikely to play a role in ACE shedding.

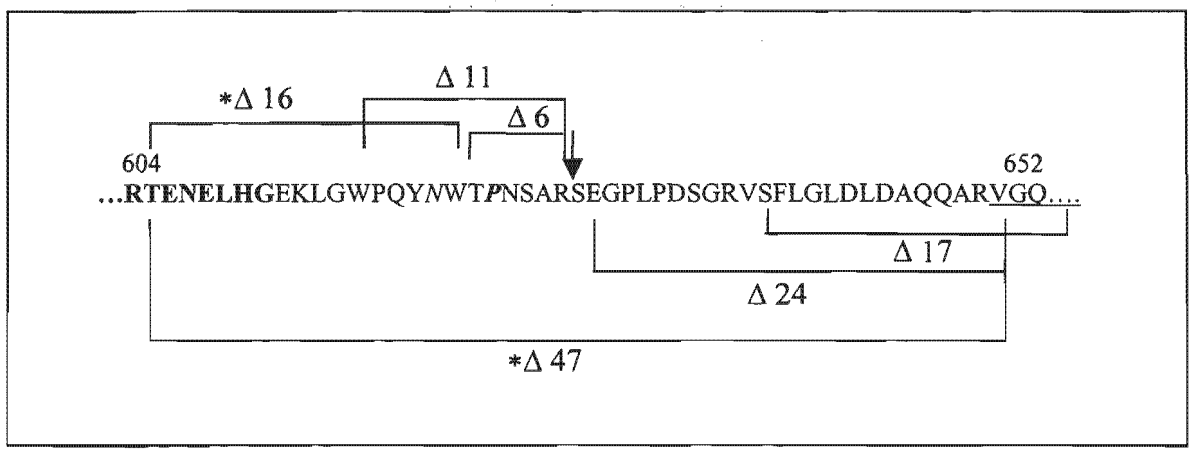


Figure 1.8.3 Stalk deletion ACE mutants Stalk sequence of ACE is indicated with TM underlined and the start of the ectodomain determined by the three -dimensional structure of testis ACE is in bold (Natesh et al, 2003). The cleavage site is indicated after R627 and the deletion mutants are ACE-JMΔ47, ACE-JMΔ24 and ACE-JMΔ17 with residues T605-R651, S628-R651 and V639-Q554 deleted C-terminal to the cleavage site, respectively. The stalk mutants with regions deleted upstream of the cleavage site are ACE-JMΔ6, ACE-JMΔ11 and ACE-JMΔ16 with residues W621-A626, W616-A626 and T605-N620, respectively. * Inactive ACE mutant (Ehlers et al, 1996; Chubb et al, 2002). P623L (Corresponding to P1199 of somatic ACE) and N620Q point mutations involved in altered shedding (Eyries et al, 2001; Alfalah et al, 2001) are in italics.

Regions of the ACE stalk were deleted to investigate whether the length of the stalk region affected shedding. ACE-JMΔ47 ACE-JMΔ24, and ACE-JMΔ17 were generated with 47, 24 and 17 amino acid residues removed, as indicated in Figure 1.8.3. The deletion of 47 amino acid residues from the TM extending into the ectodomain resulted in inactivation of ACE. This deletion thus destabilised the native conformation of ACE. ACE-JMΔ24 and ACE-JMΔ17 had the same cleavage sites as wild type-testis ACE, but exhibited different shedding kinetics. ACE-JMΔ24 was shed less efficiently than wild type testis ACE in contrast to ACE-JMΔ17, which was shed more efficiently (Ehlers et al, 1996). As the cleavage sites remained at the R627-S628 bond for both ACE-JMΔ24 and ACE-JMΔ17, 1 and 11 amino acid residues away from the TM respectively, it seems possible that the secretase positions itself primarily with respect to the ectodomain. The authors concluded that an accessible stalk region allowing a minimum distance of 8 residues between the cleavage site and the TM and 3 residues between the cleavage site and the proximal domain is required for shedding to occur (Ehlers et al, 1996).

To determine whether the amino acid residues between the cleavage site and the ectodomain determines proteolysis, an additional 3 mutants were constructed, ACE-

JMΔ6, ACE-JMΔ11 and ACE-JMΔ16, in which 6, 11 and 16 residues were deleted N-terminal to the cleavage site (Figure 1.8.3). ACE-JMΔ6 and ACE-JMΔ11 exhibited very low expression levels in CHO cells, although they were shed with similar kinetics as wild-type testis ACE, but ACE-JMΔ16 was inactive (Schwager et al, 1995; Chubb et al, 2002). Thus, deletion of the region from W616 to A626 did not affect the shedding of ACE, but did affect the translocation of the protein to the membrane and/ or stability of the protein suggesting that this region might play a role in overall ACE processing. It cannot be excluded that a stalk of minimal length could be needed for successful expression and translocation to the PM, but ACE-JMΔ17 was successfully expressed and shed (Chubb et al, 2002; Ehlers et al, 1996). The inactivation of ACE-JMΔ16 and its inability to be processed to the cell membrane indicates that the region from T605 to W616 is critical for the maintenance of native conformation and thus activity. This was corroborated by the determination of the three-dimensional structure of testis ACE, which indicated that the final alpha helix (α20) ends at G611 (Natesh et al, 2003). Thus, deletion of the region from T605 to W616 is likely to have disrupted the structural integrity of ACE. The deletion of 11 residues N-terminal to the cleavage site did not disrupt the shedding of ACE and ACE-JMΔ11 had an identical cleavage site to wild type testis ACE (Chubb et al, 2002). Thus, positioning of the ACE secretase relative to the ectodomain of ACE-JMΔ11 did not shift the cleavage site C-terminally. This suggests that stringent alignment of the ACE secretase with that of the ACE ectodomain is not needed for proteolysis at the R627-S628 bond.

These results indicate that a distinction must be made when considering the effects of stalk ACE mutations on shedding between: 1) the effect of mutations on structural disruption and thus ACE activity; 2) the effect of the mutation on processing and translocation of ACE to the membrane; and 3) the actual effect on shedding itself. The accessibility of the stalk region has also been investigated by the substitution of the ACE stalk with the disulfide-linked N-terminal EGF-like domain of the LDL-R and the *O*-glycosylated region from the N-terminus of testis ACE in the constructs ACE-JMEGF and ACE-JGL, respectively (Schwager et al, 1998; Schwager et al, 1999). Cleavage-site determination indicated that ACE-JMEGF was cleaved at the G652-F653 bond, 12 amino acid residues away from the membrane, within the third

disulfide loop. Similarly, ACE-JGL was also proteolytically released and the cleavage site occurred at the A636-T637 bond (Schwager et al, 1998; Schwager et al, 1999). The construction of the mutant, ACE-JMΔ6, created a potential *N*-linked glycosylation site. The glycosylated N626 that occurred close to the cleavage site of wild-type ACE (R627-S628) shifted the cleavage site to the F640-L641 bond. It was proposed that the presence of bulky residues in the stalk do not prevent proteolysis, but shift the cleavage site to more accessible regions (Schwager et al, 1999). Thus, the concept of what defines an "accessible" stalk region needs to be revisited. It seems that within a highly structured domain, whether generated by glycosylation or disulfide bridges, there exist stretches of peptide backbone exposed enough at the protein surface, to be cleaved by the ACE secretase.

The data discussed above allows us to conclude that the ACE secretase is highly non-specific for protein sequence and structured domains within the stalk region. Nevertheless, a point mutation within the stalk sequence of somatic ACE, P1199L (equivalent to P623 of testis ACE) resulted in an increase of somatic ACE shedding. It was proposed that the substitution mutation resulted in better accessibility of the stalk to the ACE secretase (Eyries et al, 2001). It is also possible that the point mutation of P1199L could increase the processing and translocation of the ACE mutant to the PM, resulting in increased solubilised ACE. The metabolic labelling and pulse-chase of the cell-associated mutant indicated that it matures faster than wild-type ACE (Eyries et al, 2000). Interestingly, a point mutation within the testis ACE stalk region, N620Q, also resulted in altered proteolysis of ACE. This ACE mutant was cleaved by a serine protease within the stalk region while in transit through the endoplasmic reticulum (ER) (Althoff et al, 2001). A serine protease was also implicated in the shedding of the ACE-JGL mutant (Schwager et al 1999). Interestingly, the amino acid residue N620 was involved in the alternate shedding patterns of both of these ACE mutants. Similarly to the somatic ACE mutant, this N620Q point mutation construct was shed more efficiently than wild type testis ACE (Alfalah et al, 2001). As this mutant was shed before it localised with the ACE secretase in the plasma membrane we cannot conclude that it was not an ACE secretase substrate. It does however, have implications on the susceptibility of the

native stalk region of ACE to alternate secretases and the implications this would have on differential shedding patterns and possible physiological consequences.

The secondary structure of the region from L609 to D646 was predicted to form a flexible loop bound by α-helices N-terminally and C-terminally (Alfalah et al, 2001). Sequence alignment of the 2 domains of ACE indicates that homology between the two domains ends at P623 (testis ACE cDNA, Ehlers et al, 1989). As discussed before, the region from W616 to A626 seems to be involved in the correct processing of ACE to the plasma membrane either directly or indirectly by maintaining conformational stability (Chubb et al, 2002). Both published stalk point mutations (P1199L and N620Q) fall within this region. The crystal structure of testis ACE indicated that this region had no ordered secondary structure and was not part of the folded ectodomain (Natesh et al, 2003). Thus, it seems unlikely that mutations within this region could affect the overall conformational stability of ACE. However, as mutations within this region recruited different secretases, altered the location of proteolysis and affected the expression of ACE constructs, we can speculate that this region might be sensitive to alterations of protein sequence and might affect the overall processing of ACE.

The stalk of ACE was unable to direct the shedding of CD4 once fused to its ectodomain (Sadhukhan et al, 1998). However, the ACE stalk was able to direct the shedding of a glycosylphosphatidylinositol (GPI)-linked protein, membrane dipeptidase (MDP) once fused to the ecotodmain of this protein. Proteolysis occurred at the cleavage site of wild type testis ACE (Pang et al, 2001). It was suggested that the differences of shedding ability observed with these two ACE stalk chimeras could be ascribed to the fact that 77 amino acid residues from the ACE stalk comprised the CD4-ACE stalk chimera, whereas only 34 stalk amino acid residues were included in the construction of the MDP-ACE stalk mutant (Pang et al, 2001). Thus, the additional ACE stalk region of the CD4-ACE chimera could comprise part of the folded ectodomain of ACE, which might interfere with the accessibility of the stalk region (Pang et al, 2001). An alternative suggestion involves the nature of the shed ectodomain itself. It has been suggested that the ectodomain of membrane proteins comprise motifs, which target them for shedding from the plasma membrane. Thus, in

relation to the two ACE stalk chimeras discussed above, the MDP chimera might contain such a motif (Pang et al, 2001).

1.8.3.2 Ectodomain

In addition to constructing a CD4-ACE stalk chimeric protein, the ectodomain of ACE was also fused to the stalk region of CD4 (Sadhukhan et al, 1998). CD4 is not normally shed from the membrane. However, linking ACE residues from 1 to 655 to the CD4 stalk sequence, TM and cytoplasmic tail (ACE/CD4-3) resulted in proteolytic cleavage of the stalk region of CD4 (Figure 1.8.4). Increasing the length of the CD4 juxtamembrane region linked to the ectodomain of ACE by 61 amino acid residues, resulted in increased release (ACE/CD4-5). Therefore, the ectodomain of ACE can direct the shedding of an unshed protein, and shedding of the chimeric proteins were affected by the length of the stalk sequence fused to the ectodomain. As

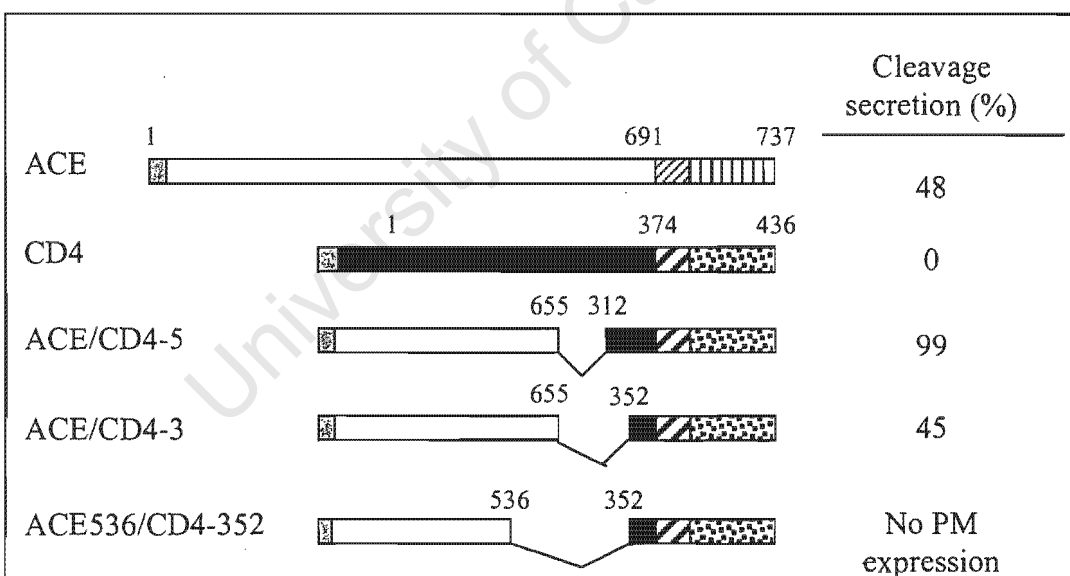


Figure 1.8.4 CD4 stalk/ACE ectodomain chimeric proteins Rabbit testis ACE is indicated above with the ectodomain shown as the clear bar, the TM as the diagonally striped box and the cytoplasmic tail indicated by the vertically striped bar. Similarly, CD4 is shown beneath with the ectodomain shown as the black bar and TM and cytoplasmic tails aligned with those of testis ACE. Three chimeric proteins are shown with their corresponding amino acid residues indicated above and the cleavage secretion (%) indicated alongside (Sadhukhan et al, 1998).

the presence of additional CD4 juxtamembrane sequence increased shedding, one can speculate that interactions between regions from different proteins within the three-dimensional structure of chimeric proteins, affect the shedding machinery by altering the overall native conformation of the ectodomain. When 119 amino acid residues from P536 to Y655 were removed from the ACE ectodomain of the chimeric protein, ACE/CD4-3, the protein was not translocated to the PM (Figure 1.8.4).

This region corresponds to P500-Y619 of human testis ACE and includes H6, α17, H7, α18, α19 and α20 (Natesh et al, 2003). This region comprises the chloride-binding amino acid residue R522. Thus, deletion of this region disrupted the conformational stability of ACE. The rational design of chimeric proteins must therefore take into account: 1) the deletion of protein sequence involved in protein processing and translocation to the PM; and 2) disruption of native conformations through the creation of unfavourable (or favourable) interactions between the protein sequence, and thus domains, of different proteins. The ability of the ACE ectodomain to direct the shedding of an unshed protein suggests that the ectodomain comprises a recognition motif for the ACE secretase. The existence of such a motif was investigated using other chimeric proteins (Althoff et al, 2001). From the various chimeras that are shed and not shed, it is difficult to come to an unequivicol conclusion concerning the existence of the secretase recognition motif (Table 1.8.1). The substitution of the stalk of the shed protein, Pro-TNF-α, with the stalk of IL-6R, another shed protein, abolishes the shedding of Pro-TNF-α (Althoff et al, 2000). However, the stalk of IL-6R was shown to direct the proteolysis of the unshed protein gp130 (Althoff et al, 2001). Thus, the recognition motif in the ectodomain of Pro-TNF-α cannot direct shedding once the stalk region comprises sequence of IL-6R whereas the recognition motif directing the shedding of the gp130/IL-6R chimera is only able to do so once the stalk region is comprised of that same protein sequence.
Additional evidence to support the proposal of a recognition motif for secretases was obtained by studies of somatic ACE shedding. Somatic ACE was cleaved less efficiently than testis ACE and, once the C domain was deleted, the truncated somatic ACE molecule was shed 10-fold more efficiently (Beldent et al, 1995). The two domains of somatic ACE have high sequence identity and similar substrate specificity (Soubrier et al, 1988, Wei et al, 1991a). Thus, it was proposed that: 1) the N domain

sterically hindered access to the stalk region; and/or 2) it lacked a recognition motif for the ACE secretase and occluded the one present in the C domain (Beldent et al, 1996; Pang et al, 2001). To test the latter, a somatic ACE mutant was constructed which lacked the C domain. This construct, which had the N domain directly fused to the stalk, TM and cytoplasmic tail of somatic ACE, was not shed from the membrane (Pang et al, 2001). This suggested that the N domain lacked the recognition motif for the ACE secretase.

Table 1.8.1 Chimeric proteins and their shedding status

Ectodomain	Stalk	Shedding	Reference
[a]Pro-TNF-α	[a]IL-6R	No	Althoff et al, 2000
[b]β-Glycan	[a]β-APP	Yes	Arribas et al, 1997
[b]β-Glycan	[a]Pro-TGF-α	Yes	Arribas et al, 1997
[b]gp130	[a]Pro-TNF-α	yes	Althoff et al, 2001
[b]gp130	[a]Pro-TNF-α	yes	Althoff et al, 2001
[b]gp130	[a]IL-6R	yes	Althoff et al, 2001
[b]gp130	[b]LIFR	yes	Althoff et al, 2001
[b]CD4	[a]ACE	No	Sadhukhan et al, 1998
[b]MDP	[a]ACE	yes	Pang et al, 2001
[a]ACE	[b]CD4	yes	Sadhukhan et al, 1998

[a] Normally shed
[b] Not normally shed

1.8.3.3 Stalk or Ectodomain

To simplify the data of the shedding status of chimeric proteins, a few examples have been listed in Table 1.8.2. If the recognition motif in the ectodomain were the sole determinant for shedding, and protein structure was not altered in the chimeric protein, then the fusion of an ectodomain from a shed protein to a stalk from any protein should result in shedding of the chimeric protein. This is the case with the ACE/CD4-stalk chimera. Similarly, an ectodomain from an unshed protein fused to the stalk of any other protein should abrogate shedding from the membrane eg. CD4/ACE stalk chimera. These two examples indicate that a recognition motif exists, which directs the shedding of membrane proteins (Figure 1.8.5).

Table 1.8.2 Possible conclusions derived from chimeric protein shedding experiments

Ectodomain shedding status	Stalk shedding status	Chimera shedding status	[a]Example	Conclusion
Shed	unshed	shed	ACE/CD4 stalk	Recognition motif needed
unshed	shed	unshed	CD4/ACE stalk	
Unshed	shed	shed	gp130/IL-6R stalk	Accessible stalk needed
Shed	unshed	unshed	?	
Unshed	unshed	shed	gp130/LIFR stalk	?
Shed	shed	unshed	Pro-TNF-α/IL-6R	

[a]Chimeric proteins were either constructed by fusing an ectodomain from a shed or unshed protein with that of a stalk region from a protein that was either shed or remained membrane bound. Examples of such chimeric proteins are listed with their shedding status indicated.

If the solubilisation of membrane proteins depended on the presence of an accessible stalk, then the fusion of a stalk sequence from a shed protein should direct the proteolytic release of any ectodomain linked to it. The shedding of the gp130/IL-6R stalk chimera is a good example (Figure 1.8.6). Similarly, a stalk from a membrane protein not normally released from the membrane should enable the chimeric protein to resist shedding independent of the nature of the ectodomain. No examples of this scenario are available. On the contrary, the stalk region from the LDL-R (not susceptible to shedding), allowed the shedding of the ACE protein once fused to its ectodomain (Ehlers et al, 1996). These findings together suggest that the ectodomain

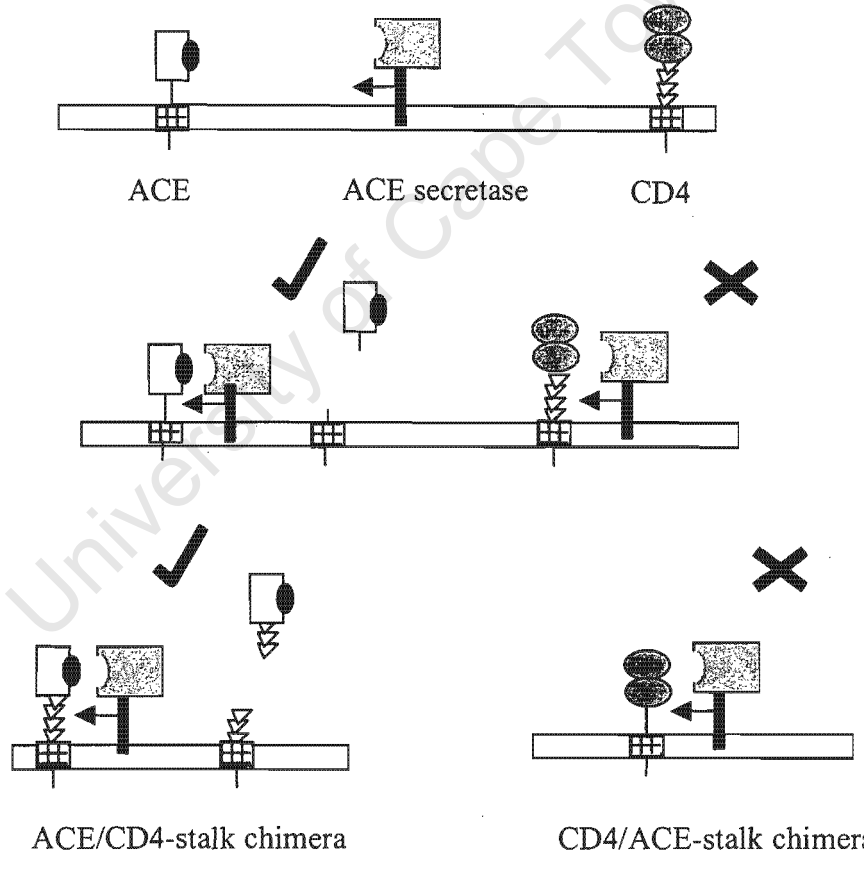


Figure 1.8.5 Ectodomain recognition motif is needed for shedding to occur ACE is shed by the ACE secretase (grey square), but CD4 is not. Once the ectodomain of ACE is fused to the stalk of CD4, ACE/CD4-stalk chimera is shed. However, the ectodomain of CD4 cannot direct the shedding of the CD4/ACE-stalk chimera. The recognition motif is shown as the black oval and the stalk of CD4 and ACE are indicated with linked triangles and a black line, respectively.

does indeed comprise a recognition motif for the membrane protein secretases, but that it also requires an accessible stalk region to direct the proteolytic release of membrane proteins. The stalk alone is unable to direct shedding and stalk "accessibility" has a broad definition embracing disulfide-bridged domains and glycosylated regions. However, this conclusion is complicated by the results shown in the last panel of Table 1.8.2. The fusion of an ectodomain and a stalk, both from unshed proteins, results in a chimera released from the membrane eg gp130/LIFR chimera (Figure 1.8.7). Similarly, the fusion of an ectodomain and a stalk, both from shed proteins, results in a chimera not released from the membrane eg Pro-TNF-α/IL-6R (Figure 1.8.8). These data suggests that gp130 comprises an ectodomain recognition motif, but that in its native form, the "accessibility" of the gp130 stalk region or recognition motif is not favourable for proteolysis. The resistance to shedding of the Pro-TNF-α/IL-6R-stalk chimera suggests that there are specific recognition motifs for different secretases. Thus, TACE, which is able to recognise and cleave Pro-TNF-α in its native conformation, is unable to cleave within a stalk derived from IL-6R. However, TACE is able to cleave IL-6R in its native conformation (Arribas et al, 1996). As mentioned before, the construction of chimeric proteins could result in altered conformations due to non-native interactions between exogenous domains (Figure 1.8.8). These alterations could be highly specific depending on the nature of the introduced sequence. Thus, combinations of different sequences (i.e. ectodomain and stalk) could result in chimeras with differing shedding status due to changes in conformation.

The use of chimeric proteins to identify the structural determinants of shedding has its limitations. The incorporation of an alien sequence into a folded protein could disrupt the native conformation of the protein. This disruption could lead to incorrect processing of the chimeric protein and thus makes its translocation to the PM to co-localise with the secretase unlikely. Conformational disruption could also lead to more subtle alterations. These could involve changes in the accessibility of the stalk sequence or the recognition domain. Binding of monoclonal antibodies (mAb) to somatic ACE changed the efficiency with which it was shed from the membrane in an epitope-dependent fashion (Balyasnikova et al, 2002). It was proposed that a mAb binding to the N domain of ACE recruited alternative proteases such as furin and

calpain (Balyasnikova et al, 2002). The binding of the mAb could change the conformation of ACE so that it is shed differently from the membrane. A N domain-specific mAb inhibited shedding by binding to the N domain (Balyasnikova et al, 2002). Thus, the maintenance of the overall conformation of the protein is vital for normal shedding to occur.

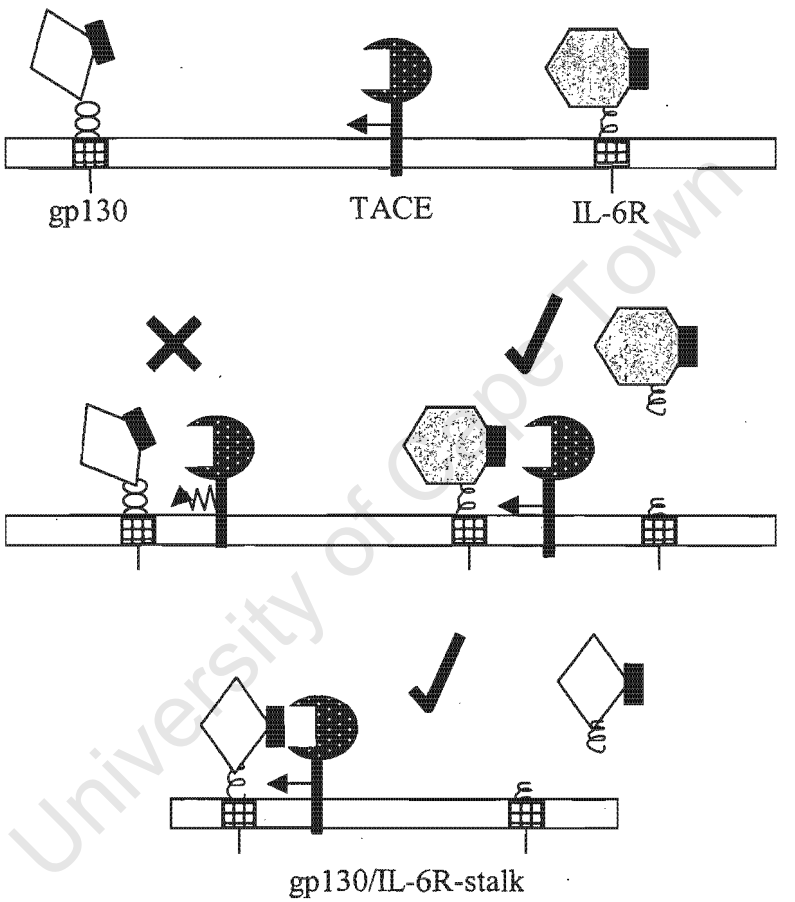


Figure 1.8.6 Accessible stalk required for shedding gp130 is not shed by TACE while IL-6R is. The chimeric protein, gp130/IL-6R-stalk, is shed due to the introduction of an accessible stalk and gp130 comprising a recognition motif in the ectodomain. The stalks of gp130 and IL-6R are shown by the linked circles and spiral, respectively while the recognition motif is shown as the black rectangle.

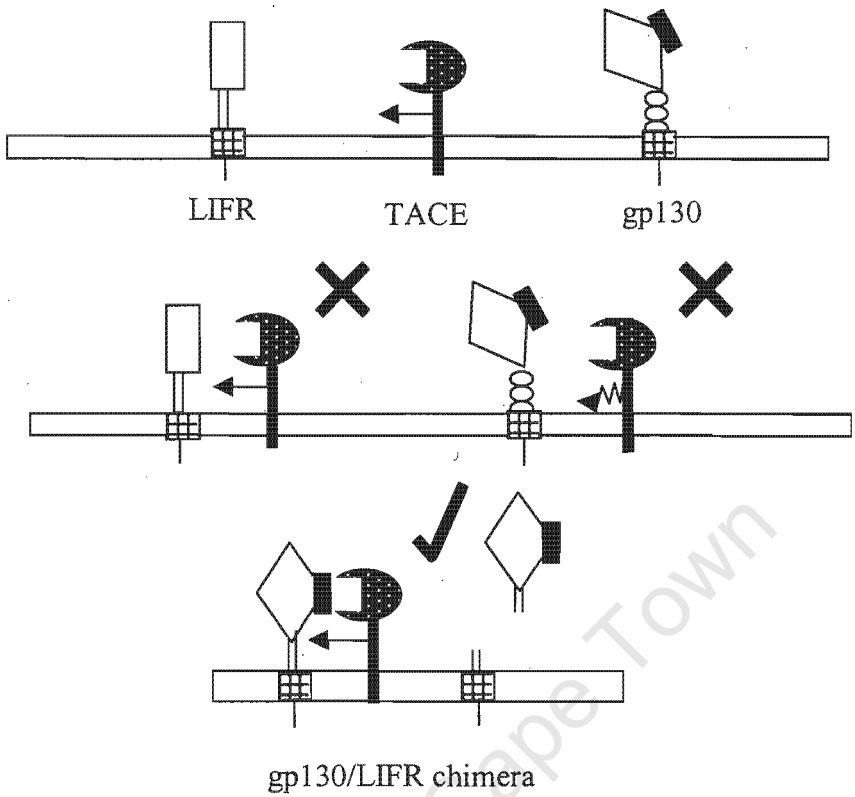


Figure 1.8.7 Favourable combinations of "accessible" stalk and ectodomain recognition motif contributes to shedding of chimeric proteins LIFR and gp130 are not shed as LIFR lacks a recognition motif (black rectangle) and gp130 has an inaccessible (or inaccessible recognition motif) stalk (linked circles). The combination of the accessible stalk of LIFR (double line) and the recognition motif of the ectodomain of gp130, results in the shedding of the chimeric protein (gp130/LIFR-stalk).

The limitations of chimeric proteins as a means of identifying the structural determinants for shedding also extend to their use for the identification of different secretases. The stalk of β-APP was able to direct the shedding of the unshed protein, β-Glycan (Arribas et al, 1997). In contrast, the ACE stalk was unable to render unshed CD4 susceptible to proteolysis. This difference in ACE and β-APP stalk-inducible shedding was used to infer that the α-secretase and the ACE secretase were distinct proteases, as they had different structural requirements. However, the ACE stalk was shown to allow for the release of MDP from the membrane (Pang et al,

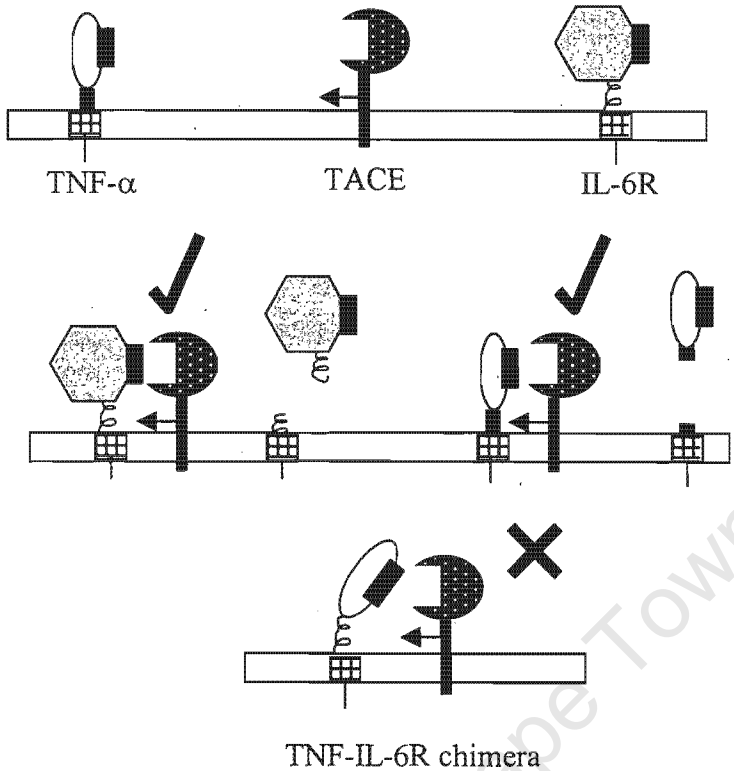


Figure 1.8.8 Conformational changes of chimeric proteins can inhibit shedding TNF-α and IL-6R are shed by TACE presumably because they both have a recognition motif in the ectodomain (black rectangle) and accessible stalks (bold line and spiral, respectively). The introduction of IL-6R stalk alters the conformation of the chimeric protein (TNF-α/IL-6R-stalk) so that either the recognition domain or stalk is rendered inaccessible to TACE.

2000). Thus, as the ACE stalk was able to convert MDP from an unshed protein to one released from the membrane like β-APP and the similar inhibition profile of the ACE secretase and the α-secretase indicates that these two metalloproteases are not distinct but the same secretase which hydrolyses two different substrates: ACE and β-APP (Pang et al, 2001; Parkin et al, 2002).

Stimulation of $TACE^{-/-}$ fibroblast cells with phorbol esters did not result in increased shedding of β-APP, indicating that TACE was involved in the regulated cleavage of APP but not constitutive shedding (Buxbaum et al, 1998). TACE was shown to be involved in the regulated shedding of APP in the Trans-Golgi network of CHO cells

(Skovronsky et al, 2000). However, specific hydroxamate inhibitors indicated that TACE was not involved in the regulated shedding of β-APP from neuronal cells (Parkin et al, 2002). The constitutive and regulated shedding of β-APP is primarily localised at the cell surface or Golgi, respectively (Hooper & Turner, 2002). However, PKC inducible shedding of β-APP did not increase APP trafficking and occurred both intracellularly and at the cell surface of CHO cells (Jolly-Tornetta and Wolf, 2000). This distinction was not investigated for the regulated shedding of ACE but no cell-associated cleavage products were identified in the ER or Golgi in the absence of phorbol (Beldent et al, 1995). Thus, constitutive shedding of ACE occurs at the cell surface. The basal and regulated shedding of ACE was not affected in $TACE^{-/-}$ cells. However, TACE was able to cleave a synthetic peptide of ACE and increased the basal shedding of ACE in co-transfection studies similar to that of APP (Sadhukhan et al, 1999; Slack et al 2001).

Thus, in $TACE^{-/-}$ fibroblasts, APP and ACE had different shedding patterns. However, residues spanning the cleavage sites were both cleaved by TACE and co-transfection with TACE cDNA resulted in increased constitutive shedding of both ACE and β-APP (Sadhukhan et al, 1999; Slack et al 2001). In neuronal cells, TACE did not affect the regulated shedding of APP and the inhibition profile of ACE and APP shedding was identical (Parkin et al, 2002). This suggests that TACE is involved in the shedding of APP under constitutive and inducible conditions dependent on cell type. The identical inhibition profiles of ACE and APP shedding, but different shedding patterns from $TACE^{-/-}$ cells, suggests that if these two proteins are shed by the same secretase, other factors come into play such as co-localisation with the secretase and protein processing pathways.

In conclusion, the shedding of membrane proteins is a highly complex process involving a number of considerations: cell type, translocation to the cell surface, processing pathways and association with specific proteins, intracellular cleavage, signalling cascades, inhibition profiles, activation mechanisms and structural determinants.

Chapter 2

Materials and Methods

Methods that are referred to in the various chapters are not repeated in Chapter 2 however, the more general methods are. Recipes for buffers used appear in Appendix II. Boehringer Mannheim supplied all restriction enzymes and Sigma Aldrich Company Inc. supplied all other reagents, unless otherwise indicated.

2.1 Molecular Biology

2.1.1 Polymerase chain reaction (PCR)

A cocktail solution was prepared containing 1 µl of a 10 mM dNTP stock, 5 µl of the 10x buffer, and 39 µl H_2O for each PCR reaction and 45 µl were aliquotted into 200 µl tubes on ice. The DNA template (1µl) and 2 µl of each primer stock (20 µM) was then added, and immediately prior to commencement of the amplification procedure 1-2 µl *pfu* DNA polymerase was added. The final reaction thus includes: 0.5 µg to 1 µg DNA template, individual primer concentration of 800 nM, 200 µM dNTP (dATP, dGTP, dCTP and dTTP), 1x *pfu* buffer, 5 U *pfu* DNA polymerase (Promega) in a final volume of 50 µl. PCR DNA amplifications were performed using a Hybaid DNASprint PCR apparatus. The PCR machine was pre-heated to ensure immediate start of the reaction. The parameters used were: 4 min denaturation step at 94°C, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at between 50°C and 70°C for 2 min, and elongation at 72°C for 3min. A final elongation step at 72°C for 5 min was performed to ensure that all DNA was double stranded, before holding at 4°C O/N. DNA loading buffer was added (6 µl), and the full 56 µl mixture was analysed on 1 % agarose gels in an electric field of 70V for 1 hr, and photographed.

2.1.2 Restriction enzyme digestion of DNA and electrophoresis

Phenol-based small scale or Qiagen medium scale DNA isolations were restriction enzyme (RE) digested in a total reaction volume of 20 µl containing between 5 and 10 units of individual RE, and approximately 1 µg DNA. The appropriate enzyme buffer was added depending on the RE used (A, B, L, M or H) at 1x concentration. The reaction mixtures were incubated at 37°C for at least 1 hour (except for digestions involving *Bcl* I), after which the reaction was terminated with the addition of 0.1 volumes of 10x sucrose loading dye. When digesting with *Bcl* I, the reaction was incubated at 37°C for 1 hour, and then at 55°C for a further hour, before the reaction was terminated. The digested DNA was separated using electrophoresis and the percentage agarose gel used depended on the size of the fragments being analysed; 0.75% and 1.5% agarose gels were used to separate large (>1000 bp) and small (<1000 bp) DNA molecules respectively. The gel was immersed in TBE buffer containing ethidium bromide (0.5 µg/ml) and electrophoresed at a constant voltage of 70 V. The DNA was then visualised using an ultraviolet light box and a Polaroid picture was taken. The relevant bands were then excised using sterile scalpel blades.

2.1.3 DNA extraction from *Escherichia coli*

2.1.3.1 Phenol-based quick small-scale plasmid DNA extraction

This method was used when isolating DNA from ampicillin resistant colony forming units (cfu) obtained after the transformation of DNA-competent *E. coli* with ligation reactions to verify the success of transformation. Usually, numerous cfus were obtained and this method was chosen, as its execution was rapid and efficient. After transformation of *E. coli* with appropriate vector/insert ligation reactions the cfus were picked from LB plates and inoculated into 5 ml LB supplemented with 100 µg/ml ampicillin and shaken O/N at 37°C. An aliquot was removed (1.5 ml) and centrifuged for 2 min at 11,000 x g before the cell pellets were resuspended in 50 µl 1x TE, pH 8. To this cell suspension, 100 µl of TE-buffered phenol and 100 µl of a mixture of isoamyl alcohol/ chloroform (24:1) was added. The cells were shaken for 3 min at ambient temperature before centrifuged at 11,000 x g for 3 min. The top, aqueous phase was removed and the plasmid DNA was precipitated by the addition of $1/10^{th}$ the volume of ammonium acetate (7.5 M, pH 4.5) and 2.5 volumes ethanol. The DNA was allowed to precipitate at –70°C for 30 min before the DNA precipitate was

sedimented by centrifugation at 11,000 x g for 30 min. The plasmid DNA pellet was washed with 70% ethanol and dried before resuspending in 20 µl water to which RNase (0.5 mg/ml) was added.

2.1.3.2 Medium scale preparation of plasmid DNA

E. coli, expressing the required bacterial expression vector, were shaken O/N in 50 ml LB supplemented with 100 µg/ml ampicillin and plasmid DNA was extracted using the midi-kit (Qiagen), according to the manufacturers instructions. DNA was quantitated by making a 1:100 dilution and the DNA concentration was determined by recording the A_{260} and A_{280} in a Anthelie Advanced spectrophotometer (Secoman). An A_{260} of 1 indicates 50 µg/ml double stranded DNA. All sequencing was done on DNA prepared using the Midi-kit with a A_{260}/A_{280} ratio of greater than 1.8.

2.1.4 Cloning DNA fragments into plasmid vectors

2.1.4.1 DNA elution

Once the relevant RE digested DNA fragments were separated by electrophoresis, the required DNA bands were excised out of agarose gels using a sterile scalpel. The agarose gel slices were fragmented using a sterile scalpel blade before placing in a GenElute agarose spin column (Sigma) which had previously been equilibrated with 100 µl 1x TE, pH 8. The DNA was eluted from the agarose gel by centrifugation for 10 min at 11,000 x g in a Sorvall MC 12C desktop microcentrifuge and the fluid eluant was collected in a 1.5 ml tube. The DNA was precipitated by adding $1/10^{th}$ volume 3 M sodium acetate (pH 5.2) and 2.5 volumes ice-cold ethanol, mixed and incubated at -70°C for 30 min. The precipitated DNA was centrifuged for 30 minutes at 11,000 x g, the DNA pellet was washed with 500 µl 70 % ethanol, and air-dried. The pellet was resuspended in 10 µl H_2O. An aliquot of DNA (1 µl) was removed and the concentration of the DNA was estimated by gel electrophoresis.

2.1.4.2 DNA ligation

The ligation of vector and insert was based on a 1:10 molar ratio and comprised a total concentration of ~10 pmoles of DNA. The ligation reaction comprised of the insert and vector DNA to which 1 µl 10x ligase buffer, 1 µl T4 DNA ligase (Roche) was added in a total volume of 20 µl. Ligation was allowed to proceed O/N at ambient

temperature, after which 10 µl was transformed directly into DNA-competent *E. coli* cells.

2.1.5 Production of competent *E. coli*

The rubidium chloride (RbCl) method was used to generate competent *E. coli* cells. A single cfu of XL1 Blue *E. coli* (Stratagene) was picked from luria agar (LA) plates supplemented with tetracycline (12.5 µg/ml) and inoculated into 5 ml terrific-(Ψ) broth and shaken O/N at 37°C. The bacterial culture was added to 100 ml pre-warmed Ψ-broth and returned to the 37°C shaker. Cell growth was determined by reading the A_{550} every 30 min. Once the A_{550} equalled 0.35 the bacterial culture was rapidly chilled on ice, divided between two 35 ml SS34 tubes and centrifuged in a pre-chilled Beckman J2-21 centrifuge at 4,000 x g for 5 min at 4°C. Each cell pellet was gently resuspended in 10.5 ml ice-cold TFB1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol), pooled and incubated on ice for 90 min. Cells were again centrifuged at 4,000 x g for 5 min at 4°C, and resuspended in 3.5 ml ice-cold TBF2 (10 mM MOPS pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol). Aliquots (100 µl) were distributed to individual 1.5 ml Eppendorf microfuge tubes, and flash-frozen in liquid nitrogen before stored at -70°C.

2.1.6 Transformation of competent *E. coli*

To transform DNA-competent bacterial cells with plasmid DNA, the cells were first thawed on ice, and then incubated on ice for a further 10 min. The plasmid DNA was then added and the cells incubated on ice for 20 min. Heat shock was performed by placing the tubes in a 42°C water bath for exactly 60 sec, followed by 2 min incubation on ice. To allow the cells to recover, Ψ-broth (400 µl) was then added and incubated at 37°C for 60 min, after which they were plated onto LA plates containing 100 µg/ml ampicillin.

2.1.7 DNA sequencing

All PCR products used for mutant construction were sequenced in both directions, using an ALFexpress DNA Automated Sequencer (APBiotech). Sequencing was done by the Specialist Sequencing Service, Department of Microbiology, University of Cape Town, using Thermo Sequenase fluorescent labelled primer cycle sequencing

kit with 7-deaza-dGTP (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions and cycle sequenced. The chain termination technique of Sanger *et al.*, 1977 was employed. Electrophoresis was done using 5% Long Ranger gel solution (FMC BioProducts), 5% gel and run according to the manufacturer's operating procedure for 13 hrs at 55° C, using a standard gel cassette, 0.5 mm spacers and controlled by ALFwin 2.1 software. Data was processed by ALFwin version 2.1 software (APBiotech).

2.2 Tissue culture

All tissue culture cells were grown in a humidified incubator at 37°C with 5% CO_2. CHO-K1 cells were maintained in complete medium [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with 20 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 20 mM *L*-glutamine and 10% foetal calf serum, supplemented with penicillin (100 µg/ml) and Streptomycin (100 µg/ml)]. For the maintenance of CHO-KI cells the FCS was heated to 56°C for 30 minutes before use.

For all experiments involving the isolation of ACE proteins or determination of ACE activity, CHO-KI cells were grown in the presence of complete medium [Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with 20 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 20 mM *L*-glutamine and 2% foetal calf serum, supplemented with penicillin (100 µg/ml) and Streptomycin (100 µg/ml) and $ZnCl_2$ (40 µM)]. In this case, the FCS was heat inactivated at 70°C for 15 min before use.

To preserve CHO-KI cell stocks, the cells were pelleted at 1,000 x g for 5 min before resuspending in heat inactivated FCS (56°C for 30 min) supplemented with 10% DMSO and frozen to -200°C in liquid nitrogen. To resuscitate frozen cells, the cells were thawed rapidly to 37°C and added to pre-warmed complete medium supplemented with 30% FCS and incubated O/N.

2.2.1 CaPO4 Transfection

All pLEN-ACE mutant vectors were stably transfected into CHO-K1 cells (adherent epithelial cells derived from a Chinese hamster [*Cricetulus griseus*] ovary; American Type Culture Collection (ATCC), CCL-61 CHO-K1; (Puck TT, 1958), using the ProFection Calcium Phosphate Mammalian Transfection System kit, according to the manufacturer's instructions (Promega). A 1:10 molar ratio of pSV2NEO (containing the neomycin-resistance cassette under the control of the SV40 early region promoter; ATCC; (South and Berg, 1982) to mammalian expression vector, pLENtACE, was used. This was achieved by mixing 10 µg pLENtACE and 0.6 µg of pSV2NEO with 62 µl 2 M $CaCl_2$ in a final volume of 500 µl water. This was slowly added to 500 µl 2× HBS (Hepes buffered saline) in a 10-ml tube, with gentle aeration. The DNA was allowed to precipitate for 30 min at ambient temperature. In the interim, 10 ml fresh complete medium had been added to CHO-KI cells at 30% confluence in 100 mm dishes and incubated at 37°C for 3 hrs. After the 3 hrs incubation, the DNA precipitate was added drop-wise to the CHO-K1 cells and incubated at 37°C for 4 hrs. The medium was removed and replaced with 3 ml of glycerol shock solution [15% glycerol in PBS] and incubated for exactly 2 minutes at ambient temperature. The cells were then washed twice with 10 ml 1x PBS (phosphate buffered saline) and grown O/N in fresh complete medium. Transfected cells were then selected using 0.8 mg/ml G418 (Geneticin, Sigma) in complete medium. Clones were selected using either cloning rings, limiting dilution or picking. (All mutant ACE constructs were transfected similarly.)

2.2.2 Cloning CHO-KI cells

2.2.2.1 Cloning rings

Cloning rings were prepared by cutting the top 1cm off blue 1000 µl pipette tips with a hot scalpel blade, and inverting onto a glass Petri dish smeared with vacuum grease. This was then wrapped in foil and autoclaved. The 100 mm dish containing neomycin resistant colonies were washed with PBS, and the cloning rings carefully placed around each colony. Cells were lifted by adding 100 µl 5 mM EDTA in PBS, incubated for 5 min at 37°C and transferred to 48-well dishes.

2.2.2.2 Limiting dilution

Neomycin resistant CHO-KI cells were lifted from 100 mm dishes using 5 mM EDTA in PBS and resuspended in 10 ml medium. 100 µl was added to 9.9 ml isoton solution, and the number of cells determined in a coulter counter. A series of dilutions were made so that ~ 1 cell/ 10 µl was generated. These diluted cells were transferred to 96 well dishes containing 50 µl complete medium. In this way, approximately one cell was seeded per well and incubated in a humidified 37°C, 5% CO_2 incubator. Each well was monitored for colony formation, and any with more than one, was discarded.

2.2.2.3 Picking of colonies

Once the neomycin resistant CHO-KI colonies had reached appropriate size, they were marked on the underside of the 100 mm dish. The cells were washed with 1x PBS and the colonies picked by immersing the tips of sterile swabs into 5 mM EDTA in 1x PBS and scraping them over each individual colony. The scraped cells were then transferred to 48 well dished containing 100 µl complete medium and grown till confluent.

2.3 Protein analysis

2.3.1 Western Blot analysis

2.3.1.1 SDS PAGE electrophoresis

The protein samples (20 µl) were prepared by adding 5 µl 5x SDS loading buffer and boiled for 5 minutes prior to loading. Proteins were separated by electrophoresis through a 10% acrylamide, sodium dodecyl sulphate (SDS) gel comprising running buffer (0.375 M, Tris pH 8.8; 0.1% SDS), 0.1% ammonium persulfate (AMPS) and 7 µl TEMED in a total volume of 10 ml, with a 3% stacking gel comprising stacking gel buffer (0.125 M Tris, pH 6.8; 0.1% SDS), 0.3% AMPS and 20 µl TEMED in a total volume of 10 ml using the Bio-Rad Gel electrophoresis apparatus according to the manufacturers instructions (Bio-Rad).

2.3.1.2 Transfer of proteins to nitrocellulose membrane

The acrylamide gels were soaked in blotting buffer for 15 min prior to assembly in the blotting apparatus (Bio-Rad). Proteins were transferred onto nitrocellulose in blotting buffer (25 mM Tris ,pH8.2; 200 mM Glycine) at 100 V for 1 hour, with cooling.

2.3.1.3 Probing of nitrocellulose membrane with antibodies

Any non-specific sites on the nitrocellulose membranes were blocked using 5% skim milk blocking buffer, Tris-buffered saline (TBS) (0.05 M Tris, pH 7.4; 0.2 M NaCl; 0.1% Tween-20) for 1 hour. A 1:1000 dilution of the rabbit anti-ACE serum in blocking buffer was then used to probe ACE, for 1 hour while shaking, followed by washing 4x with blocking buffer. The rabbit antibody was then probed using 1:2000 dilution of goat anti-rabbit IgG antibody conjugated to horse radish peroxidase (HRP), for 1 hour. After extensive washing with blocking buffer, the HRP was detected using the ECL chemiluminescence kit (Amersham).

2.3.2 Protein concentration determination

The Bradford method was used to determine protein concentration (Bradford, 1976). Samples were diluted in 800 µl sterile water and added to 200 µl Bio-Rad protein assay solution (Bio-Rad) and the A_{595} determined. A standard curve using 0, 0.5, 1, 2, 4, 8, 10 and 20 µg/ml BSA was used to convert the A_{595} to µg protein/ml.

2.3.3 Triton X114 phase separation of ACE

Triton X-114 phase separation of membrane-associated and soluble proteins was performed to confirm the presence or absence of a hydrophobic anchor region (Bordier, 1981; Hooper et al, 1987a; Ehlers et al 1996). Samples were made up to 100 µl with Triton extraction buffer (10 mM Tris, pH 7.5; 150 mM NaCl). An equal volume (100µl) of 2% Triton X114 (in Triton extraction buffer) was added and mixed thoroughly. The detergent mixture was incubated on ice (5 min) to allow for micelle dispersion and then placed at 30°C for 3 minutes for micelle aggregation. The samples were immediately centrifuged at RT for 3 minutes in a benchtop microcentrifuge. The aqueous phase (~180 µl) was carefully removed, and placed in a new eppendorf. To equalise the volumes, the detergent droplet was resuspended in ~146 µl Triton extraction buffer, and placed on ice to allow the micelles to dissolve. HHL assays were performed on 20 µl of both the detergent and aqueous phase samples, and the percentage activity in the aqueous phase determined.

2.3.4 Affinity purification of ACE

2.3.4.1 Culture medium

Soluble ACE, released into the medium by CHO-KI cells stably expressing ACE constructs, was purified to homogeneity using affinity-chromatography (Pantoliano et al, 1994; Ehlers et al 1989; Hooper et al, 1987a). The sepharose-28Å-lisinopril affinity column used consisted of the ACE inhibitor, lisinopril, bound to epoxy-activated sepharose 6B via a 28Å-long linker (Pantaliano et al, 1984; Hooper and Turner, 1987). The linker consists of aminocaproic acid coupled to aminobenzoic acid (via an *N*-hydroxysuccinimide linkage), which is in turn bound to the epoxy activated sepharose 6B (Cuatrecasas and Parikh, 1972, Pantoliana et al, 1984).

A 10 ml column was packed with Sepharose-28Å-Lisinopril. The column was pre-equilibrated with wash buffer (20 mM Hepes, pH 7.5; 0.5 M NaCl), and the medium sample (~400 ml cell-free medium) loaded over 12 hours using a Minipuls3 peristaltic pump (Gilson). The column was washed extensively O/N using 1 L of wash buffer, and the protein eluted off using 50 mM borate, pH 9.5. Fractions were collected and analysed for ACE activity using HHL as substrate. ACE-containing fractions were pooled and dialysed against H_2O at 4°C O/N using SnakeSkin dialysis tubing (Pierce, 10 000 MWCO). The dialysed ACE was then frozen to -20°C, freeze-dried O/N and resuspended in 200 µl H_2O. A small amount (~1 µg) was then analysed by SDS-PAGE.

2.3.4.2 Seminal plasma

Soluble ACE was purified from semen samples obtained from the Andrology Clinic, Groote Schuur Hospital. The semen was centrifuged at 2000 x g for 10 min to separate the seminal plasma from the spermatozoa. The seminal plasma was removed and dialysed against 20 mM Hepes (pH 7.5), 0.5 M NaCl and 0.1% Triton X-100 for 48 hrs before centrifuged at 14 000 x g for 10 min. the dialysed plasma was applied to a Sephadex G-50 column in the same buffer. The eluted fractions were tested for ACE activity using the substrate HHL. The fractions with ACE activity were applied to a 10 ml Sepharose-28Å-Lisinopril column and ACE was purified as described above.

2.3.5 ACE shedding kinetics

Stable CHO-KI cell lines, co-transfected with pLENACE or mutant ACE constructs and pSV2NEO, were grown to confluence in 6-well dishes (35 mm) for shedding-kinetics studies. ACE expression was induced O/N with complete medium (DMEM/Ham's F-12 supplemented with 20 mM Hepes, pH 7.5, 20 mM *L*-glutamine, 2% FCS, heated to 70°C for 15 minutes before use) supplemented with 40 μM $ZnCl_2$, the medium was removed at zero time and replaced with 500 μl of fresh complete medium or complete medium supplemented with 1 μM phorbol 12,13-dibutyrate (PDBu). At the indicated times (usually 0-, 0.5-, 1-, 2-, 4- and 8-hour time points) the medium (500 μl) was removed and the cells lysed in 500 μl Triton-lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.5, 0.5 M NaCl and 1 mM PMSF). ACE activity was determined using the ACE substrate HHL.

2.3.6 ACE shedding inhibition

The concentration of TAPI (100 μM) used to characterise the shedding of the ACE mutants from transfected CHO cells was well in excess of the IC_{50} (0.5 ± 0.3 μM). CHO-K1 cell lines stably expressing pLENtACE or mutant ACE constructs were grown to confluence in 6-well plates (35 mm) for shedding-kinetics studies. ACE expression was induced O/N with complete medium (DMEM/Ham's F-12 supplemented with 20 mM Hepes, pH 7.5, 20 mM *L*-glutamine, 2% FCS (heated to 70°C for 15 minutes before use) containing 40 μM $ZnCl_2$ before the medium was replaced with 500 μl of fresh complete medium or complete medium supplemented with 100 μM TNFα protease inhibitor (TAPI; (Mohler et al, 1994), 200 μM 3,4-dichloroisocoumarin (DCI), 3 mM bacitracin or 1 mM PMSF and 2μg/ml aprotinin. The cells were incubated at 37°C for 4 hrs before the medium (500 μl) was removed and the cells lysed in 500 μl Triton-lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.5, 0.5 M NaCl and 1 mM PMSF). ACE activity was determined using the ACE substrate HHL.

2.3.7 Metabolic labelling

Metabolic labelling and pulse-chase analysis of the biosynthesis and release of somatic ACE were performed as described previously (Schwager et al, 1999). Stably transfected CHO-K1 cells were grown to confluence in 6-well dishes (35 mm) in the presence of 10% FCS complete medium. ACE expression was induced O/N with 2% FCS complete medium supplemented with 40 μM $ZnCl_2$, after which the cells were washed twice with PBS and 'starved' for 30 minutes in 500 μl starvation medium [2% dialysed FCS in minimal Eagles medium (MEM) supplemented with 4 mM L-glutamine]. The medium was removed and the cells 'pulsed' with 500 μl [^{35}S] labelling medium [starvation medium supplemented with 100 μCi [^{35}S]-methionine and [^{35}S]-cysteine per ml] for 30 minutes at 37°C with 5% CO_2. The cells were washed with PBS and 1 ml fresh 2% FCS complete medium supplemented with 40 μM $ZnCl_2$, either in the presence or absence of 1 μM PDBu, was added at zero time. The metabolically labelled proteins were then 'chased' for up to 24 hours. Cell-lysate and medium samples were collected at the indicated times, centrifuged for 10 minutes in a benchtop micro-centrifuge, and 700 μl of the 'hot' supernatant was carefully collected. Fifty μl of each sample was used for ACE activity assay (using the substrate HHL). ACE in the remaining sample was affinity-precipitated by adding 50 μl of lisinopril-sepharose slurry [~50% sepharose-[28Å linker]-lisinopril affinity resin and 50% column wash buffer (20 mM Hepes, pH 7.5; 0.5 M NaCl)] and ACE binding was facilitated by continuous vortexing for 30 minutes. The beads were centrifuged, washed three times with column wash buffer and resuspended in 50 μl 2× SDS reducing buffer. The beads were then loaded onto 10% SDS-PAGE gels and the protein electrophoresed O/N. The gel was vacuum-dried onto filter paper and autoradiographed.

2.3.8 FITC labelling of CHO cells and confocal microscopy

Transfected CHO-K1 cells were seeded on flame sterilised glass coverslips in 12-well plates, cultured to 40% confluence in complete medium and ACE expression induced O/N in 1 ml 2% FCS complete medium containing 40 μM $ZnCl_2$. The cells were transferred to ice and all subsequent manipulations were done at 4°C. The cells were washed with PBS^{++} [phosphate-buffered saline (PBS) supplemented with 1 mM $CaCl_2$

and 1 mM $MgCl_2$] and then incubated with blocking buffer (3% bovine serum albumin (BSA) in $PBS^{+)}$] for 30 minutes and washed in PBS^{++}.

Cell-surface labelling:

The cells were probed with a 1:300 dilution of the rabbit polyclonal antibody (anti-recombinant human testis ACE) for 30 min, washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:500) in a humidified chamber in the dark for 30 min. After washing the cells were permeabilised with ice-cold methanol for 5 min on ice and washed with PBS^{++}. For nuclear staining the coverslips were inverted on a 50 µl drop of 2 µM propidium iodide (PI) for 10 minutes in the dark, followed by extensive washing with PBS^{++}, and a brief wash in H_2O to remove any salts. The cells were then fixed for 5 minutes using ice-cold 3% paraformaldehyde (PFA) in PBS^{++} and washed in PBS^{++}.

Intracellular labelling:

Alternatively, intracellular proteins were examined by permeabilising the cell membrane with ice-cold methanol for 5 min on ice and washed with PBS^{++} before probing with the primary and secondary antibodies as outlined above. Staining with PI was followed as before and then the cells were fixed for 5 minutes using ice-cold 3% PFA in PBS^{++} and washed in PBS^{++}.

Finally, the coverslips were mounted onto glass microscope slides by inversion on a 10 µl drop of MOWIOL mounting solution and viewed under a scanning confocal microscope (Leica DMIRBE inverted confocal microscope, Leica Microsystems Heidelberg). An FITC control slide was included with each experiment to ensure that no non-specific binding of the secondary antibody occurred (i.e. the primary antibody was excluded). The baseline laser intensity levels were set such that the CHO cells showed no signal and these settings were applied rigorously to each subsequent analysis, to ensure that the observed signal was specific to the transfected cells. Digital pictures were modified in Adobe Photoshop.

2.3.9 Cleavage site determination

The release of ACE from CHO-KI cells results in the proteolytic removal of the transmembrane domain (Ehlers et al, 1991b; Hooper et al, 1987a). To determine at which amino acid residue cleavage is occurring, a mass-spectrometry method of cleavage site determination was employed (Ehlers et al, 1996; Schwager et al, 1998). Briefly, the soluble (released) ACE proteins were purified from the medium as outlined above and the disulfide linkages were reduced, the cysteines protected with vinylpyridine and the protein hydrolysed into peptides. The peptides were analysed using MALDI-TOF mass spectrometry and the observed *m/z* correlated with the calculated masses of all potential C-terminal peptides. The C-terminal peptide was confirmed by fractionation using HPLC, N-terminal sequencing via automated Edman degradation and MALDI-TOF analysis.

2.3.9.1 Reduction and protection of disulphides

Purified soluble ACE was reduced by adding 6 M GnCl buffer [6M GnCl, 0.1 M Tris, pH 8.5], β-mercaptoethanol and incubating the mixture at 40°C for 3 hours with gentle agitation. The free thiol groups were then protected from re-forming disulphides by adding vinylpyridine (100%) for 30 minutes at RT in the dark. One drop of formic acid (100%) was used to acidify the sample prior to removal of any unreacted vinylpyridine using reverse phase high performance liquid chromatography (RP-HPLC). The sample was loaded onto a 'desalting' Aquapore RP-300 7 μm C8 column (30 × 2.1 mm; Perkin-Elmer) and developed with a linear gradient of 0-80% acetonitrile in 0.1% TFA over 20 minutes. The samples were freeze-dried.

2.3.9.2 Lys-C digestion and HPLC fractionation

The vinylpyridine-protected protein (~250 μg) was then resuspended in 100 μl Lys-C digestion buffer [25 mM Tris, 1 mM EDTA, pH 8.5], to which 5 μl (5 μg) of endoproteinase Lys-C (Boehringer Mannheim) was added and the protein digested O/N at 37°C. Alternatively, the purified protein was digested with cyanogen bromide (CNBr) using standard procedures (Gross, 1967). The peptide mixture was then analysed using MALDI-TOF mass spectroscopy and the mass of the C-terminal peptide determined. To confirm the identity of the *m/z* peak relating to the C-terminal peptide, the peptides were fractionated using HPLC and sequenced using Edman

degradation. The sample was acidified with formic acid and loaded onto a Jupiter 5 μm C_{18} HPLC column (150 × 1.0mm; Phenomenex). The peptides were separated using a gradient of 0-60% acetonitrile in 0.08% TFA over 120 min. Fractions were collected for each peak and the C-terminal peptide fraction found by MALDI-TOF analysis and sequenced using automated Edman degradation in a gas-phase sequenator 473A (Applied Biosystems, Weiterstadt, Germany).

2.3.9.3 MALDI-TOF mass spectroscopy

Matrix-assisted laser-desorption-ionisation time-of-flight (MALDI-TOF) mass spectroscopy (MS) was preformed on either the isolated C-terminal peptide or the mixed peptides directly following Lys-C digestion or CNBr hydolysis of purified ACE. The exact mass measurement (*m/z*) can then be correlated with hypothetical masses for an ACE C-terminal peptide that was cleaved at any peptide bond in the stalk region as well as the known Lys-C peptides. MALDI-TOF analysis was done using either a PerSeptive Voyager Elite Biospectroscopy Workstation (PerSeptive Biosystems, Framingham, MA) or a Bruker Reflex III time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany). The matrixes 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were used.

2.4 ACE activity assays

The methods used to determine enzymatic activity of ACE and the ACE constructs depended on objective of the experiment. All experiments aiming to determine ACE activity in the medium or cell lysates of CHO-KI cells made use of the method outlined in 2.2.4.1A. However, when determining the enzymatic activity of the purified N-domain-testis ACE chimeric proteins the method outlined in 2.2.4.1B and 2.2.4.1 was used. The aim of the latter experiment was to determine the ratio of hydrolysis of the two chimeric proteins, SomNdomBglII and SomNdomNheI, using the substrates HHL and z-PHL and thus to compare values, the methods were altered in this case.

2.4.1A HHL substrate assay for ACE activity

The level of ACE activity was determined using the Hippuryl-His-Leu (HHL) substrate and the production of cleavage product histidinyl-leucine (HL) was spectrophotometrically measured. The cell lysates and soluble ACE-containing samples (5-50 µl) were added to 120 µl HHL assay solution [5 mM HHL in 50 mM Hepes, pH 7.5, 0.3 M NaCl] and incubated for 15 minutes at 37°C. The reaction was stopped with 750 µl 0.28 M NaOH. The HL dipeptide product was conjugated to *o*-phthaldialdehyde (20 mg/ml in methanol) by adding 50 µl of a fresh stock, for 10 minutes at RT. The conjugation reaction was stopped by adding 100 µl 3 M HCl and the level of the fluorescent conjugate was determined using a Perkin-Elmer LS-5 Luminescence Spectrometer (λ_{ex} = 360 nm and λ_{em} = 485 nm). Fluorescence units were converted to nmol of HL produced using an HL standard curve (1.4, 2.8, 7, 14 and 20.9 nmol HL). 1 unit (U) of ACE activity is defined as the quantity of ACE that converts 1 mmol of HHL in 1 minute at 37°C in 50 mM Hepes, pH 7.5, 0.3 M NaCl.

2.4.1B HHL substrate assay for N-domain-testis ACE chimera activity

A stock solution of 12,5 mM HHL was prepared by dissolving 54 mg in 4 ml 0.025 M NaOH and made up to a final volume of 10 ml by adding water. This stock solution was stored at –20°C. To prepare the working stock of HHL (1 mM), 2 ml of stock buffer (0.5 M K_2HPO_4;/0.5 M KH_2PO_4, pH 8.3; 1.5 M NaCl) was added to 0.8 ml HHL stock silution (12.5 mM) and made up to 10 ml with water. Affinity-purified proteins were diluted to a concentration of 0.009 mg/ml and 120 µl of working stock of HHL (1 mM) was added to 0.9 µg of protein for 5 min at 37°C before 0.725 ml of NaOH (0.28 M) was added to stop the reaction. The HL was conjugated to *o*-phthaldialdehyde (20 mg/ml in methanol) by adding 50 µl of a fresh stock, for 10 minutes at RT. The conjugation reaction was stopped by adding 100 µl 3 M HCl and the level of the fluorescent conjugate was determined using a Varian Cary Eclipse Flourometric spectrophotometer (λ_{ex} = 360 nm and λ_{em} = 486 nm) with a slit width of 5/5 nm. Fluorescence units were converted to µmol of HL produced using an HL standard curve (0, 1.4, 2.8, 7, 14 and 20.9 nmol HL). ACE activity was defined as µmol of HL produced/min/mg of total ACE.

2.4.2 z-PHL substrate assay for testis ACE-N domain chimera activity

A 20 mM stock solution of z-phenylalanine-histidine-leucine (z-PHL) was made up by dissolving 220 mg of z-PHL in 2 ml 0.28 M NaOH and water was added to a final volume of 20 ml and aliquots (2 ml) were stored at –20°C. The working stock of z-PHL (1 mM) was generated by taking 4 ml 5x buffer stock solution (0.5 M K_2HPO_4;/0.5 M KH_2PO_4, pH 8.3; 1.5 M NaCl) and 15 ml H_2O to which 20 µl ZnSO4 (10 mM) was added before the addition of 1 ml of z-PHL stock solution (20 mM). The affinity-purified proteins were diluted to a concentration of 0.009mg/ml and 120 µl of z-PHL working stock (1 mM) was added to 0.9 µg of protein and the reaction mixture was incubated at 37°C for 5 min before the production of HL was stopped by the addition of 0.725 ml NaOH (0.28 M). The HL was conjugated to *o*-phthaldialdehyde (20 mg/ml in methanol) by adding 50 µl of a fresh stock, for 10 minutes at RT. The conjugation reaction was stopped by adding 100 µl 3 M HCl and the level of the fluorescent conjugate was determined using a Varian Cary Eclipse Flourometric spectrophotometer (λ_{ex} = 360 nm and λ_{em} = 486 nm) with a slit width of 5/5 nm. Fluorescence units were converted to µmol of HL produced using an HL standard curve (0, 1.4, 2.8, 7, 14 and 20.9 nmol HL). ACE activity was defined as µmol of HL produced/min/mg of total ACE.

2.5 Membrane isolation

CHO-KI cells expressing testis ACE were grown to confluence and ACE expression induced O/N with complete medium (50% Hams-F12/DMEM, 2% FCS; 40 µM $ZnCl_2$; 20 mM Hepes, pH 7.5; 1% penicillin/streptomycin). The cells were washed with PBS and then scraped into homogenizing buffer (Hepes, 20 mM; PMSF, 1 mM) and disrupted using a cell cracker. The cell debris was removed by centrifugation at 11,000 x g for 10 min before the post nuclear supernatant (PNS) was ultra-centrifuged at 40,000 x g for 15 min. The membrane pellet was resuspended in buffer (0.1 M borate, pH 8.3) using a Dounce homogenizer and used immediately.

2.6 Effect of inhibitors on ACE shedding from CHO-KI membranes

Membranes were isolated as outlined above and 15 µl aliquots were transferred to eppendorfs on ice. TAPI, DCI, bacitracin, PMSF and aprotinin were mixed with buffer (0.1 M borate, pH8.3) and 85 µl was added to the membrane aliquots so that

the final concentration of the inhibitors in the total volume of 100 µl equalled 100 µM, 200 µM, 3 mM, 1mM and 3 µg/ml, respectively. The membranes were gently resuspended and incubated at 37°C before the membranes were centrifuged at 11,000 x g. The supernatant was removed and the membrane pellet was resuspended in an equal volume of Triton X-100 lysis buffer. ACE activity was determined with HHL and the level of soluble ACE activity was depicted as the percentage ACE activity in the supernatant of the total ACE activity determined in both the supernatant and the membrane pellet.

Chapter 3

The Development of an ACE Secretase Assay

3.1 Introduction

In order to isolate and identify the protease responsible for the release of ACE from the membrane, a suitable and efficient assay has to be developed. The identification and characterization of novel secretases involved in the shedding of membrane proteins have used peptides spanning the cleavage site, whole cell systems, biological membrane preparations and artificial lipid structures, liposomes (Hooper et al, 1997). In order to characterize the ACE secretase, membrane fractions were isolated from pig kidney microvilli, as well as lung, placenta, and testis, and incubated at 37°C to determine the cleavage of ACE by the endogenous secretase(s) (Oppong et al, 1993). The appearance of a hydrophilic cleavage product was detected by phase separation. At 37°C the release of ACE from the pig kidney microvillar membranes was relatively rapid and washing the membranes with 0.5 M NaCl did not alter this activity, as one would expect, if this were an integral membrane protein. The microsomal membrane fractions of lung and testis exhibited similar secretase activity. Shedding was inhibited by EDTA and was magnesium dependent. However, ACE was not released from human kidney microvillar membranes and pig and human intestinal microvillar membranes, indicating the absence of the ACE secretase (Oppong et al, 1993).

As no endogenous secretase activity was present in pig intestinal microvillar membranes, this membrane fraction was used as a source of ACE substrate in further experiments (Parvathy et al, 1997). Triton X-100-solubilised, ACE secretase-containing membranes, purified from pig kidney cortex, was incubated with lisinopril-28nm-Sepharose to remove endogenous ACE before incubating with intestinal microvillar membranes at 37°C. This resulted in 47% release of ACE from intestinal membrane. The same ACE secretase fraction was incubated with [^{125}I]-labeled amphipathic form of ACE purified from pig kidney cortex and no significant radioactivity was detected in the aqueous phase after phase separation (Parvathy et al,

1997). The retention of ACE secretase activity in membranes after high salt washes and the ability of detergent solubilization of membranes to release secretase activity indicate that the ACE secretase is an integral membrane protein. The inability of the detergent-solubilised ACE secretase to cleave purified full-length ACE shows that the ACE secretase requires ACE to be inserted within a lipid bi-layer in order to carry out cleavage.

Rabbit testis ACE has been expressed in the mouse epithelial, TACE-deficient cell line, ACE89, and the normal expression, processing and shedding of ACE has been documented (Sadhukhan et al, 1999). Membrane prepared from this cell line has been used in the development of a cell free *in vitro* assay system for the characterization of the ACE secretase. It was shown that rabbit testis ACE is shed from these membrane preparations in a temperature and time dependent manner and is inhibited by a metalloprotease inhibitor, Compound 3. Once these membrane preparations were solubilised using 0.1% Triton X-100, shedding stopped and was only restored with the addition of lisinopril-sepaharose or concanavalin-A agarose. This provided additional evidence indicating that the ACE secretase required its substrate to be anchored to a solid support in order for cleavage to occur (Sadhukhan et al, 1999).

When human testis ACE cDNA was transfected into Chinese Hamster Ovary (CHO-KI) cells, the presence of an endogenous ACE secretase(s) resulted in the shedding of membrane-anchored ACE (Ehlers et al, 1991b). In order to identify and characterize the ACE secretase, membrane was prepared from this cell line and used in the development of an ACE secretase assay.

3.2 Methods

3.2.1 **Biological Membrane preparations**

3.2.1.1 **Total Membrane Isolation I**

CHO-KI cells expressing testis ACE were grown to confluence and ACE expression induced O/N with complete medium 2% FCS; 40 μM $ZnCl_2$; 20 mM Hepes, pH 7.5; 1% penicillin/streptomycin; 50% Hams-F12/DMEM). The cells were scraped into homogenizing buffer I (20 mM Hepes, pH 7.5, 0.2 M sucrose, 1 mM PMSF) and disrupted using a cell cracker. The post-nuclear supernatant (PNS) was obtained by centrifugation at 3,000 x g for 10 min before ultra-centrifuging at 40,000 x g for 40 min. The pellet was washed twice in homogenizing buffer and resuspended in buffer (0.1 M Borate, pH 8.3) to give membrane fraction 1 (MF1). Aliquots were stored at – 20°C.

3.2.1.2 **Total Membrane Isolation II**

CHO-KI cells expressing testis ACE were grown to confluence and ACE expression induced O/N with complete medium (2% FCS; 40 μM $ZnCl_2$; 20 mM Hepes, pH 7.5; 1% penicillin/streptomycin; 50% Hams-F12/DMEM). The cells were washed and then scraped into homogenizing buffer II (Hepes, 20 mM; PMSF, 1 mM) and disrupted using a cell cracker. The cell debris was removed by centrifugation at 3,000 x g for 10 min before the PNS was ultra-centrifuged at 40,000 x g for 15 min. The membrane pellet was resuspended in buffer (0.1 M Borate, pH 8.3) using a Dounce homogenizer and used immediately.

3.2.2 **Isolation of the ACE secretase**

3.2.2.1 **ACE Secretase Membrane Isolation I**

CHO-KI cell membranes, not expressing testis ACE, were prepared as in the method for Total Membrane Isolation II.

3.2.2.2 **ACE Secretase Membrane Isolation II**

CHO-KI cells not expressing testis ACE were prepared as in the method for Total Membrane Isolation II. However, the final membrane pellet was resuspended in buffer containing 20 mM Hepes, pH 7.5, 1 mM PMSF and 0.01% Triton X-100 and shaken for 1 hr at 4°C. The mixture was centrifuged for 30 min at 11,000 x g and the

supernatant was used as the source of detergent-solubilised ACE secretase. The extent of solubilisation was determined using the Bradford protein concentration assay on the membranes before, and on the supernatant after, solubilisation.

3.2.3 **ACE Secretase Characterization**

An aliquot (15 µl) of CHO-KI cell membrane containing testis ACE was made up to 100 µl with Borate buffer (0.1 M, pH 8.3) and incubated at 37°C, 4°C, or 15°C for 15 min. The reaction mixture was removed and placed at 4°C. Full-length and soluble ACE was separated using TritonX-114 phase separation or centrifugation of the membrane at 11,000 x g. After centrifugation the membrane pellet was resuspended in Triton X-100 lysis buffer (1% Triton X-100; 50 mM Hepes, pH7.5; 0.5 M NaCl and 1 mM PMSF) to equal the volume of the supernatant. After the volumes were adjusted, ACE activity was determined in each phase using Hip-His-Leu (HHL) as a substrate.

3.2.4 **ACE Secretase Assay**

To detect an increase in shedding in the presence of additional CHO-KI cell membrane, detergent-solubilised CHO-KI cell membrane (11.6 µg total protein) was added to a 15 µl aliquot of CHO-KI cell membrane containing testis ACE (21 µg total protein) and the volume of buffer was adjusted to a total volume of 100 µl. In experiments where the membranes (not containing ACE) were not detergent-solubilised, the membranes were mixed together using a Dounce homogenizer. This reaction mixture was incubated at various temperatures depending on the parameters being tested. After incubation, the soluble and membrane-bound ACE were separated from each other either by Triton X-114 phase separation or centrifugation at 11,000 x g for 5 min. HHL was used as a substrate to determine the ACE activity in the relevant fractions.

3.2.5 **Triton X-114 Phase Separation**

To separate the cleaved ACE from its membrane bound form, an equal volume of 2% Triton X-114 buffer (10 mM Tris, 150 mM NaCl, pH 7.4) was added and placed at 4°C for 5 min. The soluble detergent mixture was resolved into aqueous and detergent phases by incubation at 30°C for 3 min and centrifuged at 11,000 x g for 3 min. The aqueous phase was removed and the detergent droplet was made

up to an equal volume with buffer (10 mM Tris,150 mM NaCl, pH 7.4). Each phase was assayed for ACE activity using the substrate HHL.

3.3 Results and Discussion

3.3.1 **Testis ACE shedding from isolated CHO-KI cell membrane in the presence of inhibitors and trypsin**

To determine whether the incubation of isolated CHO-KI cell membranes containing recombinant testis ACE, would result in shedding of testis ACE by the endogenous CHO-KI cell ACE secretase, the membranes were incubated at 37°C and 4°C (Figure 3.3.1). Moreover, protease inhibitors were added to test whether a hydroxamic acid-based inhibitor, TAPI and serine protease inhibitors inhibited the endogenous ACE secretase activity in the *"in vitro"* membrane system.

Proteases are inactive at low temperatures however, surprisingly, 52% testis ACE activity was localized in the aqueous phase, suggesting it was being released from the membrane (Figure 3.3.1). It was previously reported that ~34% of ACE activity was associated with the aqueous phase when detergent-solubilised, full-length ACE was separated by Triton X-114 phase separation (Ramchandran & Sen, 1995). This is most likely due to the limitations of the detergent to completely sequester amphipathic proteins. The high aqueous ACE activity obtained at 4°C in Figure 3.3.1 thus implied that if 34% aqueous ACE activity was taken as zero shedding, then 18% of testis ACE was being shed under these experimental conditions. The shedding of testis ACE at 37°C resulted in an increase of soluble ACE by 25% and this increase was inhibited by TAPI but was unaffected by the non-specific inhibitors. These results indicated that the shedding of testis ACE at 37°C was due to the activity of the ACE secretase, as the production of ACE was inhibited by TAPI, a known ACE secretase inhibitor.

Membrane fractions were incubated for 3 hours in Figure 3.3.1. The 77% aqueous ACE activity observed at 37°C indicated that the membrane-bound substrate had been depleted during this length of incubation. Thus, if the shedding of testis ACE at 37°C by the endogenous ACE secretase was too rapid, then total membrane-bound ACE would be shed, and an increase in shedding in the presence of additional protease would be hard to detect. Thus, trypsin, which is known to solubilise ACE by cleaving within the stalk region (Ehlers et al, 1991b), was added to the membranes to

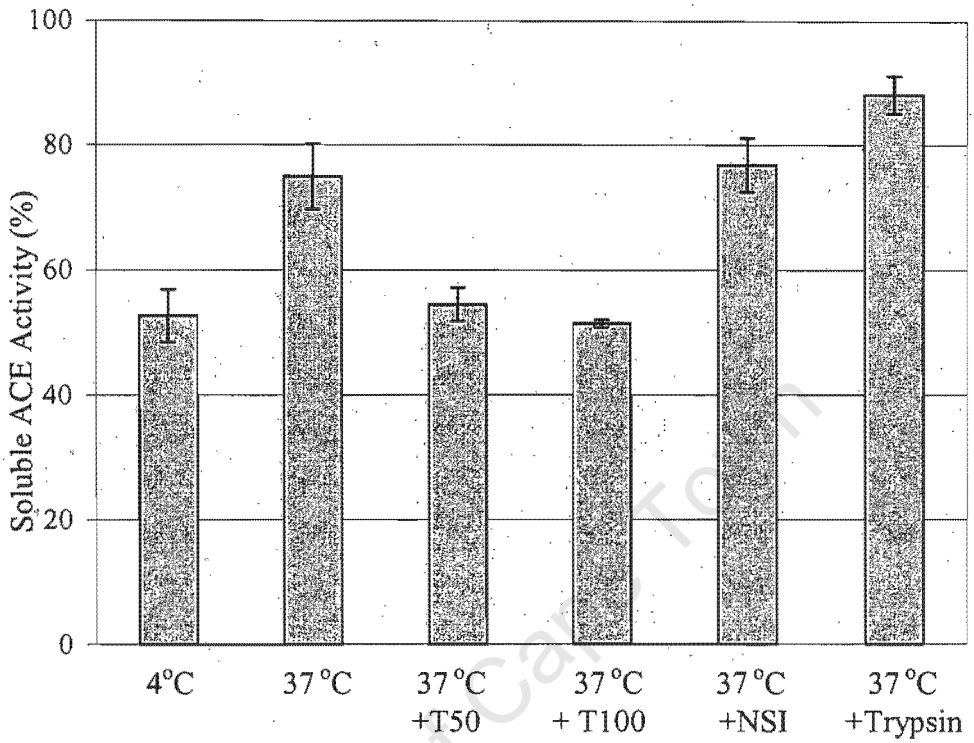


Figure 3.3.1 Release of testis ACE from isolated membrane is inhibited by TAPI Expression of testis ACE in CHO-KI cells is described in materials and methods and the membranes were isolated as outlined in Total Membrane Isolation I. Aliquots of membranes were incubated at 37°C and 4°C for 3 hrs, TAPI: (T50: 50 μM and T100: 100 μM), NSI: nonspecific inhibitors (aprotinin, 3 μg/ml/ PMSF, 1 mM) and trypsin (10 μg/ml). Each aliquot was separated into aqueous and detergent phases using phase separation and ACE activity was determined for each phase using the HHL ACE assay. The ACE activity in the aqueous phase was expressed as a percentage of total ACE activity found in both phases. (n=3 +/-SD)

investigate whether an increase in soluble ACE activity could be detected. The presence of trypsin resulted in additional soluble ACE activity (12%), but the increase was slight and this could be due to insufficient available substrate to detect the activity of trypsin (Figure 3.3.1).

3.3.2 **Rate of shedding of testis ACE from isolated CHO-KI cell membrane**

To determine when total membrane-bound ACE was depleted, CHO-KI cell membrane fractions, containing testis ACE, were incubated at 37°C and 4°C and the level of soluble ACE was determined at various time points. In Figure 3.3.2, ~32% of soluble ACE activity was obtained at 4°C and this remained constant during the 2 hrs of incubation. This indicated that shedding was not occurring at 4°C as previously indicated in Figure 3.3.1. The rate of shedding was very rapid at 37°C (Figure 3.3.2). Total membrane-bound ACE was converted to its soluble counterpart within the first 30 min of incubation at 37°C. Although only 60% aqueous ACE activity was reached after 30 min, the plateau of the graph suggests that this was the maximum soluble ACE that could be generated. It is possible that this reflects the presence of a population of membrane-bound ACE molecules that is not susceptible to cleavage by the ACE secretase (although it *is* cleaved by trypsin). Alternatively, incomplete separation of amphipathic (membrane-bound ACE) from hydrophilic (soluble ACE) proteins by Triton X-114 phase separation could result in lowered soluble ACE values.

3.3.3 **Determination of the sensitivity of Triton X-114 phase separation for the reproducible quantification of ACE shedding**

In Figure 3.3.1 maximum solubilisation of membrane-bound ACE was indicated as 77% at 37°C whereas in Figure 3.3.2, only 60% soluble ACE activity was obtained. This discrepancy was also observed with the values obtained at 4°C for the two experiments. This implied that the experimental procedure was not reproducible. To investigate this further, five independent experiments were performed with membrane prepared as outlined in Total Membrane Isolation Protocol I. As described before, CHO-KI cell membrane containing testis ACE was incubated at 37°C and at 4°C and the proteins were separated using Triton X-114 phase separation. The results in Figure 3.3.3 show that the level of aqueous ACE detected in the five independent

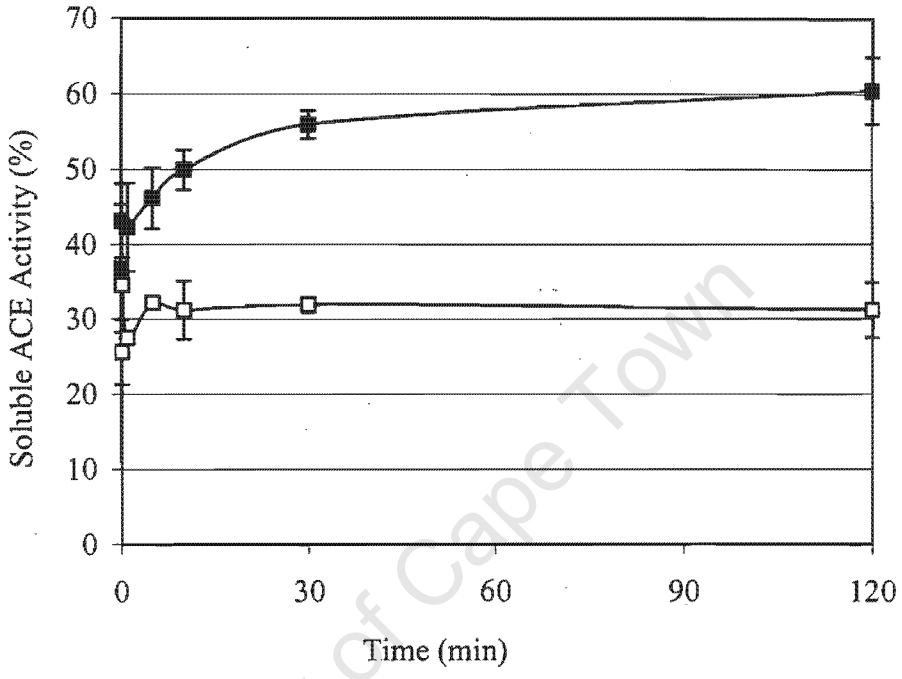


Figure 3.3.2 Kinetics of testis ACE release from isolated CHO-KI cell membrane Membranes were prepared as described before in Total Membrane Isolation I, incubated for 2 hrs at either 37°C (black squares) or 4°C (clear squares) and the reaction was stopped at time points, 0, 0.167, 1, 5, 10, 30 and 120 min. Phase separation was carried out on each sample and the ACE activity was determined for both phases using HHL as an ACE substrate. The extent of solubilisation was shown as the % ACE activity in the aqueous phase. (n=3 +/-SD)

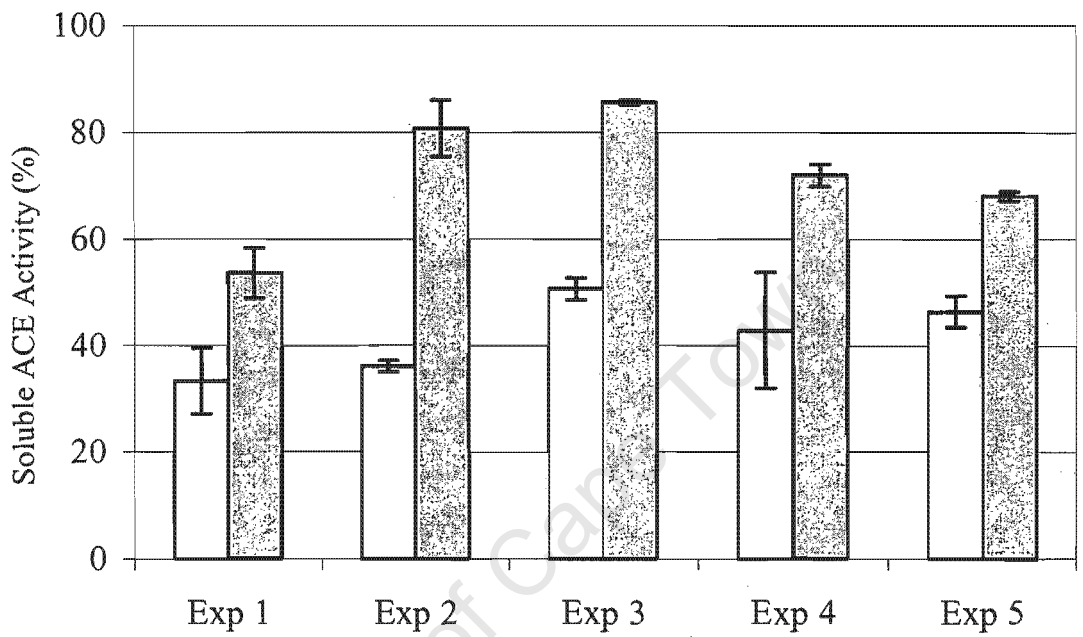


Figure 3.3.3 Aqueous ACE activity varied when CHO-KI cell membranes, expressing tACE, were incubated at 4°C and 37°C Membranes were prepared as outlined in Total Membrane Isolation I and incubated at 4°C (clear bar) and 37°C (grey bar) for 20 min and shedding was stopped by the commencement of Triton X-114 phase separation. ACE activity was determined using the HHL assay and the extent of shedding was indicated by the % aqueous ACE activity. (n=3 +/- SD)

experiments varied from one experiment to the next. A variation of ~15% at 4°C and a variation of ~35% at 37°C was observed. For each experiment at 37°C in Figure 3.3.3, shedding was observed over and above that seen at 4°C. This indicated that within each experiment the activity of the ACE secretase could be detected. However, the level of detectable secretase activity, varied between experiments. Thus, the limitation of Triton X-114 to completely separate amphipathic ACE from its soluble counterpart resulted in variation from one experiment to the next. This indicated that Triton X-114 phase separation, as a method for the quantification of soluble ACE activity, was not suitable for this assay system.

3.3.4 **Testis ACE shedding assay**

The development of an accurate ACE secretase assay, using CHO-KI cell membranes depends on an increase of aqueous ACE activity above that seen at 37°C upon addition of exogenous ACE secretase. This increase is affected by the level of soluble ACE activity detected at 4°C and at 37°C and the variation observed in the difference between these values. The results obtained thus far revealed three obstacles: (1) the high level of aqueous ACE at 4°C, (2) the variation obtained in the aqueous activity observed at 37°C and (3) the rapid rate of shedding of the endogenous ACE secretase. These problems and their possible solutions are discussed further.

3.3.4.1 **High aqueous ACE activity at 4°C**

Sadhukhan et al showed that 34% ACE activity was always present in the aqueous phase after phase separation of amphipathic ACE (Sadhukhan et al, 1999). In addition, the previous results demonstrated that the level of soluble ACE associated with CHO-KI cell membrane at 4°C by Triton X-114 phase separation did not remain constant from one experiment to the next. Thus, Triton X-114 phase separation not only generated high aqueous ACE activity, but it also affected the reproducibility of the assay. As an alternative to phase separation for the membrane-shedding assay, centrifugation was tried (Figure 3.3.4).

The separation of membranes from the supernatant by centrifugation decreased the amount of ACE activity detected in the aqueous phase at 4°C from 35% to 15% (Figure 3.3.4.). The comparison of the levels of ACE activity prior to incubation (CF

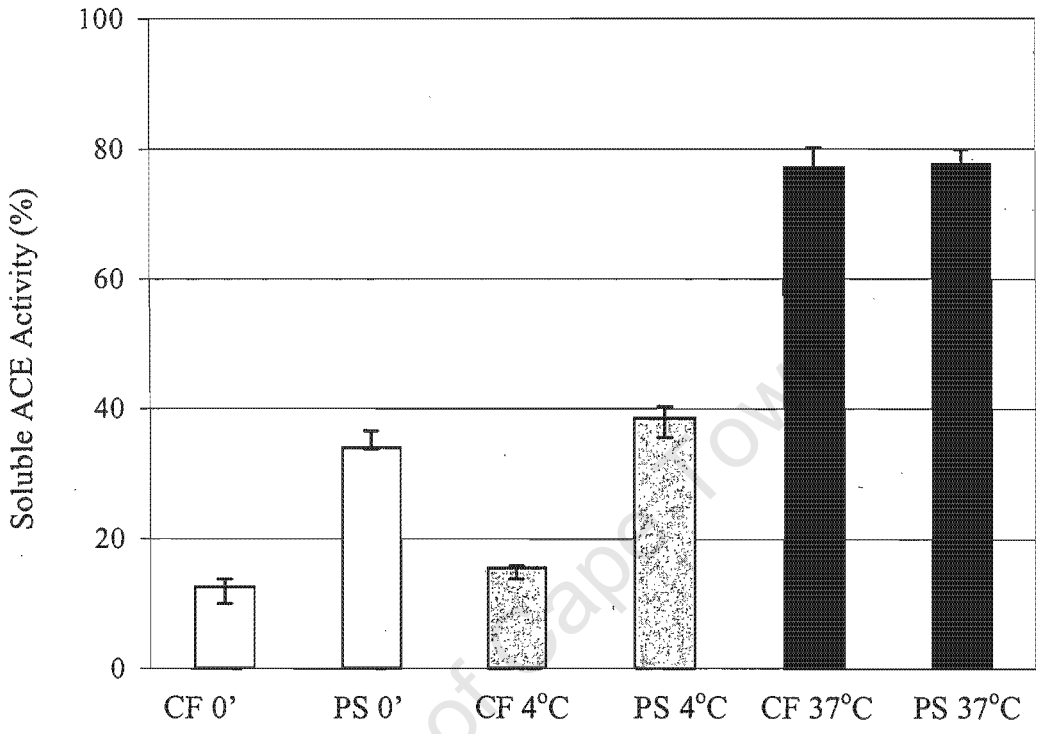


Figure 3.3.4 Comparison of Triton X-114 phase separation (PS) and centrifugation (CF) as methods for separating membrane-bound ACE from soluble ACE Membranes were prepared as outlined in Total Membrane Isolation Protocol II. After incubation at 4°C (grey bar) and 37°C (black bar) for 1hr, the membranes were divided equally and one part was subjected to Triton X-114 phase separation while the rest were centrifuged at 11,000 x g for 5 min at 4°C. The detergent droplets and the membrane pellets were made up to equal the volume of the aqueous phase and supernatant, respectively. The membrane pellet was resuspended in Triton X-100 lysis buffer to solubilise the membrane. ACE activity was determined using HHL as a substrate. Prior to incubation, at 0 min (clear bar), membranes were either centrifuged or separated using phase separation to control for the amount of aqueous ACE generated during the membrane purification protocol (CF 0' or PS 0').

0 and PS 0) to those obtained after incubation at 4°C (CF 4°C and PS 4°C) showed no significant difference, suggesting that negligible soluble ACE was generated during the 1 hr incubation at 4°C. Thus, by separating amphipathic ACE from its soluble form by centrifugation, the percentage of aqueous ACE activity obtained at 4°C was lowered. The level of aqueous ACE activity at 37°C was the same for both methods of separation, indicating that centrifugation successfully separated membrane-bound ACE from soluble ACE. Centrifugation was shown to be more beneficial than phase separation and was used in further experiments to optimise the ACE secretase assay.

To optimise the membrane preparation, further changes of the protocol were investigated. In the majority of experiments, the level of ACE activity detected at 4°C exceeded 34%. This indicated the possibility that shedding was occurring during the membrane isolation procedure. To eliminate this possibility, the membrane isolation method was shortened as outlined in Total Membrane Isolation Protocol II. The heterogeneity of membrane vesicles could also contribute to the variation in the aqueous ACE activity if shedding happened during the stages of purification. Membranes were sedimented at each stage of the Total Membrane Isolation Protocol I and soluble ACE, if present, was discarded with the supernatant. However, soluble ACE could be entrapped within multi-lamellar membrane structures during the purification procedure thus increasing the aqueous ACE activity associated with the isolated membrane. To form a homogenous population of smaller membrane vesicles, membranes were resuspended in buffer using a Dounce homogenizer.

3.3.4.2 **High variation in the level of aqueous ACE activity at 37°C**

In Figure 3.3.3, variation of aqueous ACE activity was observed between experiments. This variation was not only limited to the level of soluble ACE generated at 37°C, but also to the total ACE activity present in each experiment. To determine the correlation between these two variables, the soluble ACE activity determined for each experiment was plotted against the total amount of ACE present (Figure 3.3.5). The maximum soluble ACE activity determined after the incubation of CHO-KI cell membranes at 37°C for 20 min increased with the total amount of ACE present within that membrane sample (Figure 3.3.5). This indicated that the

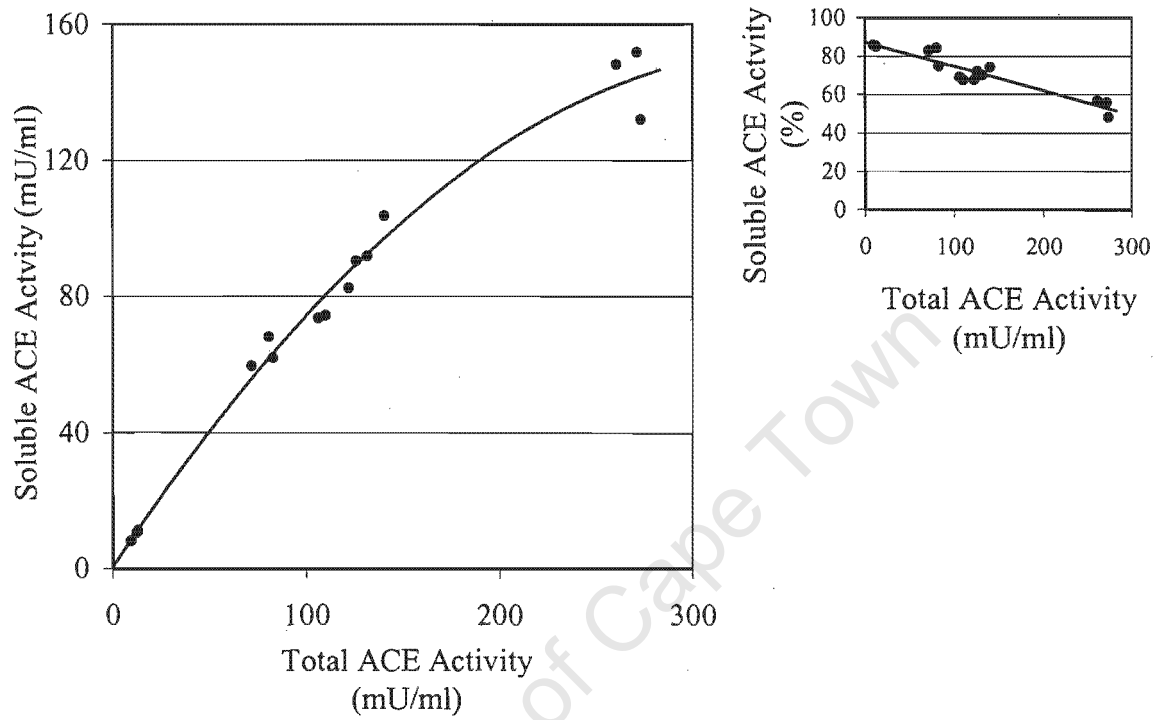


Figure 3.3.5 Correlation between the levels of aqueous ACE activity obtained after incubation at 37°C and the total ACE activity present for 5 separate experiments Membrane was prepared as outlined in Total Membrane Isolation protocol I and after incubation at 37°C, subjected to Triton X-114 phase separation. The total ACE activity is the sum of the ACE activity determined in the detergent phase and aqueous phase using HHL as a substrate. Inset graph indicates the soluble ACE activity as the percentage of the total ACE activity present.

more ACE secretase substrate available, the greater the amount of product. This suggested that the rate of shedding of ACE was proportional to the concentration of membrane-bound ACE present. In Figure 3.3.5, at higher concentrations of substrate the graph begins to plateau and at this point, zero order kinetics begins to apply when the substrate concentration no longer affects the rate of the enzymatic reaction.

The difficulty in creating a homogenous population of similar-sized membrane vesicles during the isolation of membrane from CHO-KI cells influences the amount of membrane (and therefore substrate) allocated to each experimental tube. To circumvent this problem, soluble ACE activity is depicted as a percentage of the total ACE present in each tested aliquot. In the inset graph of Figure 3.3.5, the data derived from the five experiments is shown with the soluble ACE activity depicted as the percentage of total ACE activity present. The greater the total amount of ACE present, the lower the maximum soluble ACE activity. Thus, the inter-experiment variation in aqueous ACE activity at 37°C is not only influenced by Triton X-114 phase separation, it is also likely due to the different levels of total ACE activity isolated during each independent membrane preparation experiment.

The variation in total ACE associated with each membrane preparation depends on the efficiency of transfection of the cDNA of ACE, the clonal nature of the cell line and the amount of passages the cell line has undergone. The level of ACE expression by CHO-KI cells was not controlled for in the mammalian expression system used in this assay. Thus, the variation of total ACE associated with the membranes could not be controlled. However, the development of the ACE secretase assay, although limited by the amount of substrate available, is not dependent on the amount of ACE present in each experiment. The assay is based on the increase of soluble ACE detected in the presence of exogenous ACE secretase. An excess of ACE substrate is therefore required to detect the exogenous ACE secretase activity. However, as we express the level of ACE solubility as a percentage of the total substrate present, a large excess of total ACE would overwhelm the detection of the increase in soluble ACE activity. Therefore, to detect the exogenous ACE secretase activity, total substrate concentration should not exceed the detection limit, while providing sufficient substrate during the period of incubation. To ensure sufficient substrate for

the detection of exogenous ACE secretase, time and/or temperature of the incubation can be altered.

3.3.4.3 **Rapid rate of shedding of ACE**

Shedding occurred rapidly at 37°C (Figure 3.3.2). Only 15% substrate remained available after 5 min incubation for proteolysis by the exogenous secretase (Figure 3.3.2). Furthermore, the level of available substrate varied from 10% to 45% in each individual experiment (Figure 3.3.3). Thus, an increase in soluble ACE activity with additional ACE secretase would be hard to detect within the first 5 minutes of incubation. The rate of shedding of the endogenous secretase needed to be decreased, so that at a given time point, enough membrane-bound ACE substrate remained available for cleavage by the exogenous ACE secretase. In order to slow down the proteolytic reaction, shedding was carried out at various temperatures.

ACE shedding, from isolated membranes, was temperature dependent as indicated in Figure 3.3.6. At 15°C shedding occurred at a much slower rate, such that at 5 min only ~5% membrane-bound ACE had been converted to soluble ACE above that obtained at 4°C. This suggested that although the ACE secretase was active at this temperature, within 5 min there was approximately 45% ACE substrate available for proteolysis by the exogenous ACE secretase. The increase of 45% soluble ACE activity above that obtained at 4°C was considered sufficient to enable the detection of additional ACE secretase activity.

3.3.5 **ACE secretase assay**

3.3.5.1 **Preparation of the exogenous ACE secretase**

3.3.5.1.1 **Homogenisation of CHO-KI cell membranes**

To test whether the addition of 'native' CHO-KI cell membranes to membranes containing ACE would result in an increase in soluble ACE activity, CHO-KI cell membranes were mixed with membranes containing testis ACE using a Dounce homogeniser. This homogenising methodology was used to ensure a homogenous population of membrane vesicles and thorough mixing of the two membrane samples. The combined membrane samples were incubated at 15°C to slow the rate of the

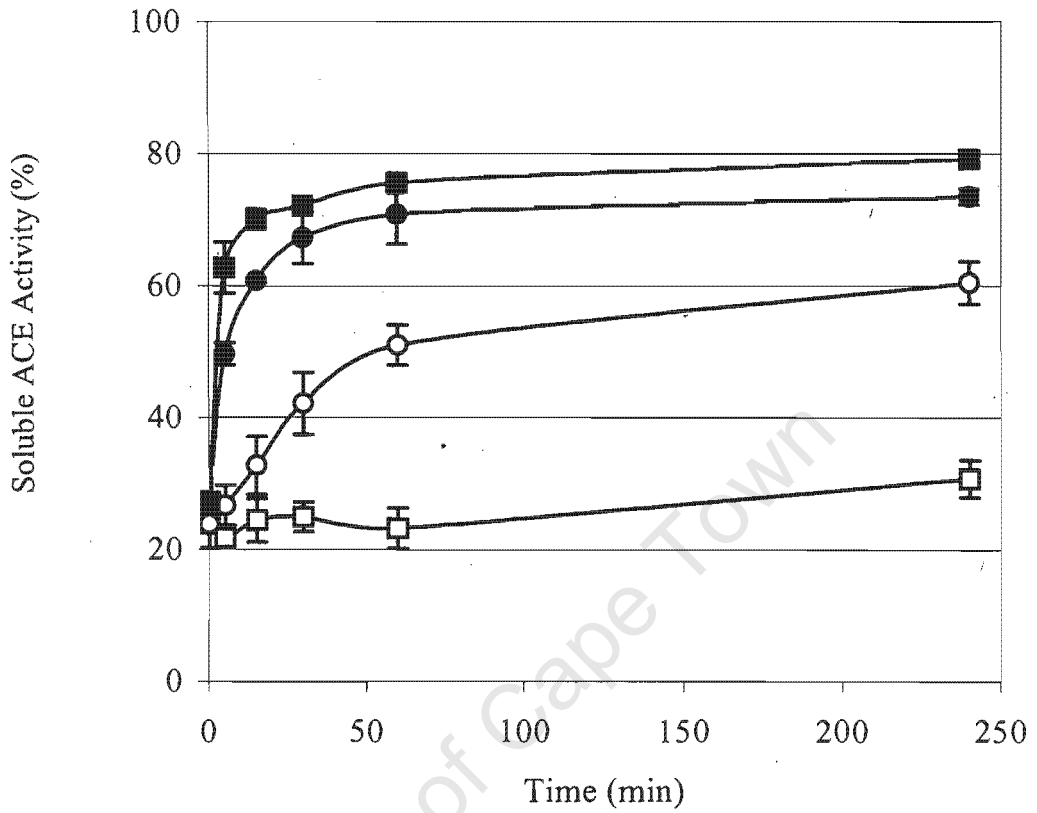


Figure 3.3.6 Affect of temperature on the rate of shedding of testis ACE Membranes were prepared as outlined in Total Membrane Isolation Protocol II and incubated at 4°C (open squares), 15°C (open circles), 25°C (black circles) and 37°C (black squares) for 0, 5, 15, 30, 60, and 240 min. Membrane-bound ACE was separated from soluble ACE by centrifugation at 11,000 x g for 5 min. The membrane pellet was resuspended in Triton X-100 lysis buffer to equal the volume of the supernatant and both samples were assayed for ACE activity using HHL as a substrate. Soluble ACE is indicated as the % ACE activity found in the supernatant of the total ACE activity found in the supernatant and the membrane pellet. (n=3 +/-SD)

reaction of the ACE secretase and thus allow for sufficient substrate for the detection of the exogenous ACE secretase (Figure 3.3.7). There was no difference in soluble ACE activity between membranes incubated in the absence or presence of 'native' membrane. Thus, addition of CHO-KI cell membrane, the source of exogenous ACE secretase, did not increase the soluble ACE activity at 15°C obtained in the absence of exogenous ACE secretase. The inability to detect the activity of the exogenous secretase could be attributed to 1) the dilution of ACE by the addition of lipids affecting the ability of the secretase to bind to its substrate and 2) the inability of Dounce homogenisation to effectively mix the two membrane populations for co-localisation of the secretase and its substrate within the membrane.

3.3.5.1.2 **Effect of Triton X-100 on the shedding of testis ACE**

Parvathy et al published a procedure in which membranes containing the ACE secretase were detergent-solubilised before addition to ACE–containing membrane vesicles. This assay detected the appearance of soluble ACE as it had the added advantage that ACE secretase was not present in the substrate-containing membranes (Parvathy et al, 1997). In these experiments, the membranes providing a source of ACE secretase were fully solubilised with Triton X-100 and this resulted in shedding when added to purified membranes containing ACE (Parvathy et al, 1997). The final concentration of Triton X-100 in these experiments was not stated, but Sadhukhan et al showed that 0.1% Triton X-100 solubilised membranes, containing both the ACE secretase and its substrate, so that shedding was only rescued upon addition of lisinopril-sepharose, likely by anchoring ACE to a solid support and thereby inducing the correct conformation and orientation of ACE for cleavage to occur (Sadhukhan et al, 1999). Therefore, a delicate balance exists between the addition of enough detergent to solubilise the ACE secretase membrane preparation, and the amount of detergent added to solubilise the membrane providing the ACE substrate. In our experiments, this balance was even more crucial, as we aimed to detect an increase in shedding, and detergents are known to affect membrane fluidity and thereby would affect the shedding process. To test at what detergent concentration shedding was inhibited, membranes containing testis ACE were purified as in Total Membrane Isolation Protocol II and incubated at 37°C in the presence of increasing Triton X-100 concentrations. Membrane fractions were subjected to either Triton X-114 phase

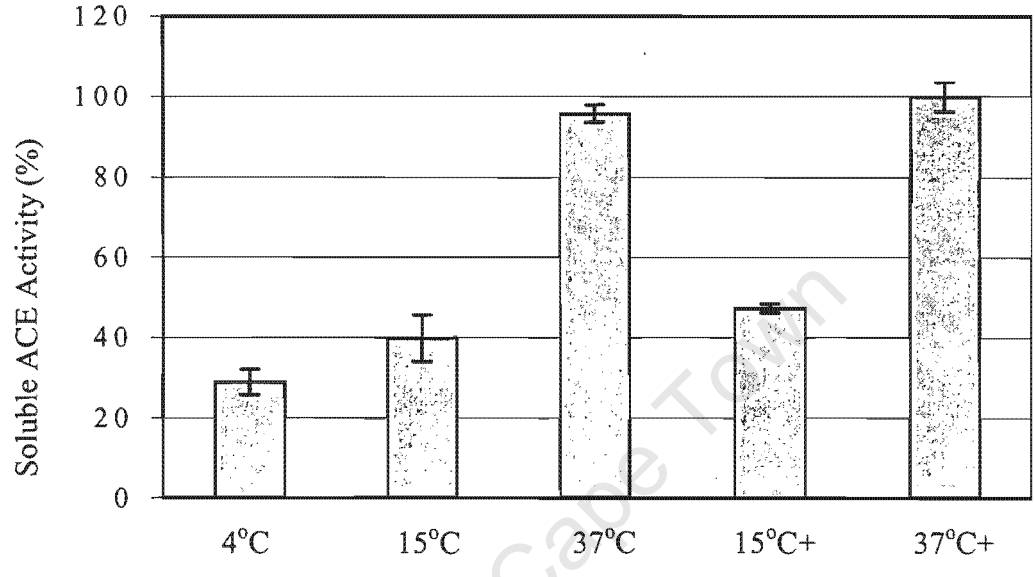


Figure 3.3.7 Effect of homogenisation of CHO-KI cell membrane on testis ACE shedding Membranes were prepared as described in Total Membrane Isolation Protocol II and equal volumes of membranes were added of the two membrane populations: CHO-KI cell membranes with and without testis ACE. These were mixed using a Dounce homogeniser. The membranes were incubated at 4°C, 15°C and 37°C for 5 min in the presence (15°C +, 37°C +) or absence (4°C, 15°C, 37°C) of additional CHO-KI cell membrane. After incubation, soluble ACE was separated from membrane-bound ACE by centrifugation at 11,000 x g for 5 min. The membrane pellet was resuspended in Triton X-100 lysis buffer and the ACE activity determined using HHL as an ACE substrate. Soluble ACE is depicted as a percentage of total ACE activity. (n=3 +/-SD)

separation or centrifugation (Figure 3.3.8). Detergent concentrations of greater than or equal to 0.05% resulted in reduced shedding, as indicated by the decrease in soluble (non-amphipathic) ACE activity detected by Triton X-114 phase separation (Figure 3.3.8 A). This decrease in shedding was presumably due to membrane disruption by the detergent. A concentration of 0.01% Triton X-100 however, did not affect shedding and thus, did not affect the integrity of the membrane. The 100% "soluble" ACE (i.e., non-membrane-bound but full-length) activity seen in Figure 3.3.8 B was likely due to the inability of centrifugation to pellet the solubilised membranes, as anticipated. Thus, 0.05% Triton X-100 was not an appropriate concentration to use to solubilise the ACE secretase, as it would lead to disruption of ACE-containing membrane.

3.3.5.1.3 **Detergent solubilisation of exogenous ACE secretase**

To solubilise CHO-KI cell membrane for the purification of the ACE secretase, 0.01% Triton X-100 was used. CHO-KI cell membranes were shaken at 4°C for 1 hour in the presence of 0.01% detergent. This process resulted in only 30% solubilisation of total membrane proteins as determined using the Bradford protein concentration assay. To optimise the solubilisation process, the shaking time at 4°C was increased to 6 hours and to 48 hours, and this resulted in 50% solubilisation (Data not shown). Similarly, when CHO-KI cell membranes were solubilised in the presence of 0.1% Triton X-100 for 1 hr, only 50% of the membrane proteins were solubilised. This seemed to indicate that Triton X-100 detergent is unable to completely solubilise CHO-KI cell membrane proteins under these conditions. This could be due to the volume (300 µl) used for detergent solubilisation, as the membranes could be too concentrated for the detergent to fully solubilise the membrane proteins. An increase in total volume was not tested, as a high concentration of detergent-solubilised ACE secretase is needed because the shedding of ACE is determined in low volumes (100 µl). To maintain the pH and molarity of the buffers used to determine shedding from the membrane while adding large amounts of ACE secretase, the volume during solubilisation was kept to a minimum.

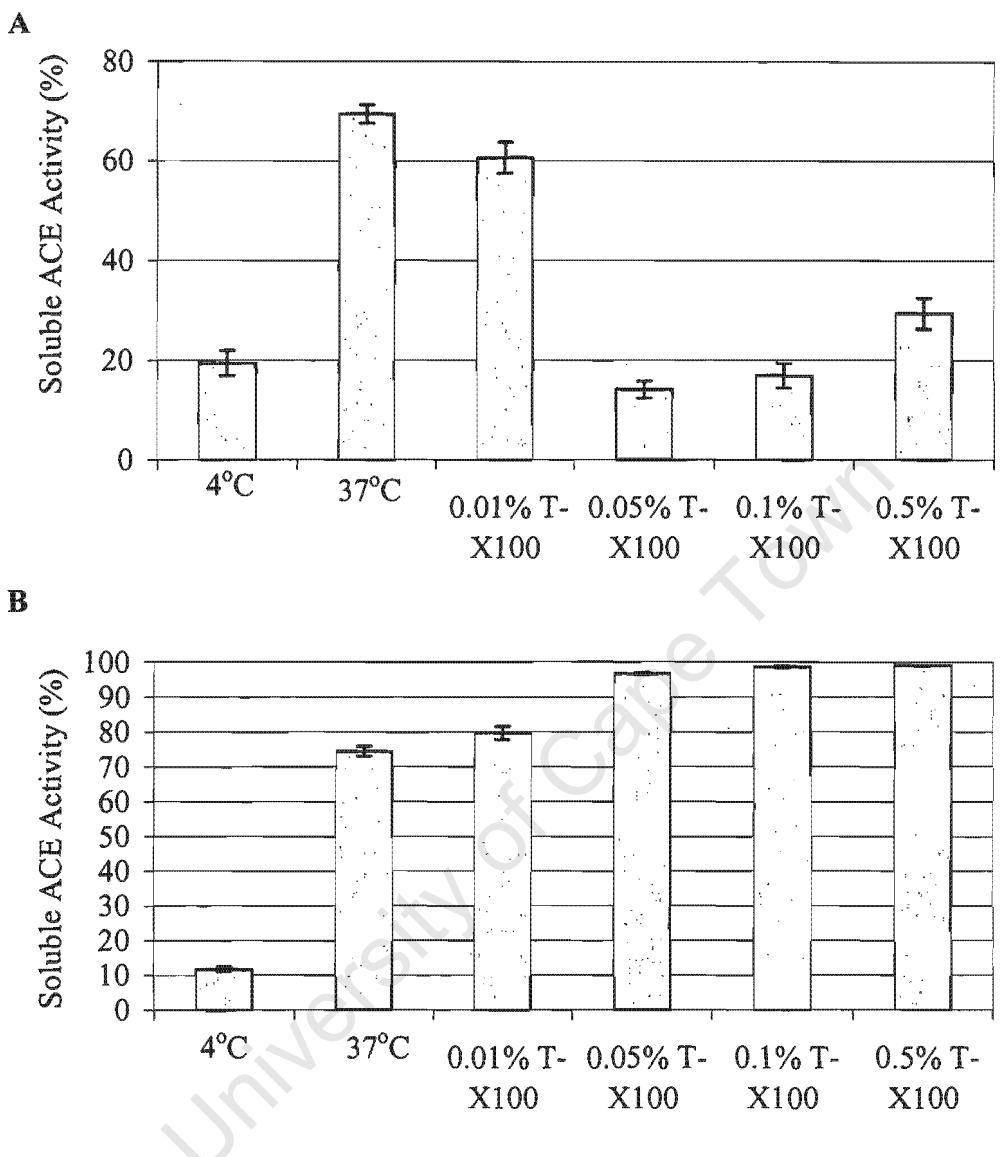


Figure 3.3.8 Effect of Triton X-100 on the shedding of testis ACE from purified CHO-KI cell membrane CHO-KI cell membrane containing testis ACE was prepared as outlined in Total Membrane Preparation Protocol II and incubated at 4°C as a negative control and 37°C in the presence of increasing concentrations of Triton X-100 (%) for 10 min. Membrane-bound ACE was either separated from its soluble counterpart by Triton X-114 phase separation (A) or centrifugation (B) and the ACE activity was determined using HHL as an ACE substrate. Shedding is depicted as the % soluble ACE activity of the total ACE present. (n=3 +/-SD)

3.3.5.2 **Effect of detergent-solubilised ACE secretase on the shedding of testis ACE from CHO-KI cell membrane**

Once the method for the solubilisation of the ACE secretase had been optimised, the effect of detergent-solubilised 'native' CHO-KI cell membrane on the shedding of ACE was determined. To determine whether the 0.01% detergent present in the detergent solubilised membrane preparation was disrupting the substrate-containing membrane and thus affecting shedding of full-length ACE, Triton X-100 buffer (0.01%) was added to the membrane-containing ACE. This addition did not affect the shedding of ACE at either 15°C or 37°C in 5 min, as there was no difference between the soluble ACE activity in the absence or presence of the detergent (Figure 3.3.9). This indicated that at this concentration, Triton X-100 does not disrupt the CHO-KI cell membrane, which would prevent the ACE secretase from recognising and cleaving its substrate. However, when the membrane was incubated for 30 min at 15°C in the presence of detergent buffer, a decrease in shedding was observed. It is possible that this could be a spurious result, as the same effect was not seen at 37°C when the membranes were incubated for 30 min. If this detergent concentration was influencing shedding then this difference should be more marked at the higher temperature.

To determine whether the addition of detergent-solubilised membrane as a source of exogenous ACE secretase would result in an increase in soluble ACE activity, 0.01% detergent-solubilised membrane was incubated with membrane supplying full-length-ACE. Addition of solubilised CHO-KI cell ACE secretase at 15°C did not result in an increase in soluble ACE activity (Figure 3.3.9). The reason could be that the concentration of ACE secretase was too low because of the low detergent solubilisation of CHO-KI cell membranes as mentioned before. The time of incubation also did not result in an increase of ACE shedding at 15°C, as there was no significant difference between the shedding in the presence of additional CHO-KI cell membrane when the membranes were incubated for 5 min or 30 min compared to when the membranes were incubated on their own. It remains possible, despite published reports, that the ACE secretase is optimally active only in cis, i.e., when the secretase and ACE substrate are co-localised in the same membrane.

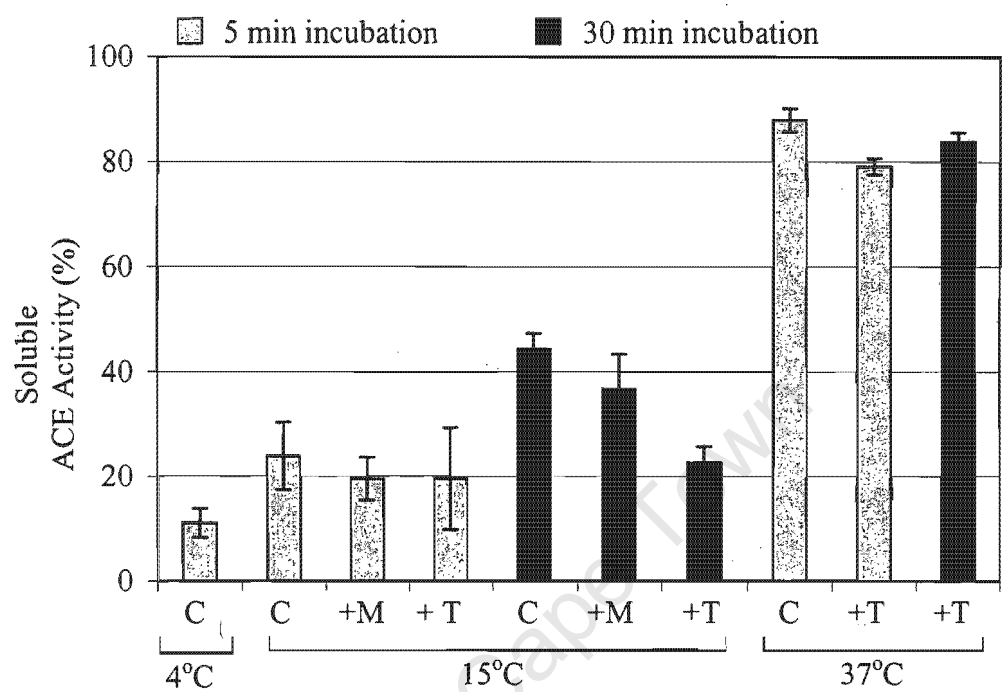


Figure 3.3.9 Addition of detergent-solubilised CHO-KI cell membrane does not increase testis ACE shedding during two different incubation periods Membranes were prepared as described in Total Membrane Isolation Protocol II and incubated at 4°C, 15°C and 37°C for 5 min (C). CHO-KI cell membranes not containing ACE, were solubilised with 0.01% Triton X-100 for 6 hrs at 4°C and 11.6 μg (+M) of solubilised protein was added to CHO-KI cell membrane containing ACE (21 μg total protein) and incubated at 15°C for 5 min (grey bar) or 30 min (black bar) To control for the effect of Triton X-100 on the shedding process, 0.01% Triton X-100 buffer (+T) was added to the CHO-KI cell membranes containing ACE and incubated at 15°C for 5 min (grey bar) and 30 min (black bar) or incubated at 37°C for 5 min (grey bar) and 30 min (black bar). Soluble ACE was separated from the membrane by centrifugation at 11,000 x g. The membranes and supernatant were assayed for ACE activity using HHL as a substrate. (n=3 +/-SD)

3.4. Conclusion

In conclusion, the use of CHO-KI cell membranes containing recombinant testis ACE as a substrate for detergent-solubilised ACE secretase does not provide optimal conditions for the ACE secretase assay, mainly because of the presence of the endogenous membrane-bound ACE secretase present in all membrane preparations. A more feasible scenario is one where ACE is contained in synthetic membrane vesicles, such as liposomes, purified biological membranes such as pig intestinal brush border membranes where no endogenous ACE secretase is present or purified, detergent-solubilised full-length ACE bound to a solid support such as lisinopril-sepharose (Oppong & Hooper, 1993; Sadhukhan et al, 1999).

To lower the rate of shedding of the ACE secretase, membranes were incubated at 15°C. It is possible that the ACE secretase activity is lowered to such a degree that an increase cannot be detected in the presence of exogenous ACE secretase. Thus, if no endogenous ACE secretase were present, this assay could be performed at 37°C and thereby eliminate the effect of lowered temperature on the shedding process.

The lack of increase in shedding in the presence of additional detergent-solubilised ACE secretase at 15°C could be due to the low solubilisation of CHO-KI cell membrane proteins obtained with the method used in this assay. Thus, if the ACE secretase needs to be detergent solubilised to be active, as indicated by the lack of detectable increase in activity when the two membrane populations are mixed without detergent (Figure 3.3.7), then a Triton X-100 concentration of 0.01% is too low to release this activity and 0.05% is too high, as it leads to membrane solubilisation and inhibition of ACE shedding (Figure 3.3.8).

The presence of lisinopril-sepharose can act as a solid support to which the ACE molecules can bind, thus circumventing the need to use low concentrations of detergent for the solubilisation of CHO-KI cell membranes (Sadhukhan et al, 1999). This might allow for the use of high concentrations of detergent, which may allow for the solubilisation of total membrane proteins and the release of the ACE secretase activity from the membrane fraction. However, the addition of lisinopril-sepharose to detergent-solubilised CHO-KI cell membrane necessitates the need to return to Triton X-114 phase separation to separate membrane-bound ACE from its soluble

counterpart. As shown, this method of separation has its limitations in producing reproducible results when trying to determine an increase in shedding above what is obtained at 4°C.

Lipid microdomains found in the plasma membrane have recently been reviewed (Zajchowski & Robbins, 2002). The authors speculate that there are different sub-populations of these lipid microdomains or "lipid rafts" in most cell types and that their lipid and protein composition varies not only between sub-populations, but also between different cell types. These lipid rafts are characterised by insolubility in the presence of detergent and are thought to be integral to the sorting of membrane proteins and cell signalling (Zajchowski & Robbins, 2002). It is possible that the ACE secretase from CHO-KI cells are located within these lipid rafts and thus the addition of Triton X-100 does not result in the release of ACE secretase activity. Previous studies into the solubilisation of the ACE secretase activity used membranes prepared from pig kidney cortex and it is possible that the lipid raft populations in these cell types differ from that found in CHO-KI cell membranes. Recently, the shedding of APP has been linked to lipid rafts indicating that secretases are localised within these membrane compartments (Ledesma et al, 2000; Ikezu et al, 1998). In this study, alternative sources of the ACE secretase were not tested and thus we can speculate that this membrane assay could be used for the detection of ACE secretase activity present in membranes prepared from different cell types.

Although this attempt to develop an *in vitro* assay for the activity of the ACE secretase has not yielded the desired result, it has resulted in the optimisation of membrane preparation from CHO-KI cells. The method for membrane isolation has been made more efficient, reducing the time taken to 30 min (outlined in Total Membrane Isolation II). This study has also indicated the limitations of Triton X-114 phase separation as a technique for the quantification of ACE shedding and the effect of Triton X-100 on the release of full-length ACE from CHO-KI cell membranes. More importantly, we have designed a simple method for the characterisation of ACE shedding by looking at the effect of temperature on this process. We have shown a correlation between ACE shedding and temperature and this can be used to characterise the effect of mutations introduced into ACE molecules on shedding. Although, we have optimised the shedding kinetics of ACE and ACE mutants from

intact CHO-KI cells (Ehlers et al, 1996; Schwager et al, 1998, Schwager et al 1999), the advantage gained through membrane isolation is that we can now differentiate between mutations that affect cellular processing and trafficking of ACE to the membrane and those that only affect shedding from the membrane. Similarly, this is an ideal method for the study of the effect of inhibitors of the ACE secretase on ACE shedding. In some cases, inhibitors are non-specific and affect cellular processes such as cell adhesion, protein trafficking or cell growth. These "side effects" will influence the results obtained on ACE shedding, as we need to maintain cell viability and homeostasis. However, by using isolated membrane we limit these effects, as we are not working within a cellular environment, making our results more specific. We have attempted to do this on ACE chimeras constructed in our laboratory and the results will be discussed in Chapters 4 and 6.

Chapter 4

The rate of shedding of the two isoforms of ACE differ even though they share identical cleavage sites

4.1 Introduction

Both isoforms of ACE are type I transmembrane proteins with identical stalk, transmembrane and cytoplasmic regions (Ehlers et al, 1989). A soluble form of enzymatically active somatic ACE (i.e. lacking the transmembrane and cytoplasmic regions) has been identified in blood plasma, urine, amniotic fluid and seminal plasma (Yokoyama et al, 1982; Lanzillo et al, 1995, El-Dorry et al, 1983; Beldent et al, 1993; Corvol et al, 1995). The physiological significance of the generation of soluble somatic ACE remains unclear although most or all of the physiological functions of ACE have been ascribed to the membrane-bound form (Esther et al, 1997). Although no naturally occurring soluble form of testis ACE has been identified, it has been shown that the expression of testis ACE by CHO-KI cells results in the release of ACE into the culture medium (Sen et al, 1991; Ehlers et al, 1991b). The shedding of testis ACE by CHO-KI cells is regulated by phorbol esters and it is hypothesised that the shedding of this molecule is a mechanism for rapid down-regulation of cell-surface expression (Ehlers et al, 1995).

The cleavage site of testis ACE occurs after R627 within the stalk region, 24 amino acid residues away from the transmembrane domain (Ehlers et al, 1996; Ramchandran et al, 1994). Attempts to identify the requirements for ACE shedding have involved the deletion of 47 amino acid residues of the juxtamembrane region and this ACE deletion mutant was rendered inactive (Ehlers et al, 1996). This indicated that the deleted region comprised residues vital for ACE structure and thus activity. Thus, the previously reported cleavage site of somatic ACE at the R1137-L1138 bond (somatic

ACE numbering), seemed unlikely for 2 reasons: 1) cleavage 100 amino acid residues away from the membrane would render the C domain inactive [noting that the N domain contributes only 25-10% catalytic activity to the somatic ACE enzyme (Esther et al, 1997; Wei et al, 1991a)] and 2) cleavage at this site would not result in the 6.9 kDa proteolytic product comprising the TM and cytoplasmic tail of ACE reported by Parvathy et al (1997). The different reported cleavage sites of the two isoforms has been attributed to the N domain acting as a steric hindrance causing the ACE secretase to cleave somatic ACE at an alternative, less favoured cleavage site. This would explain the lowered efficiency at which somatic ACE is shed from the membrane (Beldent et al, 1995).

The enzymatic and structural similarity between somatic and testis ACE, the reported differences in shedding, and the inability of the ACE isoforms to replace one another functionally, underscores the need for further investigation to understand the effect of the N domain on the shedding of somatic ACE (Kessler et al, 2000). In this study we investigated the shedding of somatic ACE from isolated membranes of CHO-KI cells and intact CHO-KI cells. The soluble protein was purified from the culture medium and its cleavage site was determined by mass spectrometry. Similarly, we determined the cleavage site of soluble somatic ACE purified from seminal plasma to see whether the cleavage of naturally occurring ACE differed from that of the transfected cell lines.

4.2 Methods

4.2.1 **Sub-Cloning of somatic ACE**

The cDNA of somatic ACE was sub-cloned into the vector pLEN for expression in CHO-KI cells (Figure 4.2.1). The cDNA of somatic ACE in pBluescript (pBS) (2960 bp) was digested with *Bam* HI and *Nhe* I and the 3085 bp band was eluted from the 1% agarose gel using the GenElute Agarose Gel DNA kit (Sigma). This DNA fragment was ligated to the 7177 bp band generated by the similarly digested pLEN-tACE and comprised the 3' end of the cDNA of testis ACE encoding the stalk, transmembrane domain (TM) and cytoplasmic tail and the mammalian expression vector, pLEN. The ligation reaction was transformed into XL1-Blue *E.coli* competent cells and the ampicillin resistant colony forming units (cfus) were inoculated into 5 ml Luria broth (LB) aliquots and grown O/N before the plasmid DNA was extracted using a quick, phenol-based, plasmid DNA mini-preparation method. The plasmid DNA was digested with *Bam* HI and *Nhe* I to determine the presence of the N domain in pLEN-tACE and the cfu containing the correct DNA construct was inoculated into 50 ml LB and grown O/N. The plasmid DNA was extracted using the Qiagen Plasmid Midi kit and the digestion of the final DNA preparation with *Bam* HI and *Nhe* I confirmed the presence of the somatic ACE cDNA in pLEN (AII.1).

4.2.2 **Expression and analysis of somatic ACE**

CHO-KI cells were grown to 40% confluence and co-transfected with pLEN-sACE and pSV2 Neo by methods detailed in Chapter 2, section 2.2.1. The expression of somatic ACE at the cell surface was confirmed by confocal microscopy and its processing and shedding was studied using metabolic labelling and pulse chase experiments. The shedding of this isoform was further studied from the isolated membranes of CHO-KI cells (section 2.5) and from intact cells (2.3.5). The soluble form was purified from seminal plasma (section 2.3.4.2) and the culture media of the stably transfected CHO-KI cells (section 2.3.4.1) and the cleavage site was determined by MALDI TOF mass spectrometry (section 2.3.9).

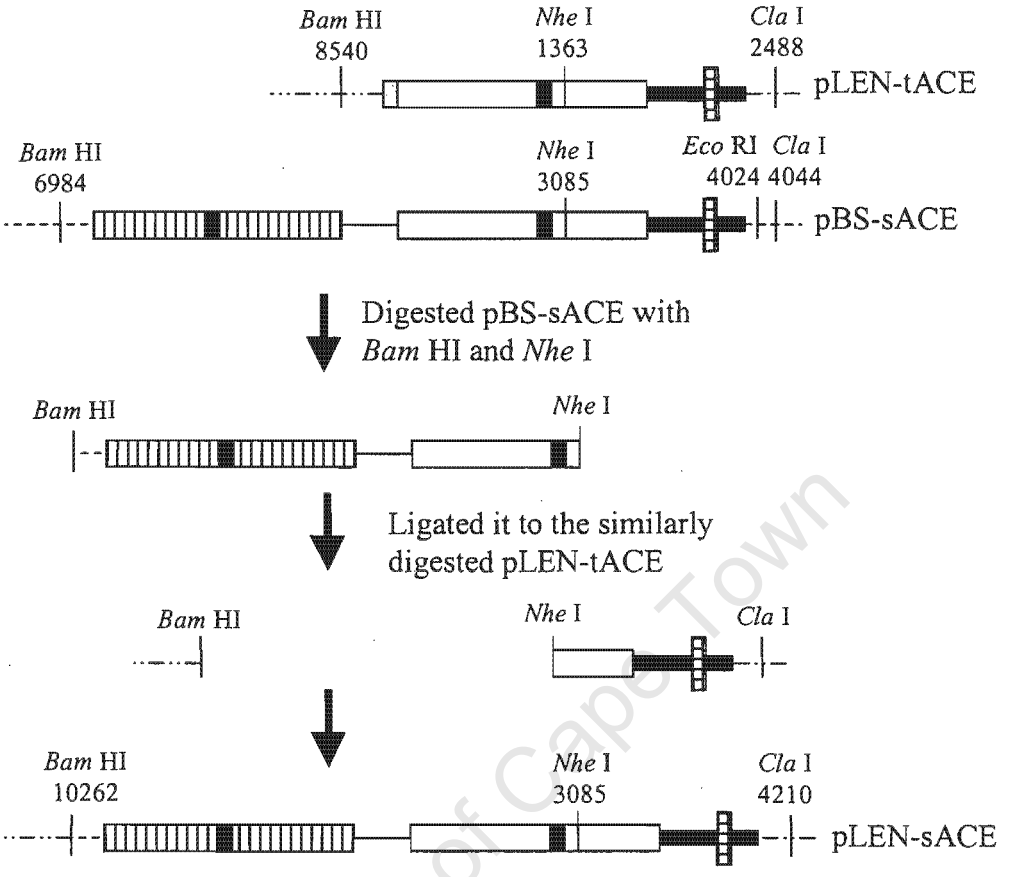


Figure 4.2.1. Sub-cloning somatic ACE from pBluescript (pBS) into the mammalian expression vector, pLEN Testis ACE in pLEN is indicated above (pLEN-tACE, 8567 bp) with somatic ACE in pBS (pBS-sACE, 6984) shown below it. The similarity between the testis ACE and the C domain of somatic ACE is indicated by the open bars, with the unique *O*-glycosylated region of testis ACE indicated by the grey box, the N domain shown by the striped bar, the active sites indicated by the black box, the stalk and cytoplasmic tail shown as the bold line and the transmembrane domain indicated with a hatched box. Both, pBS-sACE and pLEN-tACE were digested with *Bam* HI and *Nhe* I and the 3085 bp and 7177 bp bands, respectively, were ligated to form pLEN-sACE (10262 bp). The size (bp) of pLEN-sACE and pBS-sACE is approximated based on the numbering of Soubrier et al, 1988, Ehlers et al, 1989 and Ehlers et al, 1991. Refer to Appendix AI.2 and AI.3 for vector maps.

4.3 Results

4.3.1 Expression of human somatic ACE in CHO cells and kinetics of release

4.3.1.1 Cell surface localisation of somatic ACE

The cDNA of human somatic ACE was stably transfected into CHO-KI cells. The stably transfected cell line was grown on coverslips and ACE expression was induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM) before the cells were sequentially probed with a rabbit polyclonal testis ACE antibody and a FITC-conjugated goat anti-rabbit secondary antibody. The green fluorescent signal was visualised by confocal microscopy. CHO-KI cells stably transfected with the cDNA of testis ACE were treated similarly and included as a comparison for the expression of somatic ACE (Figure 4.3.1).

Somatic ACE was expressed on the cell surface and did not differ from that of testis ACE (Figure 4.3.1). The membrane-permeabilised cells in column III confirmed the intracellular expression of both somatic ACE and testis ACE. CHO-KI cells not transfected with the cDNA of ACE had no intracellular or cell-surface endogenous expression of this enzyme (Row A). Column I indicates the corresponding nuclei of the CHO-KI cells tested for cell-surface ACE expression by staining with propidium iodide (PI).

4.3.1.2 Metabolic labelling and pulse-chase analysis of somatic ACE release

CHO-KI cells expressing somatic ACE were grown in the presence of [^{35}S]methionine and [^{35}S]cysteine for 30 min to radioactively label all cellular proteins and this label was chased for 24 hrs in the presence or absence of PDBu. The medium and the cells were lysed at the time points indicated (Figure 4.3.2). The protein was affinity purified with lisinopril-sepharose, resolved on SDS PAGE and autoradiographed (Figure 4.3.2).

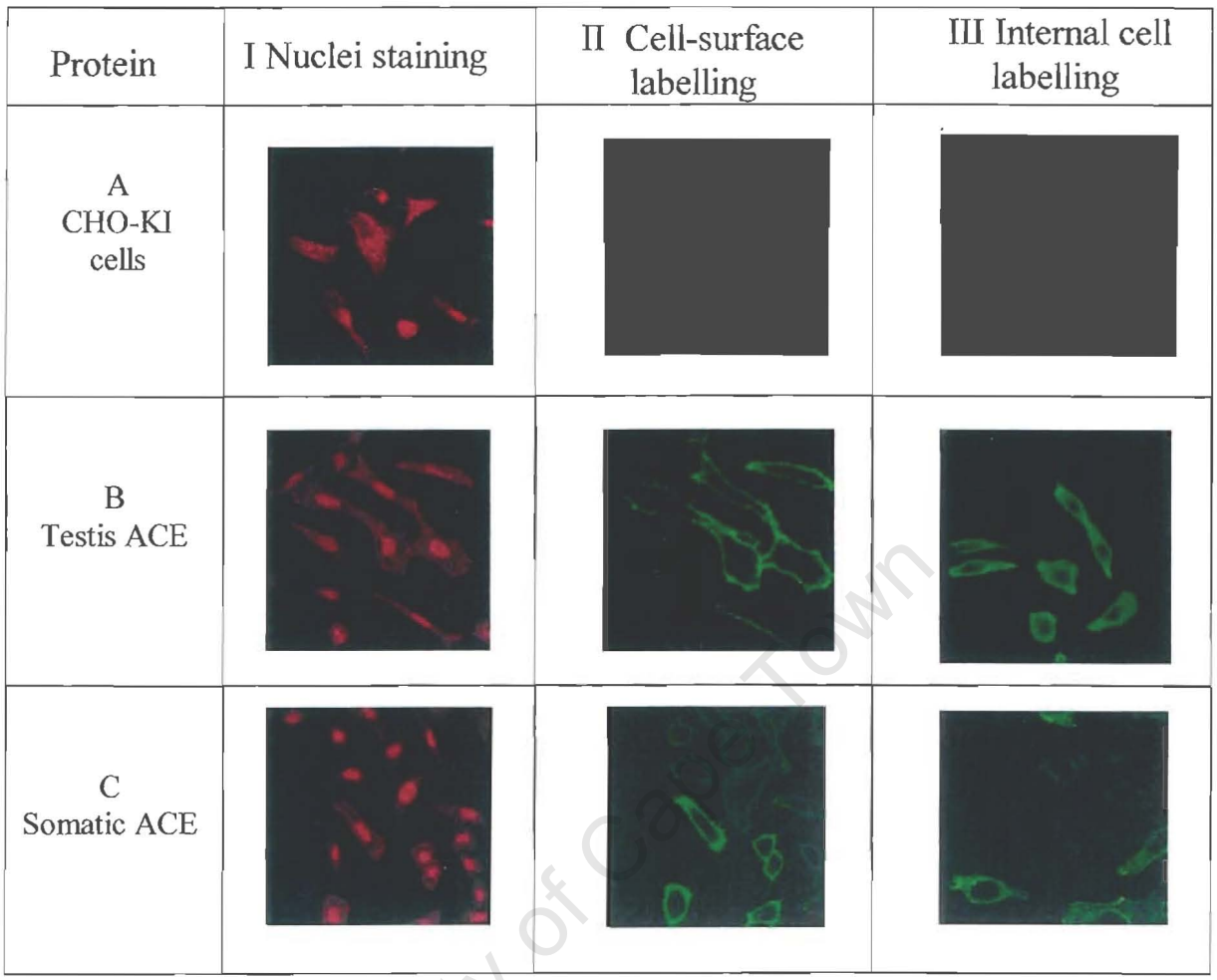


Figure 4.3.1. Cell surface localisation of somatic and testis ACE CHO-KI cells, not expressing (row A) or expressing either the cDNA of testis (row B) or somatic ACE (row C) were grown on coverslips before being probed with a rabbit anti-testis ACE polyclonal antibody. The cells were washed before the addition of FITC-labelled goat anti-rabbit secondary antibody and the cells were fixed to detect cell surface localisation of the ACE isoforms (column II). To detect internal expression of the proteins, the cell membranes were permeabilised with cold methanol before probing with the primary antibody (column III). To detect all CHO-KI cells present on the coverslips, the nuclei were stained with propidium iodode (PI) (column I).

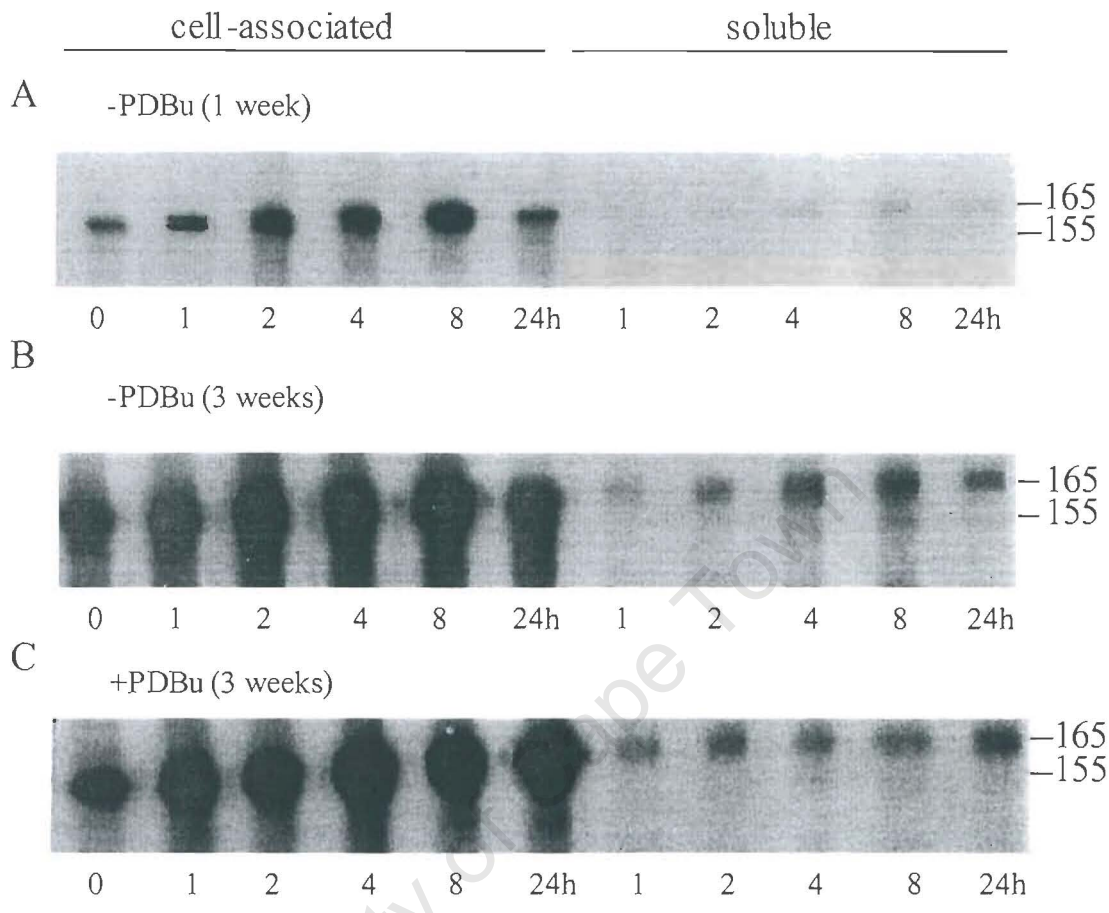


Figure 4.3.2 Metabolic labelling and pulse-chase analysis of the expression and shedding of somatic ACE CHO-KI cells stably transfected with the cDNA of human somatic ACE were grown in the presence of [^{35}S]methionine and [^{35}S]cysteine for 30 min before the culture medium was removed, the cells washed and grown in fresh complete medium in the absence (A, B) and presence (C) of PDBu to chase the label for 24 hrs. The culture medium was removed and the cells were detergent-lysed at the time points indicated and cell-associated and soluble ACE were purified using affinity chromatography, subjected to SDS PAGE and autoradiographed for 1 week (A) or 3 weeks (B, C). The estimated molecular masses are indicated on the right.

In panel A, cell associated somatic ACE is indicated on the left, showing a 155 kDa band at zero time and the appearance of a 165 kDa band after 1 hr. This doublet (155 and 165 kDa bands) is likely to be the unprocessed, immature, and the fully processed, mature form, respectively. This result was also seen for testis ACE where there was the appearance of a 90 kDa and 105 kDa band (Schwager et al, 1998). The 155 kDa band disappeared after 24 hrs indicating that in that time period all the labelled immature protein was processed to its mature active form. This mature form appeared in the medium after 4 hrs in panel A and 1 hr in panel B after being autoradiographed for 1 week and 3 weeks, respectively. Thus, only the mature form was shed into the medium. Schwager et al showed that testis ACE appeared in the medium after 2 hrs after being autoradiographed for 1 week (Schwager et al, 1998).

In panel C, the cells were grown in the presence of PDBu (and autoradiographed for 3 weeks) and there is an increase in band intensity after 1 hr of the soluble form of somatic ACE compared to panel B when the cells were grown in the absence of PDBu. The increase was less compared to testis ACE shedding in the presence of PDBu. The soluble form of testis ACE appeared in the medium after 1 hr and the band intensity was much higher. In addition, the 105 kDa band of the testis ACE cell-associated samples declined rapidly after 2 hrs in the presence of PDBu indicating rapid release of the mature form of testis ACE into the medium (Schwager et al, 1998). This decrease in cell-associated mature somatic ACE was not observed in panel C. This indicates that somatic ACE is shed at a slower rate than testis ACE and is not upregulated to the same extent in the presence of PDBu.

4.3.1.3 **Shedding kinetics of somatic ACE**

To determine the rate at which somatic ACE is shed from CHO-KI cells, two methods were used. The first method used intact cells whereby the somatic ACE, stably transfected cell line was cultured in the presence and absence of PDBu. The culture medium was removed and the cells were lysed at different time points and ACE activity was determined using the substrate HHL. The second method made use of the technique developed in Chapter 3, section 3.2.1.2, whereby the membranes were isolated from CHO-KI cells and incubated at different temperatures for increasing lengths of time. The soluble ACE was separated from the membrane-bound form by centrifugation before the ACE activity was determined for these two samples:

supernatant and membrane pellet. The level of soluble ACE is depicted as the percentage of ACE activity in the supernatant of the total ACE activity determined in both samples. The results of these two methods are outlined in 4.3.1.3.1 and 4.3.1.3.2, respectively.

4.3.1.3.1 **Shedding of somatic ACE from intact CHO-KI cells**

For comparison, the shedding of human testis ACE from CHO-KI cells was investigated. The shedding of testis ACE was upregulated 4-5-fold in the presence of PDBu and there was a concomitant decrease in cell associated activity as would be expected if testis ACE was rapidly shed from the cell surface (Figure 4.3.3).

Soluble somatic ACE activity (Figure 4.3.3 A) was approximately 2- to 3-fold lower than that of testis ACE for all time points in the absence of PDBu. There was an approximate 1.5-fold increase in the shedding of somatic ACE in the presence of PDBu. Thus, the stimulation of somatic ACE shedding by PDBu was about 10-fold lower than that of testis ACE. This low rate of shedding of somatic ACE was corroborated by the determined cell associated activity, as there was no change in the level of membrane-bound ACE for all the time points tested (Figure 4.3.3 B). This would be expected if the shedding of somatic ACE was inefficient so that the level of cellular ACE remained constant.

4.3.1.3.2 **Shedding of somatic ACE from isolated membranes**

CHO-KI cells transfected with the cDNA of somatic ACE were cultured and the membranes were isolated (section 2.5). The membranes were incubated at 37°C, 15°C and 4°C to compare the rate of release of somatic ACE at different temperatures (Figure 4.3.4). In addition, the shedding of somatic ACE was compared to the release of testis ACE previously determined in Chapter 3, Figure 3.3.6.

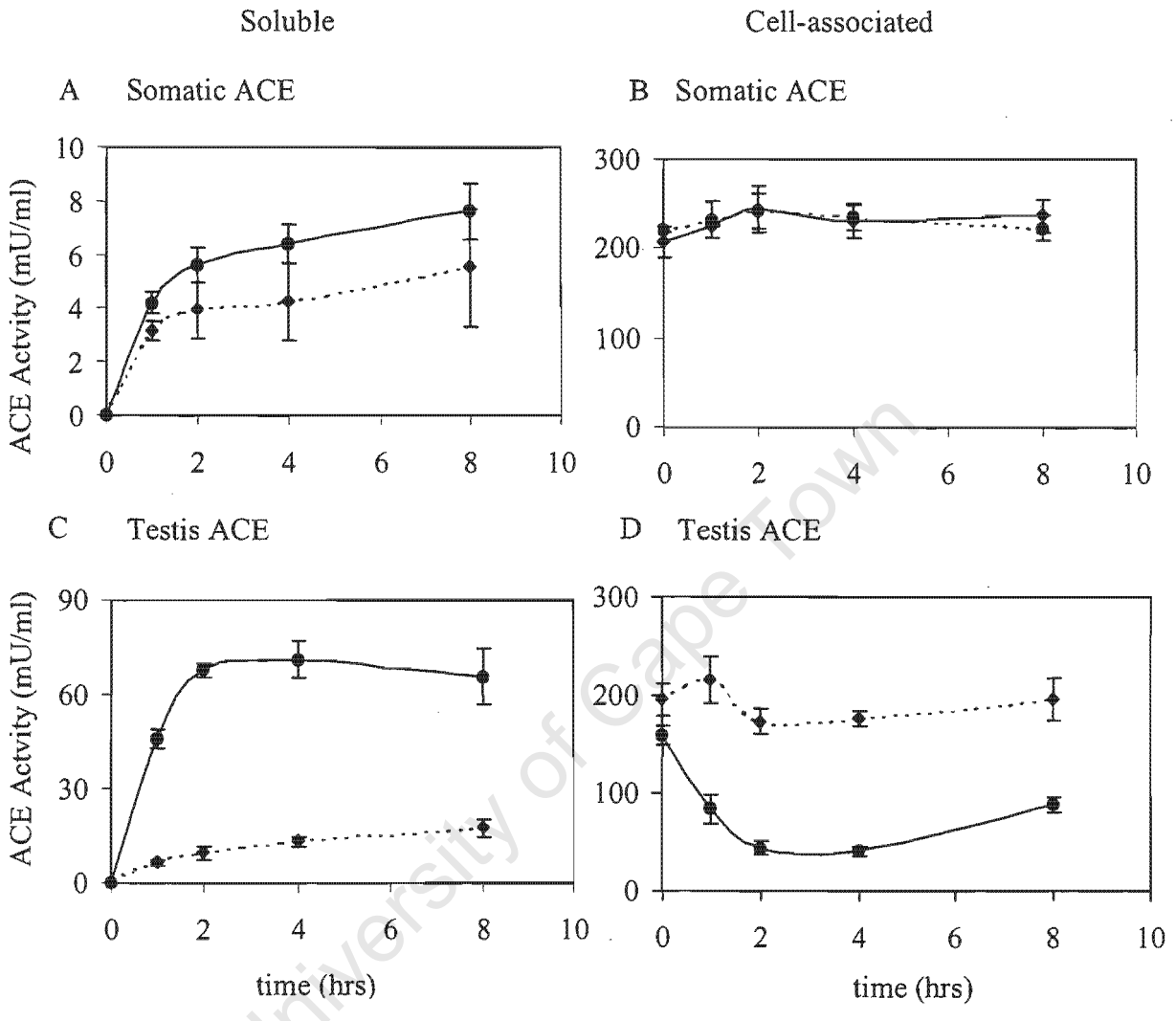


Figure 4.3.3 Shedding kinetics of somatic and testis ACE from intact cells CHO-KI cells stably expressing the cDNA of somatic (A, B) and testis (C, D) ACE were grown to confluence and ACE expression was induced O/N with 2% FCS complete medium supplemented with 40 μM $ZnCl_2$. The cells were then grown in the presence (solid line) and absence (broken line) of PDBu (1 μM) and the medium (A, C) was removed and the cells (B, D) were lysed at the times indicated. ACE activity was determined for the medium and cell lysates using the ACE substrate, HHL. (n=3 +/- SD)

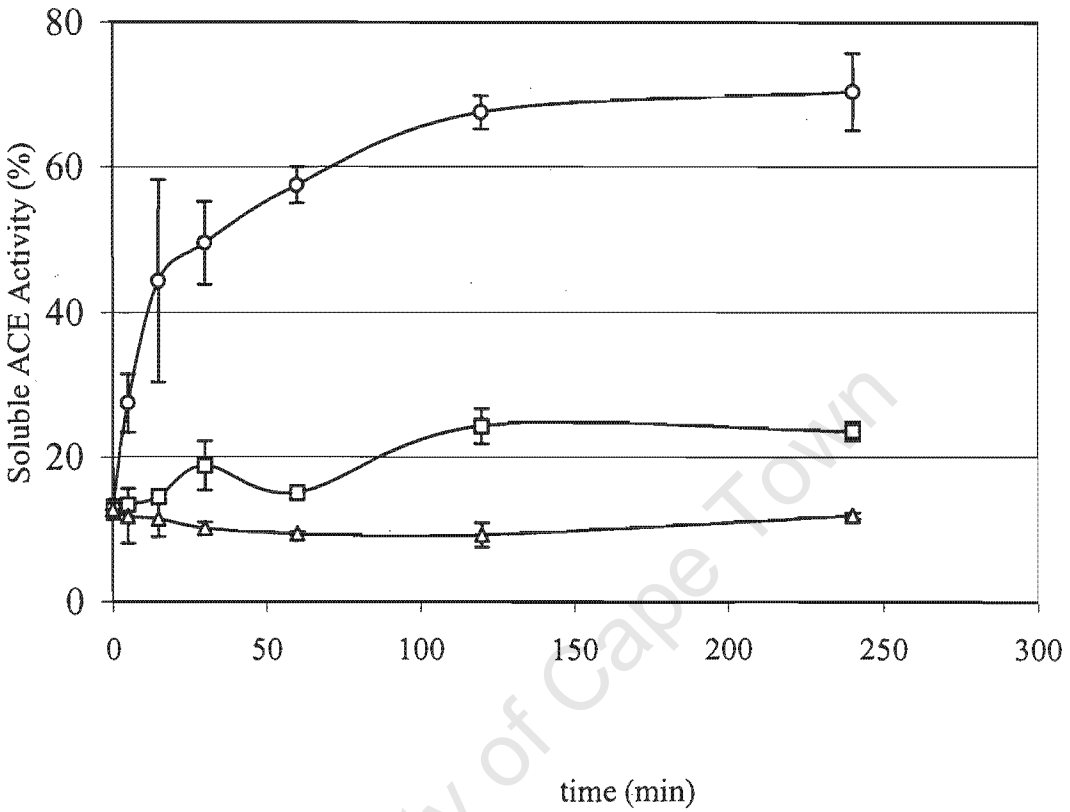


Figure 4.3.4 Shedding of somatic ACE from isolated CHO-KI cell membrane Membranes were isolated from CHO-KI cells transfected with the cDNA of somatic ACE and incubated at 37°C (circle), 15 °C (square) or 4 °C (triangle) for the time (min) indicated on the x-axis. The soluble ACE was separated from the membrane by centrifugation and the supernatant and the membrane pellet was tested for ACE activity using the substrate, HHL. The soluble ACE activity is depicted on the y-axis as the percentage ACE activity in the supernatant of the total ACE activity present in both samples. (n=3 +/-SD)

At 4°C, there was no change in soluble ACE activity (Figure 4.3.4). Thus, somatic ACE was not shed at 4°C. The rate of shedding of somatic ACE was lower than that of testis ACE at 37°C. The release of somatic ACE at 37°C resembled that of testis ACE release at 15°C. Testis ACE was shed very rapidly from the membrane and maximum soluble ACE activity (~60%) was reached within the first 5 min of incubation at 37°C (Figure 3.3.6). In comparison, only ~25% of soluble somatic ACE was detected after 5 min of incubation at the same temperature. This difference was more marked at 15°C where somatic ACE shedding only reached 25% after 4 hrs of incubation. Thus, somatic ACE was shed less efficiently than testis ACE in an '*in vitro*' system and these results were in agreement with those carried out on intact cells.

4.3.1.4 **Inhibition studies of the release of somatic ACE by the ACE secretase**

Shedding of testis ACE from isolated membranes of CHO-KI cells was specifically inhibited by TAPI, a hydroxamic acid-based inhibitor (Figure 3.3.1). This indicated that the production of soluble ACE was due to the specific action of a zinc metalloprotease, termed the ACE secretase(s). TAPI has been shown to specifically inhibit the shedding of ACE from isolated membranes of pig kidney cells (Hooper et al, 1997). To confirm that the shedding of somatic ACE from CHO-KI membranes was due to ACE secretase activity, membranes from CHO-KI cells expressing the cDNA of human somatic ACE were incubated in the presence of TAPI. The membranes were also incubated in the presence of a serine protease inhibitor, DCI, because this inhibitor has been shown to inhibit the shedding of ACE mutants (Alfalah et al, 2001; Schwager et al, 1999). Serine protease inhibitors, not implicated in the inhibition of shedding, phenylmethanesulfonyl fluoride (PMSF) and aprotinin were also tested. An ACE stalk mutant, ACE-JMEGF, remains tethered to the membrane by a disulfide bond and requires the addition of a reducing agent to be shed (Schwager et al, 1998). The involvement of protein disulfide isomerase (PDI) in the shedding of ACE-JMEGF is investigated in Chapter 7, section 7.3.2.2. PDI is a chaperone involved in disulfide bond formation of nascent proteins. To investigate whether PDI is involved in the shedding of ACE in general, membranes comprising full-length testis and somatic ACE were incubated in the presence of bacitracin, a PDI inhibitor.

The results of this experiment are shown in Figure 4.3.5 where testis ACE shedding is indicated in Figure A and somatic ACE in Figure B. Within 1 hr, 90% of membrane-bound testis ACE and 60% somatic ACE were converted to soluble ACE at 37°C. This data confirmed that somatic ACE was shed less efficiently than testis ACE. The inhibition profiles of these isoforms are the same: TAPI inhibits shedding to basal level while DCI, NSI and bacitracin had no effect. These results confirm that the ACE secretase(s) is responsible for the shedding of somatic ACE because of the specific inhibition of somatic ACE shedding by TAPI.

4.3.2 **Determination of the cleavage site of soluble somatic ACE**

Somatic ACE was purified by affinity chromatography from the culture medium of CHO-KI cells stably transfected with the cDNA of human somatic ACE. The purity of the protein was confirmed by SDS PAGE (Figure 4.3.6, lane 3). The soluble enzyme was digested with the endoproteinase Lys-C, which cleaves at the C-terminal end of lysine residues. The total endoproteinase Lys-C digest of somatic ACE was subjected to MALDI-TOF mass spectrometry and a peptide with the *m/z* 1691.1 was identified (Table 4.3.1). The *m/z* of this peptide is very similar to that of the calculated *m/z* of the peptide LGWPQYNWTPNSAR (1690.8) This peptide, which corresponds to the C-terminal peptide of soluble somatic ACE, ends in R1203, the amino acid residue (R627) identified as the cleavage site for testis ACE. To confirm that this peptide is the C-terminal peptide, the total somatic ACE Lys-C digest was fractionated by HPLC and a peptide was purified, which after 5 cycles of N-terminal sequencing, gave the sequence LGWPQ... This peptide was subjected to mass spectral analysis and a *m/z* of 1691.9 was obtained. This confirmed the identity of the C-terminal peptide.

In addition, mass spectral analysis was done on soluble somatic ACE obtained from two additional, independent purifications and in each case a single major peak was observed for the *m/z* 1691. The peptide immediately adjacent to the C-terminal peptide with the expected mass of 1951.2 has been readily identified in all mass spectral analyses of testis ACE and mutated ACE molecules with an intact stalk region and acts as a reliable marker for endoproteinase Lys-C digestion of the ACE protein. This peptide was identified in the total digest of soluble somatic ACE with

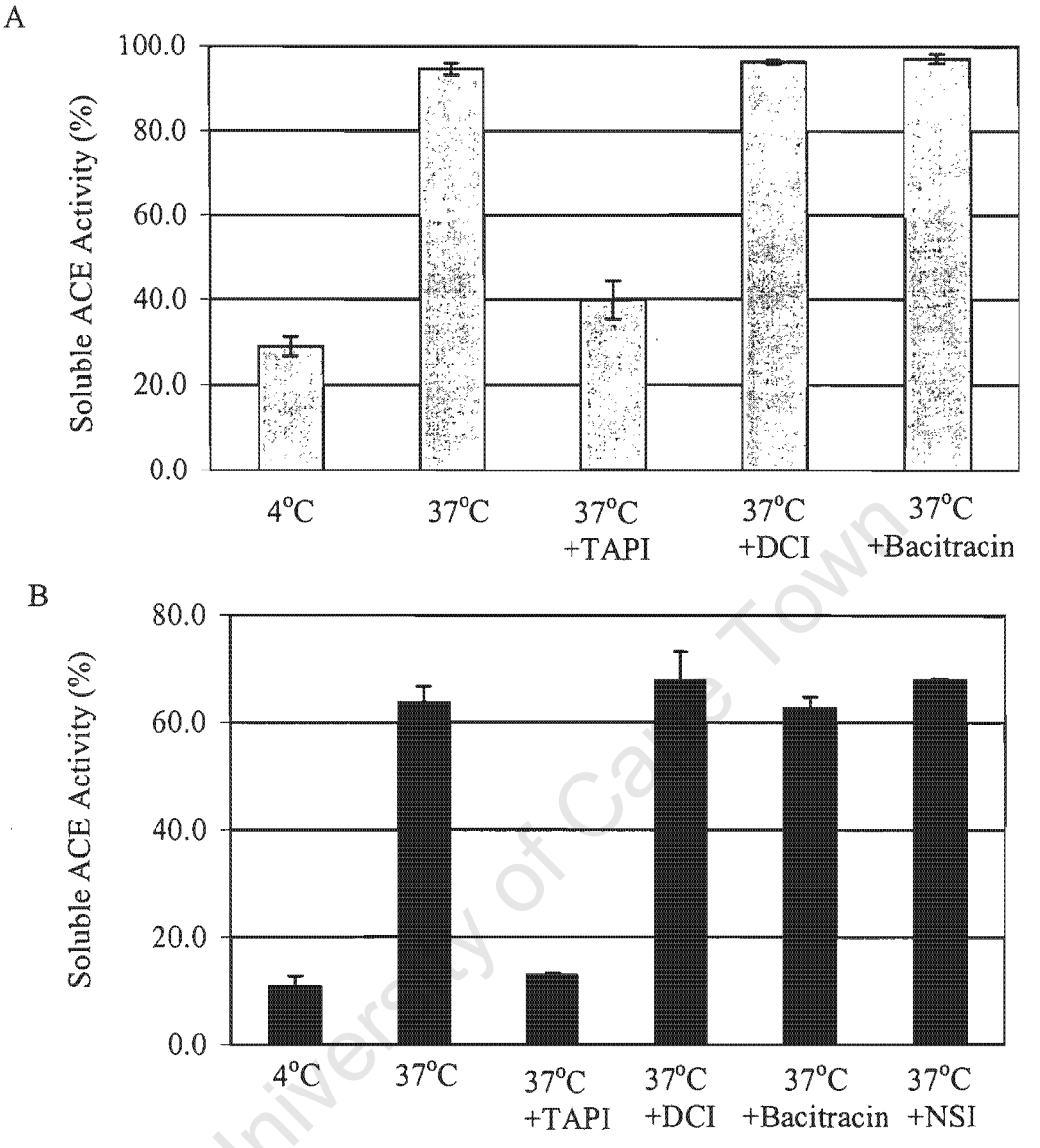


Figure 4.3.5 Effect of inhibitors on the shedding of testis ACE and somatic ACE from isolated CHO-KI cell membrane CHO-KI cell membranes isolated from cells expressing the human cDNA of testis ACE (A) (grey bars) and somatic ACE (B) (black bars) were purified using the Total Membrane Isolation Protocol II discussed in Chapter 3.2 and the membrane was incubated at 37°C in the absence and presence of TAPI (100 μM), 3,4-dichloroisocoumarin (DCI) (200 μM), bacitracin (3 mM) and non-specific serine protease inhibitors (NSI), aprotinin (3 μg/ml) and PMSF (1 mM), for 60 min before the soluble ACE was separated from the membrane-bound form by centrifugation (11, 000 x g) for 5 min. The soluble ACE activity is depicted as the percentage of ACE activity associated with the supernatant of the total ACE found in the membrane pellet and supernatant. Membrane was also incubated at 4 °C to determine basal shedding during the 60 min incubation. (n=3 +/-SD)

the observed mass of 1950.1. In Table 4.3.1, the peptide comprising the reported cleavage site at R1137 (Figure 4.3.7), from residue E1133 to K1143 is indicated with the expected *m/z* 1176.4 (Beldent et al, 1993). This peptide was identified in the total digest of somatic ACE with the observed *m/z* of 1175.6, indicating that proteolysis is unlikely to occur at R1137. Similarly, no peptides C-terminal to R1203 were identified indicating that cleavage at R1227 was also unlikely.

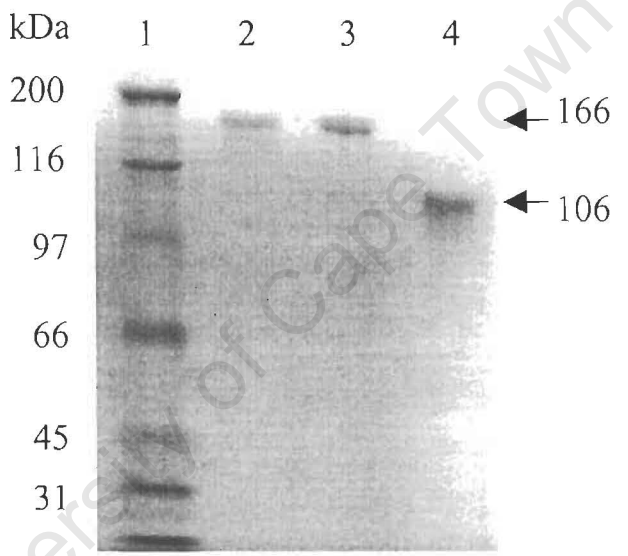


Figure 4.3.6 Purified somatic ACE resolved by SDS PAGE Somatic ACE was purified by affinity chromatography from the culture medium of CHO-KI cells (lane 3) and from human seminal plasma (lane 2), electrophoresed on a SDS gel, and stained with Coomassie Brilliant Blue. Similarly, testis ACE was also purified from the conditioned medium of CHO-KI cells and included in lane 4.

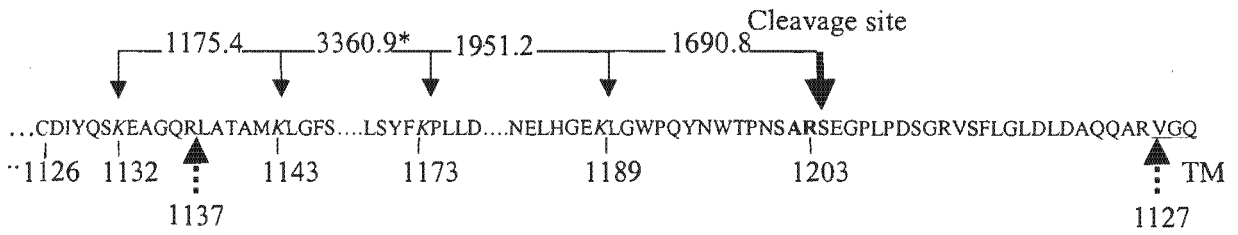


Figure 4.3.7 Protein sequence of the juxtamembrane region of somatic ACE The protein sequence depicts the region of somatic ACE proximal to the transmembrane (TM) domain (underlined), indicating with arrows (normal) the lysine residues, numbered according to somatic ACE sequence, at which endoproteinase Lys-C, cleaves and the calculated *m/z* of the peptides generated are shown above the sequence. The published sites of shedding of somatic ACE are shown with broken arrows and the thick arrow at R1203 indicates the cleavage site determined by this study.

To determine whether naturally occurring soluble somatic ACE was processed at the same site as that of the recombinant form, somatic ACE was also purified from human seminal plasma, a rich source unlike that of blood plasma. The seminal ACE had the same electrophoretic mobility as that of the somatic ACE purified from the culture medium of CHO-KI cells (Figure 4.3.6, lane 2). The purified protein was digested with endoproteinase Lys-C and the total digest was analysed by mass spectrometry. The penultimate peptide with the *m/z* of 1951.6 was identified but the expected C-terminal peptide with the *m/z* 1690.8 was not observed. Instead a new peak with the *m/z*, 1533.9 was obtained which corresponded to a C-terminal peptide ending at residue A1202. Once again no peptides ending in R1137 and R1227, the previously reported cleavage sites, were identified.

Table 4.3.1 Observed $[M+H]^+$ Ions of C-terminal Somatic ACE Peptides Generated by Endoproteinase Lys-C Digestion[a]

Peptide (residue no.)	expected m/z	observed m/z		
		hsACE	semACE	tACE
1133-1143	1176.4	1175.6	1175.5	1176.8
1144-1173	3360.9	glyc[b]	glyc[b]	glyc[b]
1174-1189	1951.2	1950.1	1951.6	1951.2
1190-1203	1690.8	1691.1	-	1691.4[c]
1190-1202	1533.7	-	1533.9	-

[a]Soluble (released) somatic ACE was purified from the conditioned medium of transfected CHO cells (recombinant human somatic ACE, hsACE) and from human seminal plasma (semACE). In each case, proteins were digested with endoproteinase Lys-C and the total digests were analysed directly by MALDI-TOF mass spectrometry. Only peptides identified in the C-terminal half of the protein up to and including the peptide 1133-1143, which contained a previously reported alternative cleavage site (Beldent et al, 1993), are shown (Figure 4.3.7). All values are calculated for protonated average molecular weight *m/z*. Peptides 1190-1203 and 1190-1202 were the only peptides identified not ending in a lysine residue; no peptides C-terminal to these peptides were found.

[b]Glycosylated peptide.

[c]C-terminal peptide of testis ACE determined previously (Ramachandran et al, 1994; Ehlers et al, 1996).

4.4 Discussion

The release of somatic ACE and testis ACE from transfected cell lines has previously been documented (Beldent et al, 1995; Ehlers et al, 1991b; Sen et al, 1991). Similarly, studies on the release of somatic ACE from membranes purified from tissues expressing ACE have also been published (Oppong and Hooper, 1993). The proteolytic release of somatic ACE from the membrane is responsible for the generation of the soluble form of somatic ACE found in bodily fluids. The shedding of testis ACE from spermatozoa is still unresolved although it has been shown that ACE activity has been detected in the medium of capacitated spermatozoa and this release is independent of the acrosome reaction (Kohn et al, 1995). These data provide evidence that somatic ACE and testis ACE, both type I transmembrane proteins, form part of the growing number of membrane proteins that are proteolytically released from the membrane by a group of proteases called secretases or sheddases (Ehlers and Riordan, 1991b; Hooper et al, 1997). A number of these proteases form part of the ADAMs family of proteases (Black and White, 1998). The shedding of somatic ACE and testis ACE is inhibited by the metalloprotease inhibitor, TAPI, suggesting that the ACE secretase is a member of the ADAMs family of proteases.

It has been reported that somatic ACE is cleaved 10-fold less efficiently than the C domain (essentially testis ACE) and we have corroborated this finding with the present study, confirming that somatic ACE is a poor substrate for the CHO-KI secretase (Beldent et al, 1995). This lower shedding efficiency of somatic ACE could be due to the N domain acting as a structural hindrance to the ACE secretase, thus causing the protease to seek an alternative less favourable site for cleavage (Beldent et al, 1995). They reported two cleavage sites at R1137 and R1227, 100 and 1 amino acid residues N-terminal to the transmembrane domain, respectively (Beldent et al, 1993). In this study, it has been shown that somatic ACE is cleaved at the same site as testis ACE, 24 amino acid residues away from the membrane after R1203.

The method that we have used has previously been documented and makes use of chemical or enzymatic hydrolysis of the purified protein, reverse phase HPLC

fractionation and identification of the C-terminal peptide by MALDI TOF mass spectrometry (Ehlers et al, 1996; Schwager et al 1998; Schwager et al 1999). It is possible that proteolysis occurs at the previously published sites (R1137 and R1227). However, the size of the soluble enzyme would be 7.5 kDa smaller than that generated by cleavage at R1203 and no such protein species is observed on the SDS PAGE gels or the autoradiograph shown in Figures 4.3.6 and 4.3.2.

In addition, the fragment of somatic ACE left attached to the membrane after proteolysis has been shown to be 6.9 kDa and this mass does not coincide with cleavage at R1137 (Parvathy et al, 1997). Finally, proteolysis 100 amino acid residues away from the membrane would lead to the inactivation of the C domain of somatic ACE as indicated by the inactivation of testis ACE with the deletion of 47 amino acid residues of the juxtamembrane region (Ehlers et al, 1996). Moreover, the three-dimensional structure of testis ACE confirmed that the ectodomain of the C domain extends to G615, 53 amino acid residues C-terminal to the cleavage site at R1137 (Natesh et al, 2003). Thus, cleavage at R1137 would disrupt the structural integrity of the ectodomain of the C domain. As mentioned before, the C domain constitutes 80-90% of the catalytic activity of somatic ACE. Similarly, endogenous soluble somatic ACE purified from seminal plasma is most likely also generated by cleavage after R1203, but that secondary *in vivo* carboxypeptidase processing results in the C-terminal amino acid residue, A1202. This secondary C-terminal processing was also found with APP (Esch et al, 1990).

Deletion of the N domain from somatic ACE resulted in a 10-fold increase in shedding. This led to the suggestion that the N domain was affecting the accessibility of favourable cleavage sites (Beldent et al, 1985). We have shown that the lowered efficiency of shedding of somatic ACE is not due to proteolysis at unfavourable cleavage sites. However, the presence of the N domain, although not implicated in structurally constraining the ACE secretase to cleave at alternative sites, could still interfere with the shedding of somatic ACE. When the N domain was directly fused to the stalk of ACE (the C domain deleted), the resultant construct was not shed (Pang et al, 2001). This led to the proposal that the N domain, which has 60% sequence identity with the C domain, lacked a recognition motif for the ACE secretase. The concept of a recognition motif for the ACE secretase is not a new one. Sadhukhan et

al showed that the ectodomain of the C domain of ACE could direct the shedding of CD4 from the membrane even though this protein was not normally shed (Sadhukhan et al, 1998). Thus, it is possible that the presence of the N domain in somatic ACE, which lacks the secretase recognition motif, acts as an "inhibitor" of shedding, allowing somatic ACE to remain membrane-bound to carry out its physiological function of blood pressure maintenance.

To date, the physiological significance of the ectodomain shedding of ACE remains unknown. The maintenance of blood pressure homeostasis and kidney function can be attributed to the membrane-bound form of somatic ACE (Esther et al, 1997). Levels of somatic ACE in the blood plasma have been reported to be very low and although seminal plasma is rich in ACE activity the pathology of $ACE^{-/-}$ mice made no mention of prostate dysfunction and abnormalities (Cushman et al, 1971; Krege et al, 1995; Wei et al, 1991b). Thus, the high levels of seminal ACE generated by the prostate, is unlikely to be of functional significance. In addition, mice devoid of endothelial somatic ACE have normal blood pressure maintenance and renal function compared to wild type mice (Cole et al, 2002). Moreover, enough ACE was also shown to be present in the lung to convert blood angiotensin I to angiotensin II in a single transit (Ng et al, 1967). Thus, the production of circulating somatic ACE does not seem important if the passage of blood plasma through an ACE-bearing organ is sufficient for normal blood pressure regulation in healthy subjects. It is intriguing to speculate that the physiological significance of the shedding of ACE lies with that of the testis isoform and that the proteolytic processing of the somatic isozyme is too inefficient to play any significant role in the physiological function of this ectoprotein.

Testis ACE on the other hand is shed more efficiently from the membrane due to the presence of the secretase recognition domain and the lack of the N domain. It has been hypothesised that testis ACE is vital to male fertility because it allows for the binding of spermatozoa to the oviductal epithelium and oocyte (Hagaman et al, 1998). The binding to the oviductal epithelium could aid the survival of fertility-competent sperm and its release and continued movement through the female reproductive tract could be aided by the rapid shedding of ACE from the plasma membrane upon capacitation of the spermatozoa. This is, of course, pure speculation, but this could explain why testis ACE is shed more efficiently than its somatic counterpart.

In conclusion, we have shown that recombinant and naturally occurring somatic ACE are cleaved at the identical scissile bond as testis ACE, and that this cleavage site, 24 amino acid residues away from the membrane does not render the ACE molecule inactive. This hydrolysis by the ACE secretase therefore, does not occur less efficiently in the somatic isoform due to alternate, less favourable cleavage sites. The presence of the N domain, which likely lacks its own secretase recognition motif, could act as a steric hindrance, making access to the stalk difficult, or could occlude the recognition motif in the C domain as a way of inhibiting the rapid shedding of this molecule from the membrane.

Chapter 5

The presence of an additional C domain affects the shedding of testis ACE

5.1 Introduction

The two isoforms of ACE have different rates of shedding from the membrane even though they have identical cleavage sites as investigated and discussed in Chapter 4. Somatic ACE comprises two domains, the N- and C-terminal domains and these have 60% sequence identity. Testis ACE is identical to the C domain except for the 36 amino acid residues at the N-terminus (Ehlers et al. 1989). This region does not influence the shedding of testis ACE from the membrane (Schwager et al, 1999). However, once the N domain was deleted from somatic ACE, the shedding of the truncated C domain increased 10-fold (Beldent et al, 1995). It seems likely, therefore, that the N domain may affect the shedding of somatic ACE.

There is evidence that the N domain is more stable than the C domain. Voronov et al, showed that thermal denaturation of bovine somatic ACE at 55°C inactivated the C domain while the N domain remained active (Voronov et al, 2002). The differential thermal denaturation of the two domains within the same protein suggests independent folding of the two domains of ACE. The stability of the N domain is likely derived from its higher proline and α-helix content. The N domain also has a greater number of potential *N*-glycosylation sites that may act as a protective barrier to proteases and a structural stabiliser, preventing thermal denaturation (Voronov et al, 2002). A naturally occurring N domain has been identified in the ileal fluid of patients undergoing surgery and this is thought to be the product of proteolytic processing within the bridge region (Deddish et al, 1994). Thus far, no endogenous C domain generated by the limited proteolysis of somatic ACE, has been identified. This suggests that the N domain is less susceptible to proteolysis than the C domain. However, testis ACE is not readily degraded by proteases. This is demonstrated by its release from the membrane by trypsin without complete degradation and loss of

enzyme activity (Ehlers et al, 1991b). Testis ACE does have a unique *O*-glycosylated region that could play a role in its stability (Ehlers et al, 1992).

These data infer that the N domain has properties that differ from those of the C domain and confer stability to the parent molecule, somatic ACE. The two domains are linked by a stretch of 14 amino acid residues termed the bridge region and proteolysis can occur within this region after treatment with endoproteinase Asp-N (Sturrock et al, 1997). The predicted structure of the bridge region suggests high surface probability thus, it is possible that the N domain folds around the C domain, protecting it from proteolysis while exposing the bridge sequence at the surface. This is corroborated by the fact that antibodies raised against somatic ACE are more specific for epitopes of the N domain than those of the C domain (Danilov et al, 1997)

The shedding of membrane proteins depends on an accessible stalk region of certain length and a recognition motif for the ACE secretase (Ehlers et al, 1996; Sadhukhan et al, 1998). It has been proposed that the N domain lacks a recognition motif for the ACE secretase (Pang et al, 2000). The differences in shedding of the two isoforms of ACE could be attributed to the structural arrangement of the two domains in native somatic ACE: the N domain could occlude an ACE secretase recognition motif in the C domain and/or the N domain sterically interferes with the accessibility of the stalk region. Both hypotheses infer that the presence of an additional domain interferes with the proteolytic processing of ACE. To further investigate the role or significance of the N domain in the processing of ACE, the N domain of somatic ACE was replaced with a second C domain. This construct was expressed in CHO-KI cells and its shedding investigated.

5.2 Methods

5.2.1 **Construction of the 2C-domain ACE mutant**

To determine whether the presence of the C domain of somatic ACE would affect the shedding of testis ACE, two C domains were linked together with the sequence of the bridge region of somatic ACE. The bridge region connecting the two domains of somatic ACE was amplified from nt 1918 to nt 2335 using a forward primer that introduced an *Eco* RI site (nt 1912) immediately upstream to the bridge region and a reverse primer that introduced a *Cla* I site at its 5' extension (Figure 5.2.1). To introduce the *Eco* RI site in frame, the amino acid residues, P602 and L603 could not be included in the amplification of the bridge region. This PCR product (421 bp) was digested with *Eco* RI and *Cla* I and ligated to similarly digested pBS and sequenced using the ALFexpress DNA Automated Sequencer (APBiotech).

Once the correct sequence was confirmed, the PCR product was excised from pBS by digesting with *Eco* RI and *Cla* I and inserted into the similarly digested ACE construct, pBS-ΔR627 (Figure 5.2.2) (Ehlers et al, 1996). This ACE mutant has R627 deleted, which occurs within the stalk region of ACE. For the correct construction of this deletion mutant, an *Eco* RI site was introduced at nt 1984 by silent mutation. The presence of this *Eco* RI site allowed the fusion of the PCR product to the C-terminus of testis ACE (Figure 5.2.1). This construct, intermediate 1, was then digested with *Bgl* II to excise the region between the two *Bgl* II sites and ligated to the similarly digested testis ACE construct, pBS-Δ36N (Figure 5.2.2). This construct was used as it lacked the *O*-glycosylated, 36 amino acid residue-N-terminal region of testis ACE and thus was identical to the C domain of somatic ACE. All DNA manipulations were carried out in pBS as the restriction enzyme sites used for the cloning procedure occurred within the vector sequence of pLEN. To subclone the 2C-domain ACE construct into the mammalian expression vector, pLEN, intermediate 2 (Figure 5.2.1) was digested with *Bam* HI and *Cla* I and ligated to the similarly digested pLEN-tACE (Appendix I.2). All the DNA manipulations made use of the molecular cloning methods outlined in the methods section in Chapter 2. Digestion of the final DNA preparation with *Bam* HI, *Cla* I and *Eco* RI confirmed the correct construction of pLEN-2C-domACE (Appendix II.2).

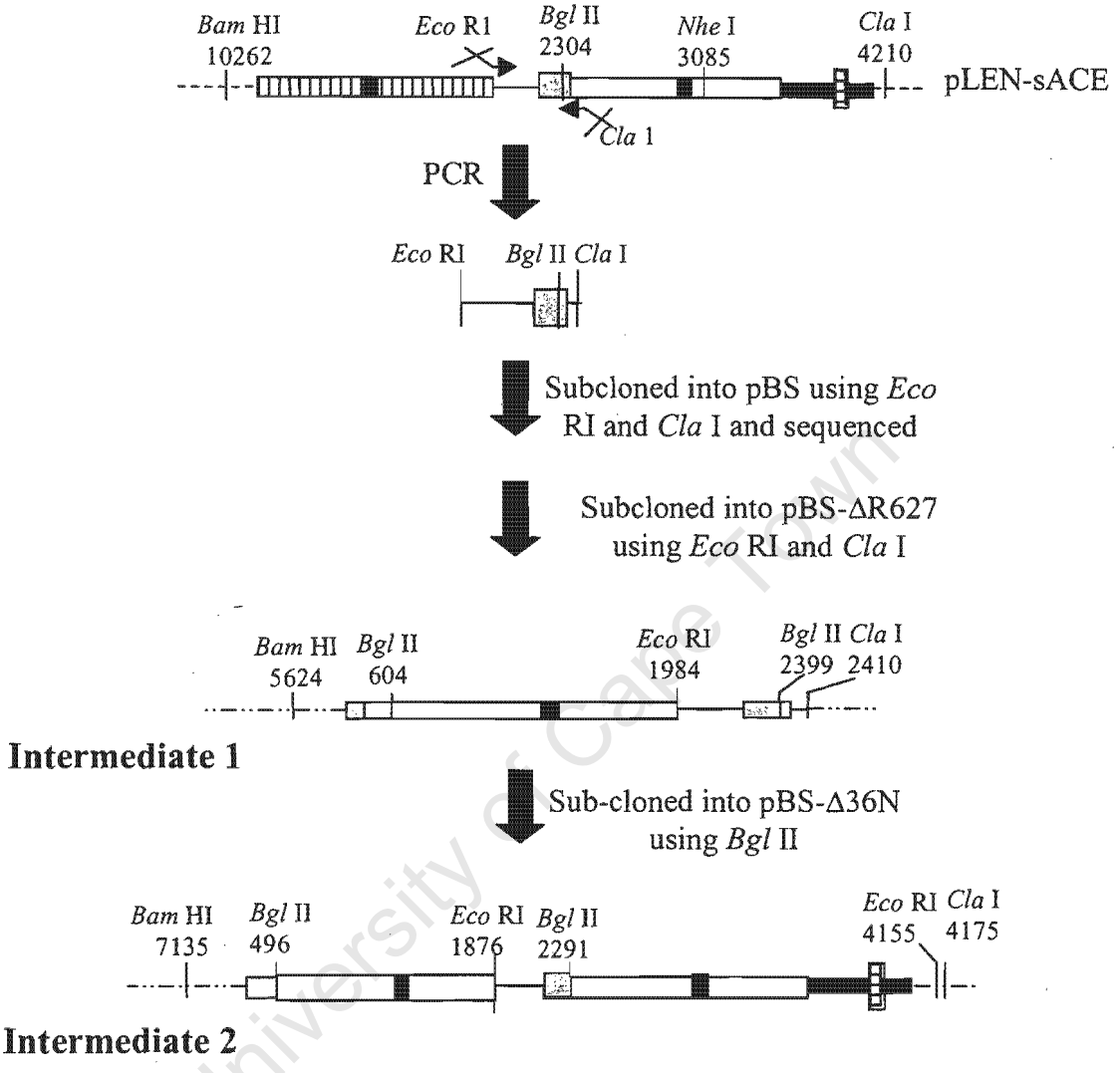


Figure 5.2.1 Construction of pLEN-2CdomACE The schematic representation of somatic ACE is depicted above with the N domain (striped bar), C domain (open bar), active site (black box), stalk and cytoplasmic tail (black bar) and the transmembrane region (hatched box) indicated. The bridge region, including the N-terminus of the C domain (up to nt 2335) of somatic ACE (pLEN-sACE) was amplified and the PCR product was subcloned into the ACE construct, pBS-ΔR627 (Figure 5.2.2), using *Eco* RI and *Cla* I after subcloning into pBS using the same sites and sequencing the PCR product. This construct, Intermediate 1, was digested with *Bgl* II and the 1795 bp DNA fragment was ligated to the similarly digested ACE construct, pBS-Δ36N (Figure 5.2.2), to construct Intermediate 2. This intermediate was sub-cloned into pLEN, by digesting with *Bam* HI and *Cla* I and ligating to the similarly digested pLEN-tACE (Appendix I.2) to construct pLEN-2CdomACE. All nt numbering is as determined by Ehlers et al, 1989 and Soubrier at al, 1988.

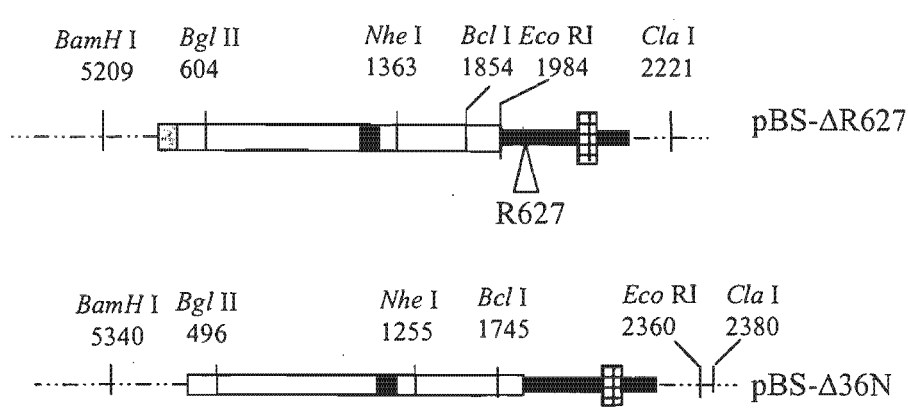


Figure 5.2.2 ACE constructs used for sub-cloning of pLEN-2C-domACE
The schematic representation of pBS-ΔR627 (A) and pBS-Δ36N (B) is indicated with the restriction enzyme sites and their positions (nt) on the ACE cDNA sequence shown above. The ectodomain of testis ACE is indicated by the open bar, the active site is represented by the black box, the stalk region and cytoplasmic tail are shown by the black bar separated from one another by the transmembrane domain depicted by the hatched box. Both ACE constructs have been cloned into pBS using *Bam* HI and *Cla* I at the 5′- and 3′- end of the cDNA, respectively. The differences between these two constructs and testis ACE is that pBS-ΔR627 has R627 deleted and an introduced *Eco* RI site at nt 1984 and pBS-Δ36N lacks the *O*-glycosylated, 36 amino acid residue region at the N-terminus of testis ACE (grey box). All nt numbering is as determined by Ehlers et al, 1989.

5.2.2 **Expression and kinetics of solubilisation of 2C-domACE**

Once the cDNA of 2C-domACE was correctly constructed and cloned into the mammalian expression vector, pLEN, it was stably transfected into CHO-KI cells. The shedding of this construct was investigated by western blot analysis and the detection of ACE activity in the culture medium, using the ACE substrate, HHL. In addition the ACE construct was purified from the culture medium and the cleavage site was determined by MALDO-TOF mass spectrometry. All methods used are outlined in the methods section in Chapter 2.

5.3 Results

5.3.1 **Shedding of 2C-domACE**

5.3.1.1 **Release of 2C-domACE from intact CHO-KI cells**

CHO-KI cells expressing the recombinant 2C-domACE protein, were grown in 35-mm dishes until confluent and then ACE expression was induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM). Fresh 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM) was added either with or without PDBu and the cells were cultured for a further 8 hours. Medium was removed and the cells were lysed using 1% Triton X-100 lysis buffer. ACE activity was determined for both the medium and the cell lysate samples at the indicated time points (Figure 5.3.1).

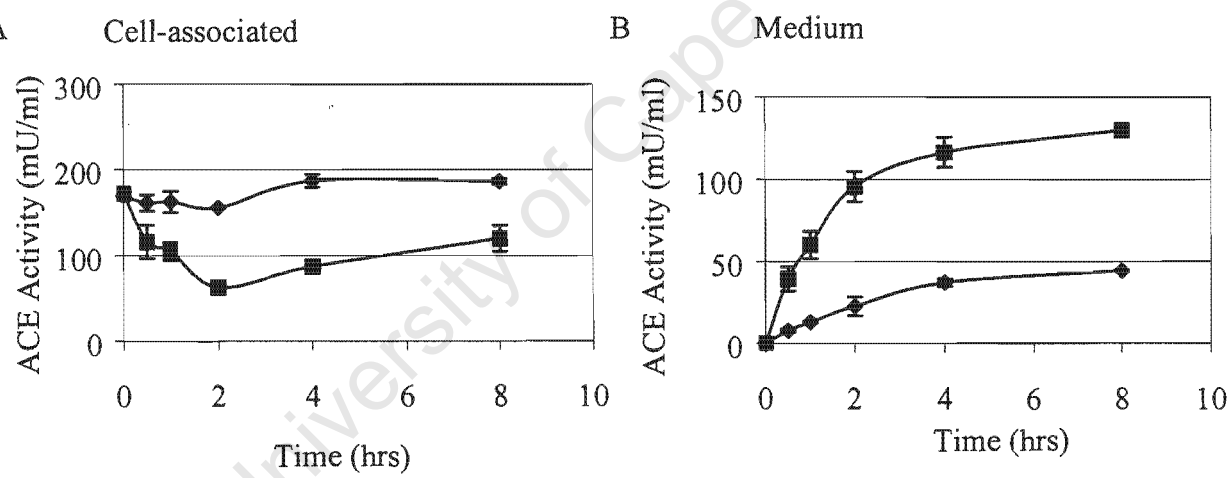


Figure 5.3.1 Shedding of 2C-domACE from intact CHO-KI cells CHO-KI cells stably transfected with the cDNA of 2C-domACE were grown to confluence and induced O/N with 2% FCS conditioned medium supplemented with $ZnCl_2$ (40 μM) before the addition of fresh medium either with (black squares) or without (black diamonds) PDBu. The culture medium was removed and the cells were lysed with 1%Triton X-100 lysis buffer at the times indicated before ACE activity was determined for the cell lysates (A) and culture medium (B) using the ACE substrate, HHL. (n=3 +/- SD)

There was an increase of ACE activity in the culture medium over 8 hrs, indicating that 2C-domACE was shed into the medium. In the presence of PDBu there was a 6-fold increase in shedding with a concomitant decrease in cell associated ACE activity relating to the rapid removal of membrane-bound 2C-domACE from the plasma membrane. To compare the shedding of this construct with wild type testis ACE, the ACE activity in the medium (mU/ml) was converted to the percentage of total ACE activity associated with the cell lysates and medium (Figure 5.3.2). 2C-domACE was shed as efficiently as wild type testis ACE suggesting that the presence of an additional C domain does not hinder the proteolysis of testis ACE.

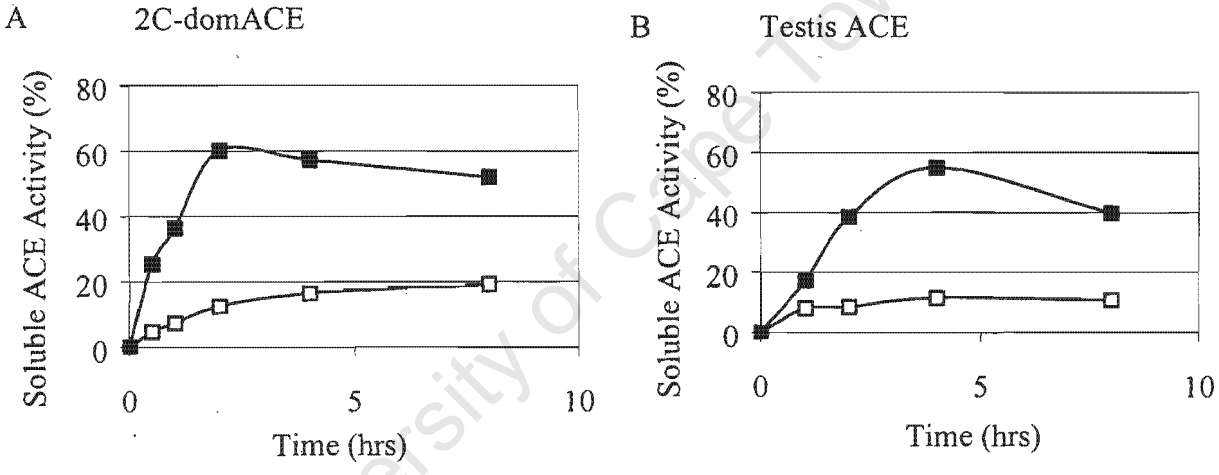


Figure 5.3.2 Generation of soluble 2C-domACE (%) from intact CHO-KI cells
CHO-KI cells stably transfected with either the cDNA of 2C-domACE (A) or testis ACE (B) were grown to confluence in a 6 well plate and induced O/N with 2% FCS conditioned medium supplemented with $ZnCl_2$ (40 μM) before the addition of fresh medium either with (black squares) or without (open squares) PDBu. The culture medium was removed and the cells were lysed with 1%Triton X-100 lysis buffer at the times indicated before ACE activity was determined for the cell lysates and culture medium using the ACE substrate, HHL. The level of soluble ACE activity is shown as the average ACE activity in the culture medium as a percentage of the total ACE present in each well determined for Figure 5.3.1. (n=3)

5.3.1.2 **Release of 2C-domACE in the presence of inhibitors**

To determine whether the release of 2C-domACE from CHO-KI cells was inhibited by the metalloprotease inhibitor, TAPI, CHO-KI cells stably expressing the protein were grown in the presence of TAPI for 4 hours. ACE activity was determined for both the culture medium and the cell lysate (Figure 5.3.3). The shedding of the recombinant protein was specifically inhibited by TAPI and stimulated in the presence of PDBu, comparable to testis ACE. This confirmed that the proteolysis of this ACE construct was due to the action of a metalloprotease.

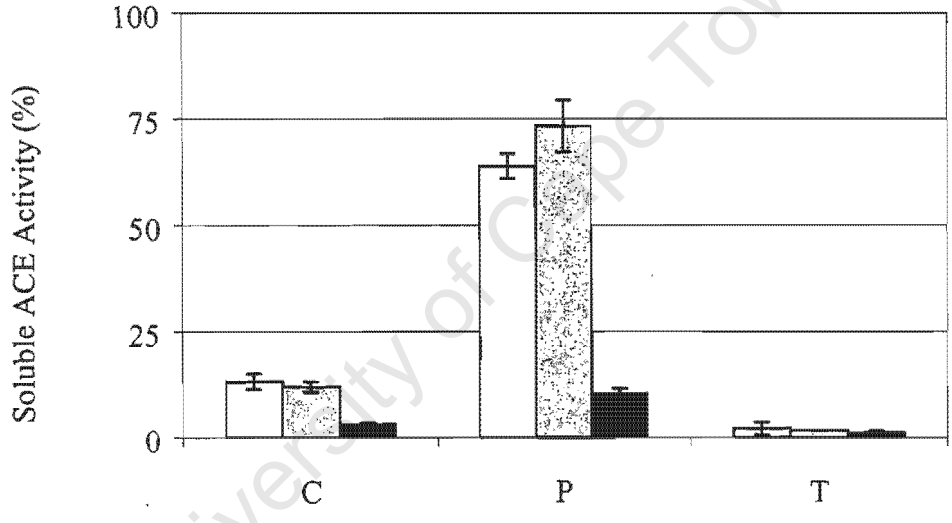


Figure 5.3.3 Upregulation and inhibition of shedding CHO-KI cells expressing 2C-domACE (open bars), testis ACE (grey bars) and somatic ACE (black bars) were grown in the absence (C) or presence of PDBu (1μM) (P) or TAPI (100 μM) (T) for 4 hrs at 37°C before ACE activity was determined for the cell lysates and culture medium using the ACE substrate, HHL. The level of soluble ACE activity is shown as the ACE activity in the culture medium as a percentage of the total ACE present in each well. (n=3 +/- SD)

5.3.1.3 **Western blot analysis of 2C-domACE**

To further investigate the upregulation and inhibition of the proteolytic products of 2C-domACE, the culture medium and cell lysates obtained from CHO-KI cells and grown in the absence and presence of either PDBu or TAPI, were resolved by SDS PAGE (Figure 5.3.4). Western blot analysis of 2C-domACE demonstrated two proteolytic products in the medium and cell lysate samples: an upper band of ~170 kDa, corresponding to somatic ACE, and surprisingly, a lower band of ~80 kDa (Figure 5.3.4). The appearance of the lower band, which corresponded to testis ACE, indicated that 2C-dom ACE was cleaved to generate two products: full length soluble 2C-domACE and soluble C domain. The lower band was smaller than the testis ACE protein (wt), which has an intact *O*-glycosylated N-terminal region, contrary to the two C domains of 2C-domACE. This would contribute to the observed size difference between the lower band of 2C-domACE and testis ACE. Thus, cleavage seems to be occurring not only within the stalk region, but also within, or close to, the bridge region of 2C-domACE.

These two cleavage events of 2C-domACE were upregulated in the presence of PDBu as indicated by the increased band intensity of both proteolytic products in the medium samples (Figure 5.3.4 A) and the concomitant decrease in cell-associated protein (Figure 5.3.4 B). The cell-associated, smaller proteolytic product disappeared upon addition of PDBu. This suggested that both C domains of 2C-domACE, distal and proximal to the TM, were shed into the medium. In addition, proteolysis was also inhibited by TAPI as indicated by the decreased band intensity of both cleavage products in the medium (Figure 5.3.4 A) and the concomitant increased band intensity visible in the cell lysate samples (Figure 5.3.4 B).

To compare the shedding of 2C-domACE with that of somatic and testis ACE, CHO-KI cells transfected with the cDNA of the two isoforms were treated similarly and western blot analysis was carried out on the samples obtained. Both isozymes were inhibited by TAPI, as indicated by the decrease in band intensity of the media samples, and shedding was upregulated in the presence of PDBu (Figure 5.3.4 II and III). The upregulation of shedding of somatic ACE was not as marked as that of

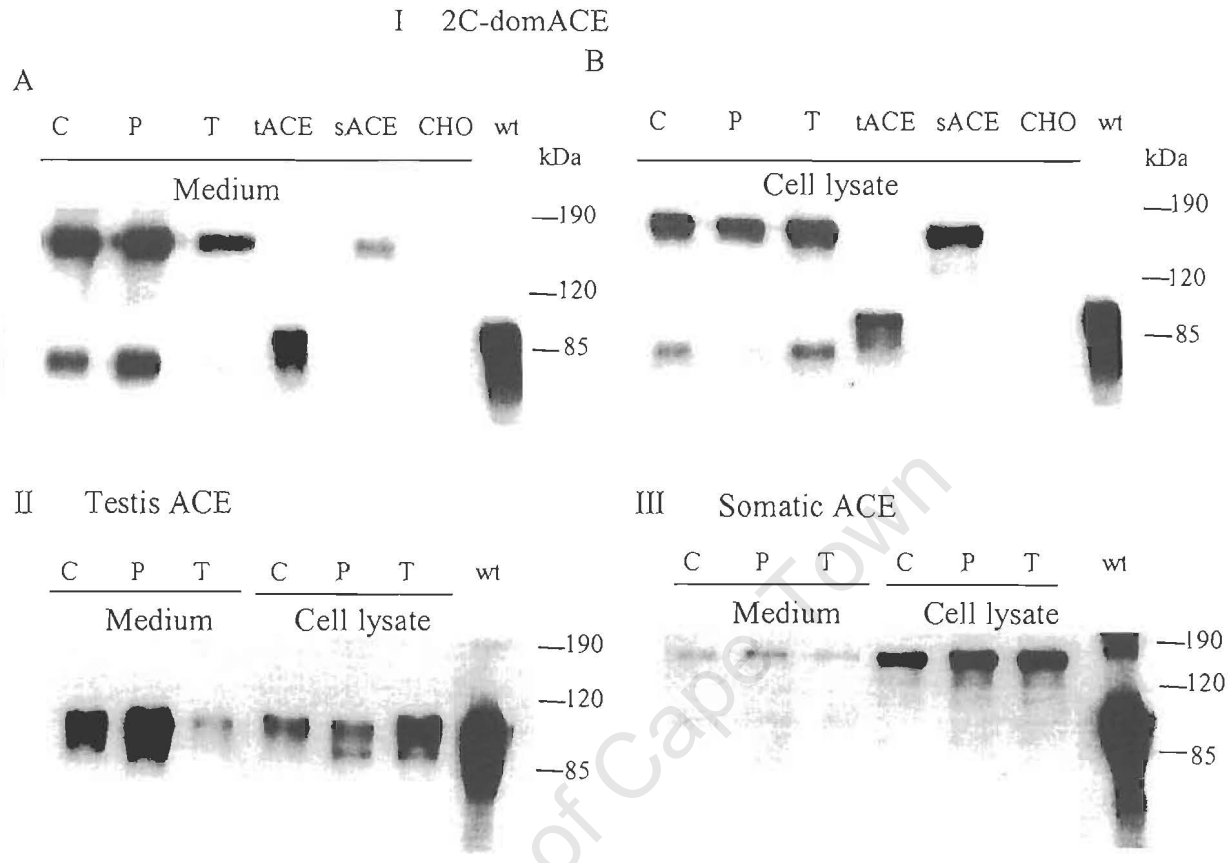


Figure 5.3.4 Western Blot analysis of 2C-domACE, somatic ACE and testis ACE CHO-KI cells stably expressing either 2C-domACE (I), testis ACE (II) or somatic ACE (III) were grown to confluence in 35 mm dishes and grown for a further 4 hrs in the absence (C) or presence of PDBu (P) (1 μM) or TAPI (T) (100 μM). Aliquots of culture medium and cell lysates were removed and proteins were separated by SDS PAGE on a 7.5% acrylamide gel before western blot analysis with a testis ACE antibody. Aliquots of medium and cell lysate samples of CHO-KI cells expressing testis ACE (tACE) and somatic ACE (sACE) and untransfected CHO-KI cells (CHO) were included in Figures IA and IB and purified soluble recombinant testis ACE (wt) was included on all western blots as indicated.

testis ACE as expected from the results discussed in Chapter 4. There was no secondary cleavage product visible in the media samples of somatic ACE. Thus somatic ACE was cleaved within the stalk region only and not within the bridge region.

5.3.2 **Cleavage site determination**

To determine the peptide bond where 2C-domACE was being cleaved, soluble protein was purified by affinity chromatography from the culture medium of 2C-domACE-CHO cells and analysed using SDS PAGE (Figure 5.3.5). Two populations of soluble 2C-domACE were obtained: A, full length 2C-domACE comprising both C domains (~170 kDA) and B, single C domains (~80 kDA). The two protein fractions, A and B were eluted from the gel, digested with endoproteinase Lys-C, and the $[M+H]^+$ ions were determined by MALDI-TOF mass spectrometry (Table 5.3.1).

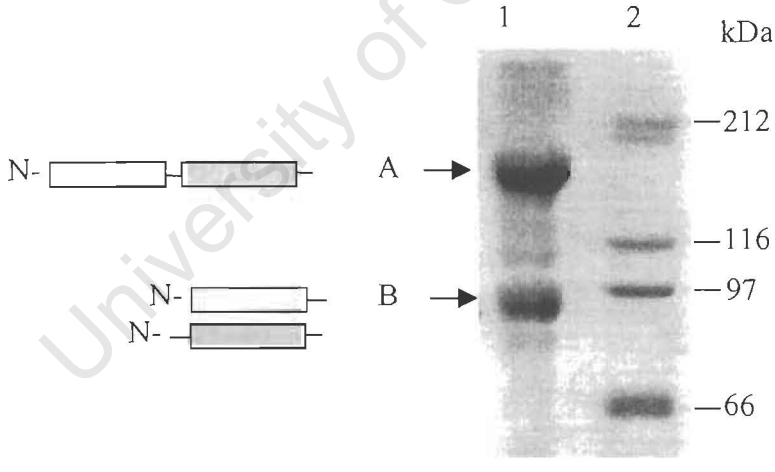


Figure 5.3.5 SDS PAGE of purified soluble 2C-domACE The purified protein was resolved on a 10% acrylamide gel and stained with coomassie blue (lane 1). Two protein bands were observed, A, representing full-length soluble 2C-domACE (170 kDa) and B, comprising the cleaved soluble C domains (80 kDa). The C domain proximal to the TM is depicted as a grey bar and the open bar represents the distal C domain. MW weight marker was included in lane 2.

The intact bridge peptide from L578 to K608 with a *m/z* of 3508.4 was identified in the peptides obtained from the eluted protein from band A (Figure 5.3.6). This suggested that fraction A comprised full-length 2C-domACE with an intact bridge region. However, the bridge peptide was not found in the peptides derived from the eluted protein of fraction B, suggesting that the bridge peptide had been cleaved into two peptides. The protein eluted from band A and band B were subjected to N-terminal sequencing to determine after which amino acid residue cleavage occurred within the bridge region. No N-terminal sequence was obtained from the protein eluted from band A suggesting that the N-terminus of full-length, soluble 2C-domACE was blocked. Two sequences were obtained from the protein from band B; 1) a major sequence, GIDVLT and, 2) a minor sequence, NYPEG. This suggested that cleavage occurred predominantly after E595 within the bridge region, and to a lesser extent after D591 (Figure 5.3.6). Alternatively, cleavage occurred after D591 and secondary N-terminal processing resulted in a N-terminal amino acid residue of G596.

Table 5.3.1 Observed $[M+H]^+$ Ions of the peptides generated by endoproteinase Lys-C digestion of soluble 2Cdom-ACE purified by affinity chromatography

Peptide residues	Calculated *m/z*	Observed *m/z*
578-608	3508.7	3508.4[a]
1176-1189	1690.8	1690.6[b]

[a] The intact bridge peptide.
[b] The C-terminal stalk peptide

Peptides with *m/z* of 1690.8 were identified in the Lys-C digest of fractions A and B (Table 5.3.1). These correspond to the C-terminal peptide, L614 to R627, of wild type testis ACE. This indicated that juxtamembrane cleavage of 2C-domACE was occurring at the same cleavage site as testis ACE within the stalk at the R1189-S1190 bond (Figure 5.3.6). The presence of the C-terminal stalk peptide in fraction B indicated that fraction B comprised soluble, TM-proximal C domain in addition to the soluble, distal C domain.

The mass spectrometry and sequencing results indicated that 2C-domACE was proteolytically processed in two ways: (1) Sequentially, first in the bridge region after E595 and/or D591 and then in the stalk region after R1189 to produce two separate soluble C domains; and (2) only within the stalk region after R1189 to generate full-length soluble 2C-domACE.

Figure 5.3.6 Sequence of 2C-domACE 2C-domACE was constructed from an ACE mutant, Δ36N, which lacked 36 amino acid residues at the N-terminus. Thus the numbering of 2C-domACE differs from that of testis ACE (Ehlers et al, 1989) resulting in L37 becoming L1. The sequence above indicates 2C-domACE from L1 to Q1216 with the start of the TM underlined. The relevant lysine residues are indicated with their amino acid numbering below and two peptides generated by Lys-C digestion are shown with the expected *m/z* indicated above: the intact bridge peptide with a *m/z* of 3508.7 and the C-terminal peptide generated by cleavage within the stalk with a m/z of 1690.8 The major cleavage sites are indicated with bold arrows and the minor site is shown by the broken arrow. The sequence of the bridge region is shown in bold.

5.4 Discussion

Somatic and testis ACE are shed from the membranes of CHO-KI cells. Testis ACE cleavage occurs within the stalk region after R627, 24 amino acid residues away from the membrane. Somatic ACE is hydrolysed at the same site (Chapter 4). The shedding of somatic ACE however occurs less efficiently than its testis counterpart. The increased shedding of the truncated C domain ACE mutant lacking the N domain suggested that the N domain sterically obstructs the ACE secretase access to the stalk region (Beldent et al, 1995). Alternatively, it has been proposed that the C domain comprises a recognition motif for the ACE secretase (Sadukhan et al, 1998). Thus, the N domain, which lacks its own recognition motif, could occlude the recognition motif of the C domain and thus down-regulate the shedding of somatic ACE (Pang et al, 2001).

To determine whether the presence of an additional domain offers steric hindrance to the shedding of the C domain, two C domains were fused together using the bridge region of somatic ACE. This ACE construct, called 2C-domACE was shed efficiently from the membrane, suggesting that the presence of the N domain does not sterically interfere with the ACE secretase. It is possible that the native conformation of somatic ACE is not mimicked in the 2C-domACE construct. However, there is evidence for independent folding of the two domains, and moreover, they exhibit similar catalytic properties in the context of native somatic ACE compared to when separated from one another (Voronov et al, 2002, Esther et al, 1997; Wei et al, 1991a). Thus, it would appear that steric hindrance created by the N domain does not play a role in the low efficiency of shedding of somatic ACE. This leaves the alternative hypothesis that the N domain either hinders access of the ACE secretase to the recognition motif or occludes the recognition motif present in the C domain of somatic ACE.

Based on the presence of a recognition motif for the ACE secretase, the shedding of 2C-dom ACE could have two explanations. Firstly, it could be characterised by two steps: (1) binding of the ACE secretase to the recognition motif and (2) cleavage of the stalk region (Figure 5.4.1). This hypothesis would correlate well with the results obtained for the shedding of somatic ACE, as the ACE secretase would bind to the

recognition motif of the C domain, proteolysis would occur within the stalk region, and full-length, soluble somatic ACE would be released.

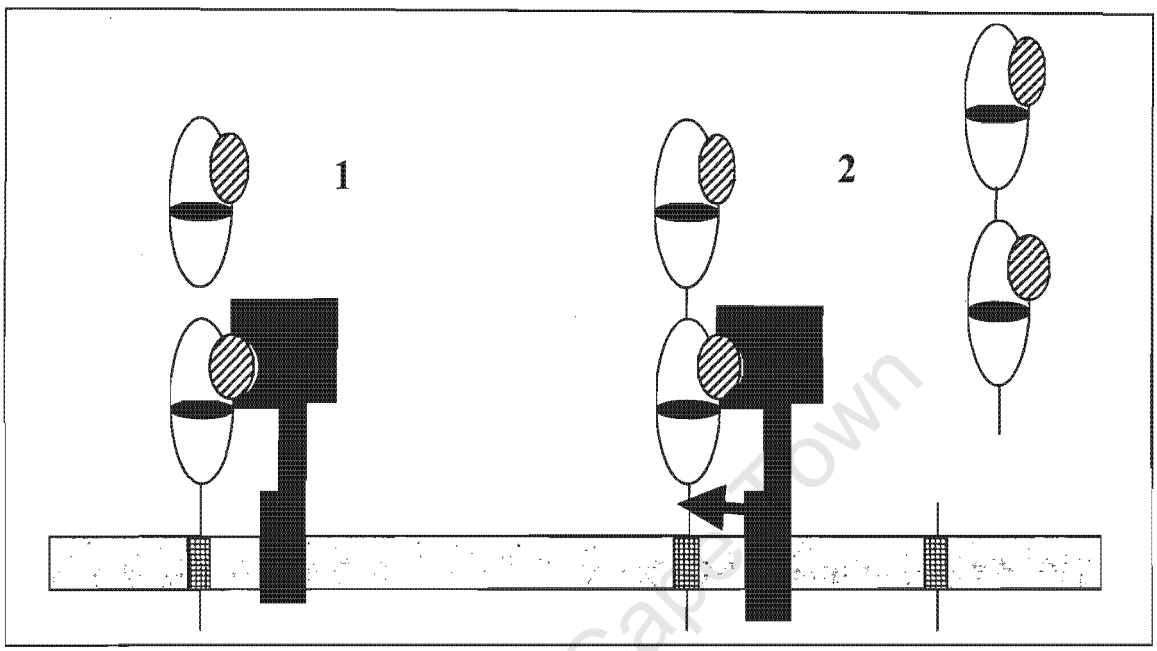


Figure 5.4.1 Schematic representation of the shedding mechanism of 2C-domACE The ACE construct, 2C-domACE comprises 2 C domains (depicted as 2 open oval shapes) each with its own active site (black oval) and recognition motif (diagonally striped oval) and associated with the membrane (grey bar) via its TM (hatched box). The ACE secretase (depicted as the black shape) binds to the recognition motif in step one and cleaves the stalk in the second step, to release the full-length soluble form of 2C-domACE.

However, the results obtained by western blot analysis also indicated that 2C-domACE was shed not only within the stalk region to release full-length soluble 2C-domACE, but also within the bridge region, whereas somatic ACE was only cleaved within the stalk region. It was shown that the endoproteinase Asp-N cleaved somatic ACE within the bridge region between T615 and D616 and that this stretch of amino acid residues was more susceptible to proteolysis than the rest of the protein (Sturrock et al, 1997). The ACE secretase, however, did not cleave somatic ACE within this interdomain region (Figure 5.3.4) and this could be because the N domain lacks the recognition motif needed for orientation. Earlier work suggests that the ACE secretase positions itself with respect to the extracellular domain, proximal to the stalk region (Ehlers et al, 1996). It is possible as shown in Figure 5.4.1, that the ACE secretase

orientates itself upon binding to the recognition motif and that this orientation allows the alignment of the active site of the ACE secretase with peptide sequence susceptible to proteolysis. Thus, if the N domain lacks this orientation motif, the bridge region of somatic ACE cannot be hydrolysed. Therefore, the second explanation of the shedding of 2C-domACE proposes that the TM-distal (outer) C domain comprises a recognition domain, to which the ACE secretase can bind. This would allow alignment between the ACE secretase active site and the bridge region facilitating proteolysis within this accessible region (Figure 5.4.2). Western blot analysis indicated that the remaining cell-associated protein fragment corresponded to the TM-proximal (inner) C domain. Thus, a second cleavage event can occur whereby orientation occurs via the inner C domain prior to cleavage within the stalk region to release the inner C domain (Figure 5.4.2). This sequential mechanism of proteolysis is corroborated by the data obtained by mass spectrometry.

In Figure 5.4.2, the depiction of the orientation of the two C domains and the bridge region was chosen to simplify the explanation of the shedding mechanism and does not aim to depict the actual three-dimensional orientation of the domains of 2C-domACE. The actual orientation of the domains of somatic ACE is unknown although it is thought that the N domain is folded around the C domain as antibodies raised against somatic ACE resulted in the recognition of epitopes mainly derived from the N domain and not the C domain (Danilov et al, 1994). Moreover, the predicted secondary structure of the bridge sequence suggested that this region has high surface probability and flexibility (Sturrock et al, 1997). Thus, although Figure 5.4.2 might not be an accurate representation of the three-dimensional interaction of the two domains, it is highly likely that the outer C domain occurs on the outside of the protein structure and exposes the bridge region at the surface.

The hypothesis of the four-step proteolysis of 2C-domACE is based on the fact that the ACE secretase has an absolute requirement for the membrane association of its substrate (Ehlers et al, 1996; Parvathy et al, 1997). Thus, it is highly unlikely that proteolysis occurs first within the stalk region to release the full-length soluble protein followed by hydrolysis of the bridge region to separate the two C domains. It is possible that binding to the recognition motif of the inner C domain allows the ACE secretase to remain bound to the protein long enough to cleave first within the stalk,

and then re-orientates itself to cleave within the bridge region. However, this would suggest that one recognition motif within the inner domain could allow for cleavage within both the stalk and bridge region.

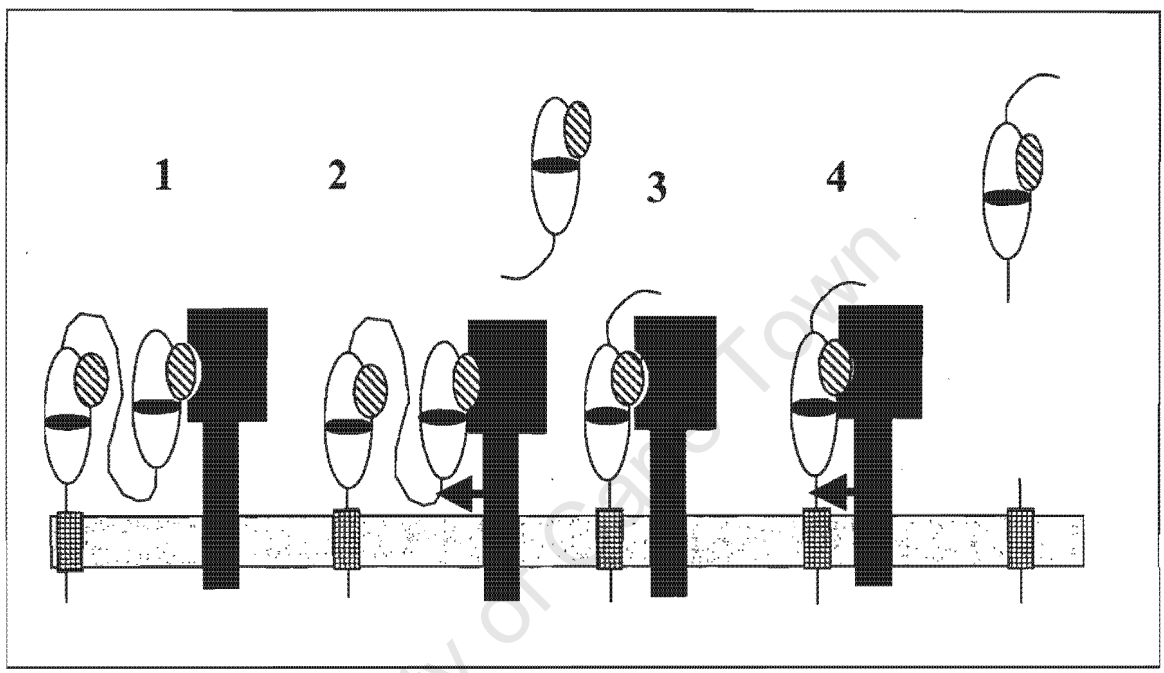


Figure 5.4.2 Mechanism of 2C-domACE proteolysis The ACE construct, 2C-domACE comprises 2 C domains (depicted as 2 open oval shapes) each with its own active site (black oval) and recognition motif (diagonally striped oval) and associated with the membrane (grey bar) via its TM (hatched box). The bridge region is indicated schematically as the curved line joining the two domains (this diagrammatic representation does not reflect the true orientation of the two C domains *in vivo*). The proteolysis of 2C-domACE can occur in a 4-step procedure whereby the ACE secretase (depicted as the black shape) binds to the recognition motif in the outer C domain in step one and cleaves the bridge region in the second step, to release the outer C domain of 2C-domACE. In the third step, the ACE secretase binds to the recognition motif of the inner C domain and cleaves within the stalk region in the fourth step to release the inner C domain.

The accessibility and the length of the stalk region have been shown to be important, and it has been postulated that the secretase positions itself primarily in respect to the proximal extracellular folded domain (Althoff et al, 2001; Ehlers et al, 1996). However, the length of the introduced juxtamembrane sequences of the gp130 chimeric proteins did not alter the shedding pattern of these proteins implying that the distance to the proximal domain is not vital for shedding to occur (Althoff et al,

2001). Moreover, the presence of a recognition motif in the distal ectodomain of ACE has been postulated to direct the shedding of ACE due to the shedding of CD4-ACE chimeras (Sadhukhan et al, 1998).

The results obtained from the construction and shedding of 2C-domACE indicated that steric hindrance incurred by the presence of an additional domain was not the reason for the lowered efficiency of shedding of somatic ACE compared to that of testis ACE. It does allow us to postulate, however, that a recognition motif in the C domain does direct the shedding of ACE from the membrane as 2C-dom ACE was cleaved both in the stalk and in the bridge region. We, therefore suggest that the ACE secretase requires the presence of a recognition motif to which it binds, and this docking mechanism allows the alignment of the active site of the ACE secretase with a region or protein sequence susceptible to cleavage. The level of disorder or random structure of the polypeptide chain is likely to define susceptibility to proteolysis. Thus, the length of this accessible protein sequence will play a role in the alignment with the active site of the ACE secretase. However, this alignment is subject to the overall structure of the protein. It is possible that in the case of the structure of gp130, the length of the substituted sequence can be accommodated to allow shedding to occur whereas the three-dimensional structure of ACE places more stringent requirements on the process of alignment. The presence of the bridge region, C-terminal to the recognition motif in the outer C domain of 2C-domain, allows shedding of the outer domain. This is due to the predicted flexibility and therefore accessibility of the bridge region and its distance from the outer recognition motif, which is similar to the distance between the stalk region and the inner recognition motif. Thus, binding to the outer recognition motif allows for the correct alignment of the bridge region with that of the ACE secretase cleavage site. As the N domain seems unlikely to comprise a recognition motif for the ACE secretase, docking of the ACE secretase to somatic ACE is unable to occur a suitable distance from the bridge region and this protein sequence is thus not cleaved. Accordingly, we postulate that membrane proteins require three distinct features in order for shedding to occur: 1) an accessible protein sequence with a level of structural disorder favouring proteolysis, 2) a recognition motif that allows the docking of the secretase, and 3) a suitable distance between the accessible protein sequence and the recognition motif which allows the correct alignment of the cleavable sequence with the active site of the

secretase. Any one or all of these features can be specific for any one secretase and the overall structure of the protein determines whether changes in cleavable sequences, or changes in chimeric proteins, are tolerated.

Chapter 6

The identification of the ACE secretase recognition motif in the C domain of ACE

6.1 Introduction

Somatic ACE consists of two active domains and although the N and C domains have high sequence identity they hydrolyse substrates with different catalytic efficiency constants. The C domain hydrolyses substance P and HHL more efficiently than the N domain and the N domain cleaves LHRH faster than the C domain (Ehlers & Riordan, 1991a; Jaspard et al, 1993; Wei et al, 1991a). However, both domains are able to hydrolyse the peptide z-PHL at the same rate (Danilov et al, 1994). The ratio of the rate of hydrolysis of HHL and z-PHL allows for the determination of N or C domain-specific activity of ACE chimeric proteins (Marcic et al, 2000b).

ACE is released from the plasma membrane by proteolysis (Ehlers et al, 1991b; Wei et al, 1991b; Oppong et al, 1993). To determine the shedding requirements for the release of membrane proteins in general, and the proteolysis of ACE in particular, mutations have been introduced within the stalk region, the cytoplasmic tail and the ectodomain of ACE with a view to deleting a specific sequence or structural element that targets ACE for cleavage. It has been shown that deletions within the stalk region, including the cleavage site of testis ACE, did not disrupt the shedding of ACE (Sadhkhan et al, 1998). A 47-residue deletion, including the juxtamembrane region and extending into the ectodomain, was not shed, but this deletion inactivated the ACE molecule (Ehlers et al, 1996). A testis ACE mutant lacking the cytoplasmic tail was shed with increased efficiency, indicating that this region of the protein was not necessary for cleavage (unpublished data).

Shedding of the CD4-ACE chimeras constructed by Sadhukhan et al suggested that the distal region of the ectodomain of ACE directed its cleavage from the plasma membrane. However, in this study determination of the exact region of the ectodomain involved in the regulation of shedding was limited, because successive deletions of the ectodomain of testis ACE not only affected the transport of the chimeric proteins to the cell surface but also resulted in the arrest of native ACE in the ER, and thus effects on shedding could not be evaluated. (Sadhukhan et al, 1998). Thus far, it has been shown that shedding of testis ACE is dependent on two factors: (1) an accessible juxtamembrane stalk region of minimum length, and (2) a region within the ectodomain that we term the recognition motif for the ACE secretase (Ehlers et al, 1996; Sadhukhan et al, 1998).

This chapter focuses on the identification of the recognition motif of testis ACE. Successive deletions of the ectodomain of testis ACE affect the correct processing of the enzyme to the cell surface. Therefore, an alternative method to identify the recognition motif was used. This method makes use of the similarity of the two domains of somatic ACE. A somatic ACE mutant that had the C domain deleted was not shed from the plasma membrane, despite being processed to the membrane (Pang et al, 2000). Testis ACE and the N domain construct are both membrane-bound, but only testis ACE is shed from the membrane. This suggested that, although the two domains are very similar in activity, and thus presumably in structure, the N domain lacks the recognition motif for the ACE secretase. To take advantage of this, regions of the ectodomain of testis ACE were replaced with the corresponding regions of the N domain in an effort to create active testis ACE-N domain chimeras that would be correctly processed to the cell surface but would not be shed (Figure 6.1). In this way we would be able to identify which region in the ectodomain of testis ACE contained the recognition motif for the ACE secretase. To test this hypothesis, we constructed four chimeric ACE proteins, and three will be discussed in this chapter.

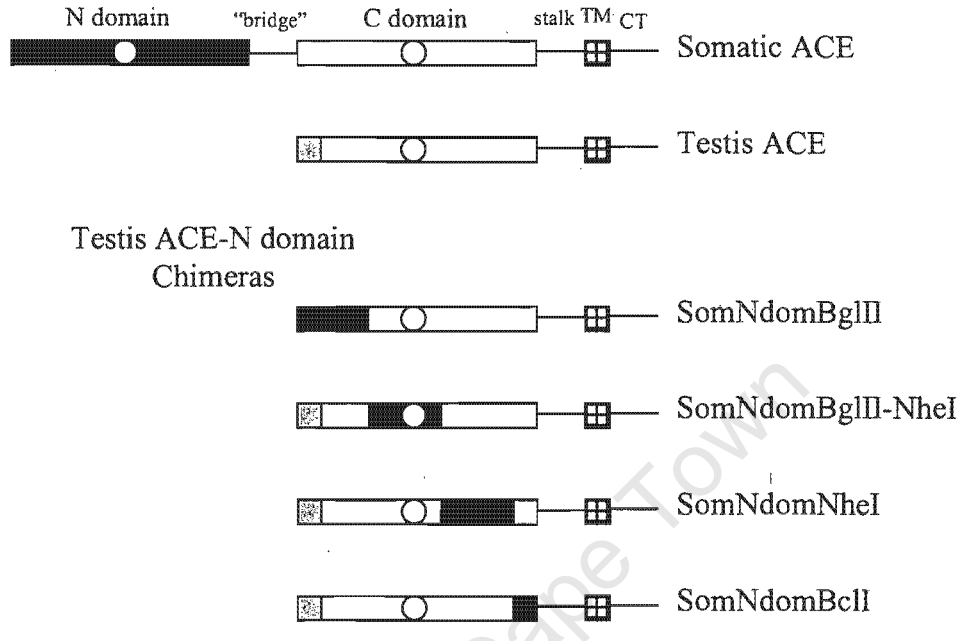


Figure 6.1 Testis ACE-N domain chimeric proteins Testis ACE is identical to the C domain (open bar) of somatic ACE except for 36 amino acid residues at the N-terminus (grey box) and ~60% identical to the N domain (black bar). Testis ACE sequence was replaced with homologous regions of the N domain to construct four chimeric proteins indicated by the black box within the open bar. The stalk, TM and cytoplasmic tail (CT) were unchanged.

6.2. Methods

6.2.1 **Construction of the testis ACE-N domain chimeras**

The testis ACE-N domain chimeric proteins are shown schematically in Figure 6.1. Due to the sequence identity of the N and C domains of somatic ACE, a few restriction enzyme sites occur at corresponding positions in the two domains (Figure 6.2.1). These restriction enzyme sites were used for the construction of the chimeras. The construction of the pLEN vector (6052 bp) involved two other mammalian expression vectors, pBR322 and pUC8 and thus, the entire DNA sequence and complete restriction enzyme map of the pLEN vector is unavailable. The cDNA of somatic ACE and testis ACE, sub-cloned into either pLEN or pBluescript (pBS), were used in the construction methodology depending on the availability of appropriate restriction enzyme sites. The positions of the restriction enzyme sites (nt) are numbered according to Soubrier et al and Ehlers et al (Soubrier et al 1988; Ehlers et al, 1989) (Appendix I.2 & I.3). The non-coding 5'region, between the start codon and the *Bam* HI site is 23 bp for somatic ACE cDNA and 50 bp for testis ACE (AI.2 & AI.3).

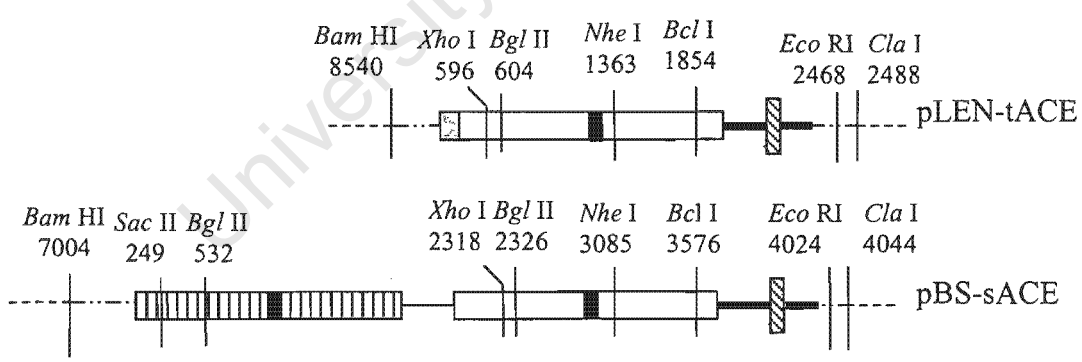


Figure 6.2.1 Corresponding restriction enzyme sites of testis ACE and somatic ACE Testis ACE (tACE) and the C domain are identical (open bar) except for 36 amino acid residues at the N-terminus of testis ACE (grey box). The start of the coding sequence (ATG) of tACE and somatic ACE (sACE) is at nt 23 and the size of pLEN and pBluescript (pBS) is 6052 bp and 2960 bp, respectively. Somatic ACE cDNA (4024 bp) was cloned into pBS using *Eco* RI and the distance between the stop codon and the *Eco* RI site is approximately 80 bp whereas the stop codon of testis ACE cDNA (2473 bp) is approximately 250 bp from the *Eco* RI site of pLEN. The positions of the restriction enzyme sites are indicated above according to the numbering of Ehlers et al and Soubrier et al and the vector maps are available in Appendix I.2 and I.3 (Soubrier et al 1988; Ehlers et al, 1989).

6.2.1.1 **Cloning of SomNdomBglII**

In order to replace the N-terminus of testis ACE with the corresponding sequence of the N domain, the cDNA of somatic ACE was digested with *Bgl* II (Figure 6.2.2). The 5230 bp band was excised from a 1% agorose gel using the GenElute Agarose Gel DNA elution kit (Sigma). Self-ligation of this fragment yielded a testis ACE-N domain chimera (pBS-SomNdomBglII) where the 5' end of testis ACE cDNA was replaced with the corresponding 532 bp from the N domain of somatic ACE including the somatic ACE signal peptide (Figure 6.2.2).

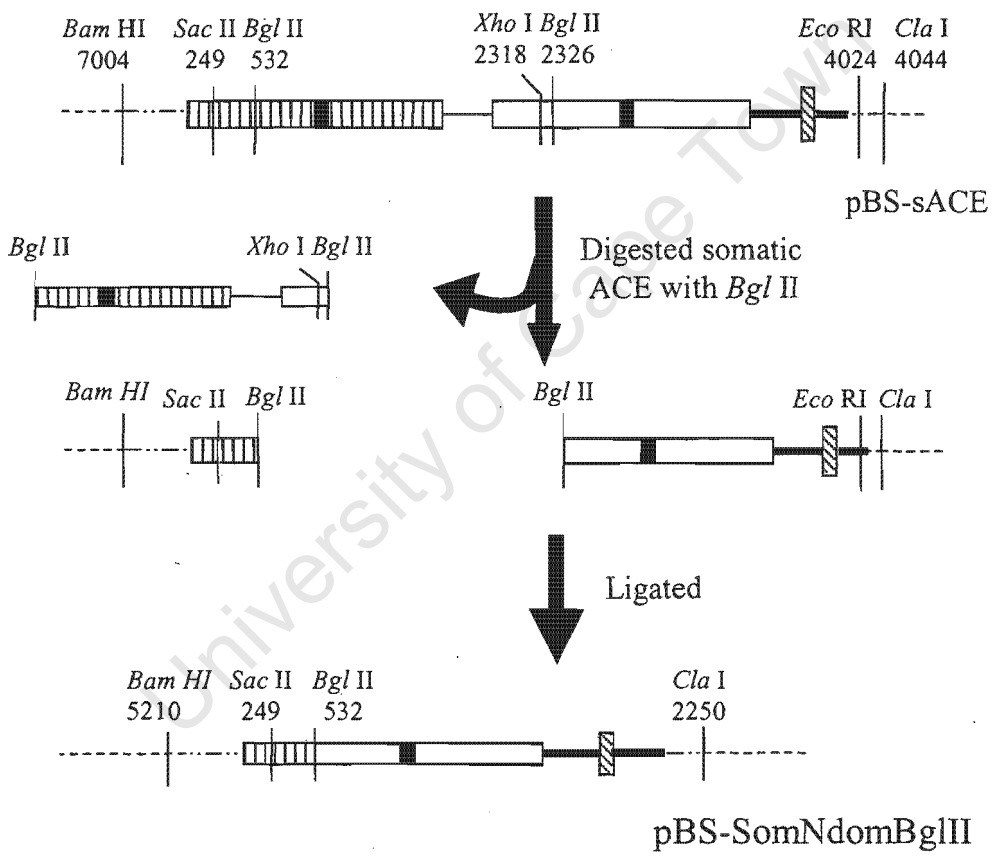


Figure 6.2.2 Construction of SomNdomBglII Somatic ACE cDNA (4024 bp) in pBluescript (pBS-sACE, 7024 bp) was digested with, *Bgl* II, which occurs in corresponding sites in both the C domain (open bar) and the N domain (striped bar). The digestion excises a 1794 bp fragment flanked by the *Bgl* II sites. The somatic ACE cDNA in pBS was then self-ligated to form SomNdomBglII, which is identical to testis ACE except for 532 bp at the N-terminus (striped box), which comprises N domain sequence. The hatched box indicates the transmembrane domain, the black box indicates the active site and bold line indicates the stalk and cytoplasmic tail. (Testis ACE nt numbering is as determined by Ehlers et al, 1989 and somatic ACE numbering is as determined by Soubrier et al, 1988).

In order to express the chimeric protein in mammalian cells, SomNdomBglII had to be excised from pBS and sub-cloned into pLEN. The chimeric construct (pBS-SomNdomBglII) was digested with *Bam* HI and *Cla* I to excise the construct from pBS and ligated to the similarly digested mammalian expression vector, pLEN-tACE (Appendix I.2). The ligation reaction was transformed into XL1-Blue *E.coli* competent cells and the ampicillin resistant cfus were inoculated into 5 ml LB aliquots and grown O/N. The plasmid DNA was extracted from the bacterial cell cultures using a quick phenol-based DNA mini-preparation method and the DNA was digested with *Sac* II to determine the presence of the N-terminal N domain sequence (Figure 6.2.2). The cfu, containing the correct DNA construct was inoculated into 50 ml LB and grown O/N and the DNA extracted using the Qiagen Plasmid Midi kit. Digestion of the final DNA preparation with Bam HI, *Bgl* II and *Xho* I confirmed the correct construction of pLEN-SomNdomBglII (AII.3.1).

6.2.1.2 **Cloning of SomNdomBglII-NheI**

In order to construct the chimera, SomNdomBglII-NheI, testis ACE sequence from nt 604-1363 had to be replaced with the corresponding region of the N domain. Although the C and N domain have 60% sequence identity, there were no corresponding restriction enzyme sites available. Thus the N domain sequence was amplified using the polymerase chain reaction (PCR) and a *Nhe* I site was introduced at nt 1291 by silent mutation (G to A substitution). To prevent the non-specific amplification of the C domain, somatic ACE was not used as a DNA template in the amplification reaction. The N domain sequence was amplified using the construct, pLEN-SomNdom (Pang et al, 2001). In this construct, the N domain of somatic ACE is fused to the juxtamembrane stalk sequence, transmembrane domain and cytoplasmic tail of testis ACE (Figure 6.2.3). The PCR product was digested with *Bam* HI and *Cla* I and ligated to the similarly digested pBS and sequenced using the ALFexpress DNA Automated Sequencer (APBiotech).

The PCR product in pBS was then excised by digestion with *Bgl* II and *Nhe* I and ligated to the similarly digested pBS-tACE to form the chimera, pBS-SomNdomBglII-NheI (Figure 6.2.3) This cDNA construct was digested with *Bam* HI and *Cla* I to excise it from pBS and insert it into the similarly digested mammalian expression vector, pLEN-tACE (Appendix I.2). The ligation reaction was transformed

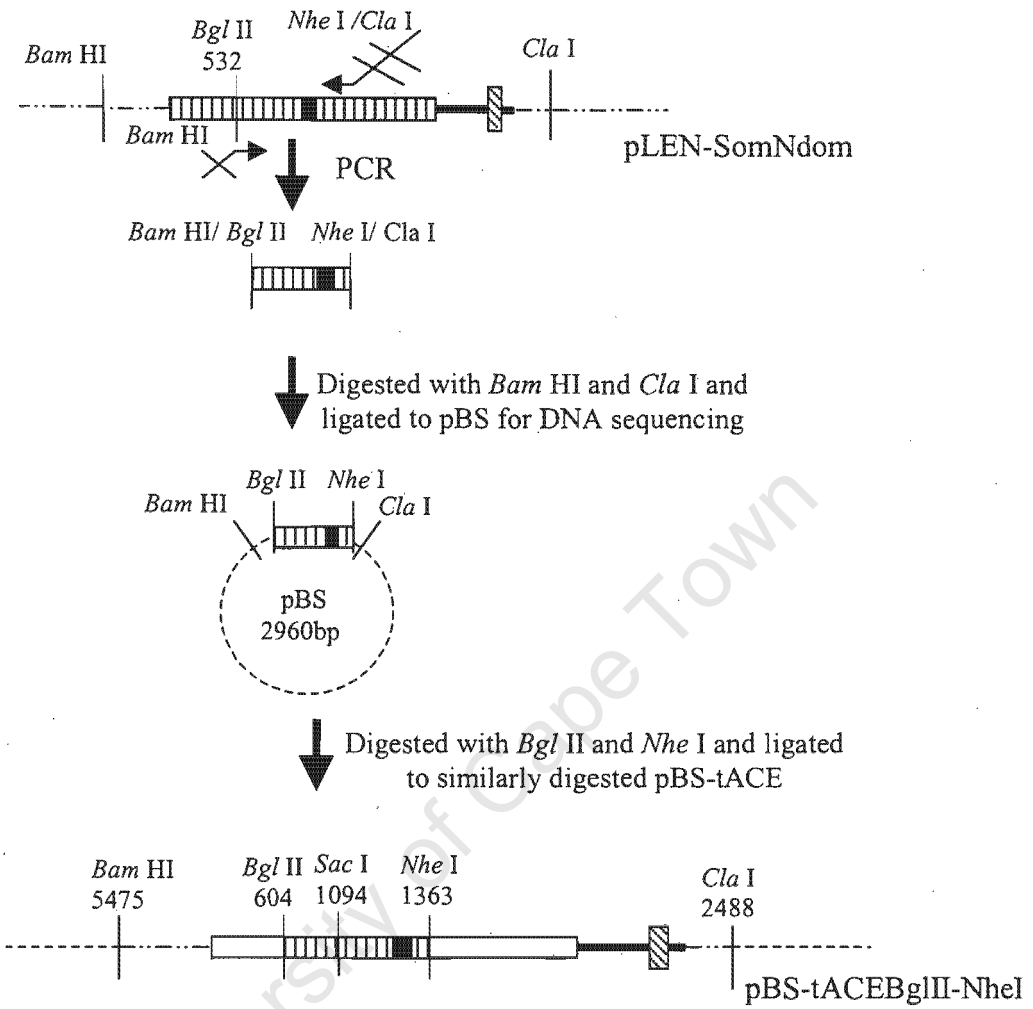


Figure 6.2.3 Construction of SomNdomBglII-NheI pLEN-SomNdom (striped bar) was amplified (nt 533 to nt 1291) and the PCR product was ligated with pBS using *Bam* HI and *Cla* I that were introduced with the forward and reverse primers, respectively. Upon confirmation of correct nt sequence by DNA sequencing, the PCR product was digested with *Bgl* II and *Nhe* I (the *Nhe* I site was introduced with a G (nt1294) to A substitution in the reverse primer) and ligated to the similarly digested testis ACE (open box) in pBS. The testis ACE-N domain chimera, pBS-tACEBglII-NheI, containing the N domain, *Bgl* II-*Nhe* I sequence (striped box), was then digested with *Bam* HI and *Cla* I and ligated to the similarly digested pLEN-tACE (Appendix I.2) to form pLEN-SomNdomBglII-NheI. Hatched box represents the transmembrane domain, black box represents the active site and bold line indicates the stalk and cytoplasmic tail. (Testis ACE nt numbering is as determined by Ehlers et al, 1989 and somatic ACE numbering is as determined by Soubrier et al, 1988).

into XL1-Blue *E.coli* competent cells and the ampicillin resistant cfus were inoculated into 5 ml LB aliquots and grown O/N. The plasmid DNA was extracted from the bacterial cell cultures using a quick phenol-based DNA mini-preparation method and the DNA was digested with *Sac* I to determine the presence of the N-terminal N domain sequence (Figure 6.2.3). The cfu, containing the correct DNA construct was inoculated into 50 ml LB and grown O/N and the DNA extracted using the Qiagen Plasmid Midi kit. Digestion of the final DNA preparation with *Bam HI*, *Cla* I and *Sac* I confirmed the correct construction of pLEN-somNdomBglII-NheI (AII.3.2).

6.2.1.3 **Cloning of SomNdomNheI**

In order to construct the chimera SomNdomNheI, pLEN-SomNdom was used as a template to amplify the region from nt 1295 to nt 1780 of the N domain (Pang et al, 2001) (Figure 6.2.4). To facilitate the cloning procedure restriction enzyme sites, *Bam* HI, *Nhe* I, *Bcl* I and *Cla* I were introduced with the 5` extensions of the primers (Figure 6.2.4). The PCR product was digested with *Bam* HI and *Cla* I and ligated to the similarly digested pBS and sequenced using the ALFexpress DNA Automated Sequencer (APBiotech). The PCR product in pBS was then excised by digestion with *Nhe* I and *Bcl* I and ligated to the similarly digested pLEN-tACE. Thus, the testis ACE sequence between *Nhe* I and *Bcl* I was replaced with the corresponding N domain sequence (Figure 6.2.4). All DNA preparations were transformed into methylation negative JM110 *E.coli* competent cells, as the *Bcl* I site is methylation sensitive. The ampicillin resistant cfus were inoculated into 5 ml LB aliquots and grown O/N. The plasmid DNA was extracted from the bacterial cell cultures using a quick phenol-based DNA mini-preparation method and the DNA was digested with *Pin* AI and *Bgl* I to determine the presence of the N-terminal N domain sequence (Figure 6.2.4). The cfu, containing the correct DNA construct was inoculated into 50 ml LB and grown O/N and the DNA extracted using the Qiagen Plasmid Midi kit. The DNA was RE digested to confirm the correct chimeric construct. Digestion of the final DNA preparation with *Nhe* I, *Bgl* I and *Pin* AI confirmed the correct construction of pLEN-SomNdomNheI (AII.3.3).

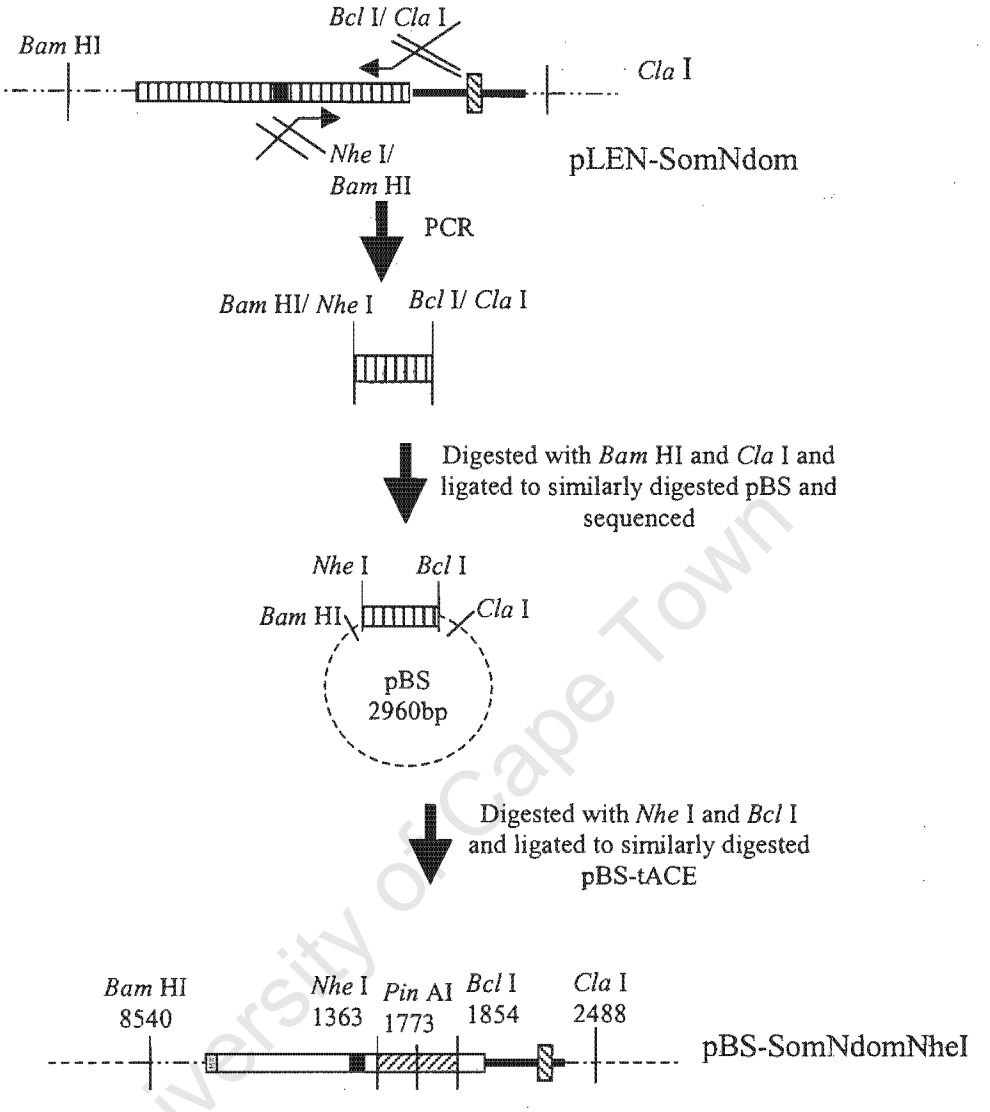


Figure 6.2.4 Construction of SomNdomNheI pLEN-SomNdom (striped box) construct was amplified (nt 1292 to nt 1780) and the PCR product was ligated into pBS using *Bam* HI and *Cla* I which were introduced with the forward and reverse primers, respectively. Upon confirmation of correct nt sequence by DNA sequencing, the PCR product was digested with *Nhe* I and *Bcl* I (the *Nhe* I site was introduced with a G1294 to A substitution in the forward primer) and ligated to the similarly digested testis ACE (open bar) in pBS. The testis ACE cDNA containing the N domain sequence (striped box) from *Nhe* I to *Bcl* I, was then digested with *Nhe* I and *Bcl* I and ligated to the similarly digested pLEN-tACE (Appendix I.2) to form pLEN-SomNdomNheI. The hatched box represents the transmembrane domain, the black box represents the active site and the bold line indicates the stalk and cytoplasmic tail. (Testis ACE nt numbering is as determined by Ehlers et al, 1989 and somatic ACE numbering is as determined by Soubrier et al, 1988).

6.2.2 **Expression and Analysis of the testis ACE-N domain Chimeric Proteins**

The three chimeric cDNAs, SomNdomBglII, SomNdomBglII-NheI and SomNdomNheI cloned into the mammalian expression vector, pLEN, were transfected into CHO-KI cells and the shedding and activity of the enzyme was determined using western blot analysis and HHL hydrolysis.

CHO-KI cells, expressing the chimeric cDNAs were grown to confluence and ACE expression was induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM). Fresh 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM) was then added and grown for a further 4 hrs in the absence and presence of either 1μM PDBu or 100 μM TAPI. The culture medium was then removed and the cells lysed in 1%Triton X-100 lysis buffer. The culture medium and cell lysate aliquots were separated on a 10% polyacrylamide SDS gel and analysed by western blot analysis with a rabbit anti-testis ACE polyclonal antibody. CHO-KI cells, expressing testis ACE cDNA, were treated as outlined above and a medium sample and a cell lysate sample were included to control for the MW of the chimeric protein. CHO-KI cells not expressing recombinant protein were treated similarly and a cell lysate sample and a medium sample were included as a control for non-specific binding of the antibody. A 50 μl-aliquot was removed from the media and cell lysate samples and tested for ACE activity using HHL as a substrate.

6.3 Results

6.3.1 **Expression of the testis ACE-N domain chimeric proteins in CHO cells**

The cDNAs of SomNdomBglII, SomNdomBglII-NheI and SomNdomNheI, cloned into the mammalian expression vector pLEN, were transfected into CHO-KI cells to determine whether testis ACE was shed after the regions of testis ACE sequence were replaced with the corresponding N domain sequence of somatic ACE. To determine the shedding of the chimeric proteins from CHO-KI cells, the transfected cells were cultured in 35-mm dishes, the culture medium was removed before the cells were lysed, and ACE activity was determined for the culture medium and the cell lysate using the ACE substrate HHL. In addition, the chimeric proteins present in the culture medium and cell lysates were visualized by western blot analysis using a polyclonal antibody raised against purified recombinant testis ACE.

6.3.1.1 **SomNdomBglII**

CHO-KI cells expressed SomNdomBglII as western blot analysis of the cell lysates indicated a protein band corresponding to wild-type testis ACE (wt) (Figure 6.3.1 A). The smaller band in the cell-associated samples was most likely the unprocessed, immature form of the chimeric protein. The appearance of a protein band corresponding to testis ACE (wt), in the culture medium samples indicated that SomNdomBglII was shed (Figure 6.3.1 A). The chimeric protein lacked the *O*-glycosylated, 36 amino acid residues located at the N-terminus of testis ACE and this would explain the decrease in size of the protein compared to testis ACE. This shedding event was upregulated in the presence of phorbol esters (PDBu) and inhibited in the presence of the metalloprotease inhibitor, TAPI, which was confirmed by the concomitant decrease of cell-associated SomNdomBglII protein after treatment with PDBu, and increase after treatment with TAPI. Determination of ACE activity using HHL (Figure 6.3.1 B) also confirmed the shedding of this chimeric protein as ACE activity was detected in the culture medium samples. There was a slight increase in shedding in the presence of phorbol esters and an inhibition in the presence of TAPI. The small increase in ACE activity in the presence of PDBu did not correlate with the significant increase of soluble ACE in Figure 6.3.1 A. This suggested that the catalytic efficiency of the chimeric protein had been altered. Therefore, replacement of the amino acid residues M-31 to P163 of testis

ACE with the corresponding protein sequence from the N domain of somatic ACE (M-29 to P141) did not affect the shedding of testis ACE but affected the ability of the chimeric protein to hydrolyse HHL.

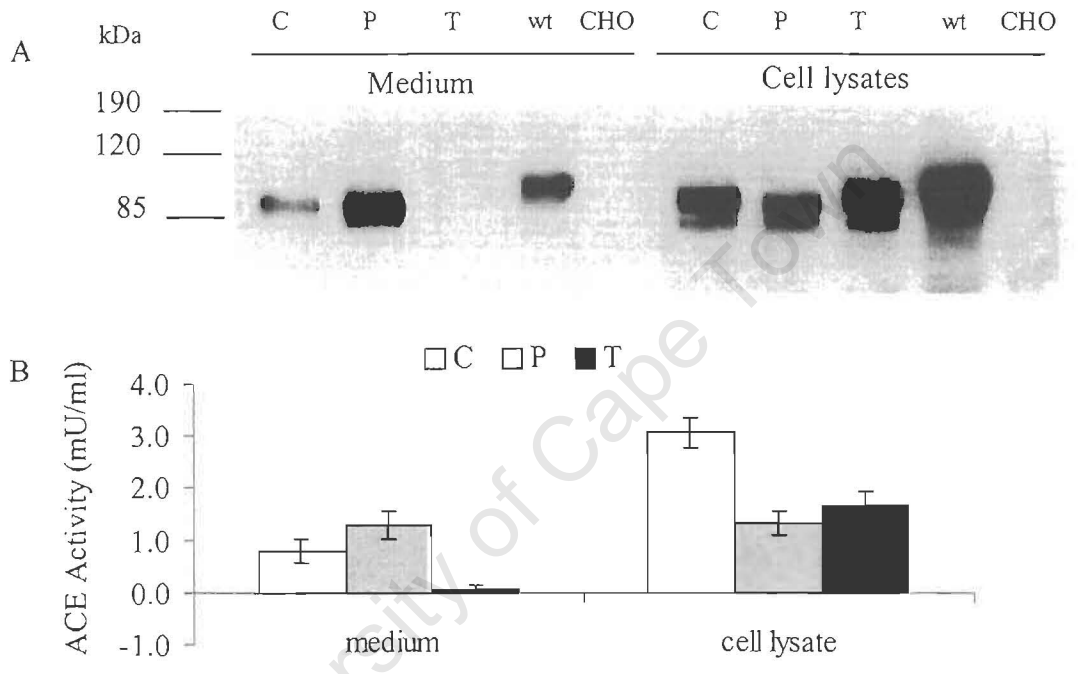


Figure 6.3.1 Determination of the shedding of SomNdomBglII from CHO-KI cells CHO-KI cells, expressing the chimeric cDNA, SomNdomBglII, were grown for 4 hrs in the absence (C) and presence of either 1μM PDBu (P) or 100 μM TAPI (T) before the culture medium and the cell lysate aliquots were separated on a 10% polyacrylamide SDS gel and analysed by western blot analysis with a rabbit anti-testis ACE polyclonal antibody (Figure A). CHO-KI cells, expressing testis ACE cDNA (wt) and, CHO-KI cells not expressing recombinant protein (CHO) were treated similarly and a cell lysate sample and a medium sample were included (Figure A). In Figure B, a 50 μl aliquot was removed from the media and cell lysate samples of SomNdomBglII, and tested for ACE activity using HHL as a substrate. (n=3 +/- SD)

6.3.1.2 **SomNdomBglII-NheI**

Shedding of the chimeric protein, SomNdomBglII-NheI, was examined in Figure 6.3.2. A protein band corresponding to the MW of testis ACE (wt) was apparent in the cell lysate samples, indicating that this protein was expressed by the transfected CHO-KI cells. However, there was no concomitant appearance of a corresponding band in the media samples, which would suggest that this protein was not shed into the culture medium. Moreover, there was no effect on the cell-associated protein in

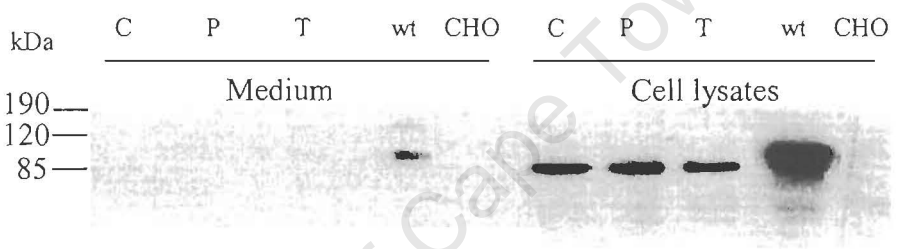


Figure 6.3.2 Determination of the shedding of SomNdomBglII-NheI from CHO-KI cells CHO-KI cells, expressing the chimeric cDNA, SomNdomBglII-NheI, were grown for 4 hrs in the absence (C) and presence of either 1μM PDBu (P) or 100 μM TAPI (T) before the culture medium and the cell lysate aliquots were separated on a 10% polyacrylamide SDS gel and analysed by western blot analysis with a rabbit anti-testis ACE polyclonal antibody. CHO-KI cells, expressing testis ACE cDNA (wt) and, CHO-KI cells not expressing recombinant protein (CHO) were treated similarly and a cell lysate sample and a medium sample were included.

the presence of PDBu and TAPI. No ACE activity was detected in these culture medium and cell lysate samples (not shown). In Figure 6.3.2, the chimeric protein resolved at a lower position on the gel than testis ACE (wt) and the sharp appearance of the protein band suggests reduced glycosylation, which may be indicative of incorrect processing of the chimeric protein. Thus, although this chimeric protein was not shed, indicating that the testis ACE region deleted in this chimera may contain the recognition element for the ACE secretase, the abrogation of enzyme activity makes this conclusion equivocal. The lack of enzyme activity indicates that the structure of the enzyme was disrupted, which may affect the recognition motif such that the ACE secretase can no longer recognize the chimeric protein.

6.3.1.3 **SomNdomNheI**

The expression and shedding of the chimeric protein, SomNdomNheI, is shown in Figure 6.3.3. Culture medium samples from CHO-KI cells grown in the absence (C) and in the presence of either PDBu (P) or TAPI (T), indicated the appearance of a soluble form of the chimeric protein. This soluble form was upregulated by PDBu and down-regulated by TAPI. The cell lysate samples showed a band corresponding to the cell-associated form but no concomitant decrease or increase in band intensity was observed in the presence of PDBu or TAPI, respectively, as would be expected if shedding was upregulated or inhibited in the presence of these compounds. In Figure B, the enzymatic activity of the media and cell lysate samples are indicated. A slight increase in ACE activity was detected in the media samples in the presence of PDBu and a decrease was detected in the presence of TAPI, as expected. However, the cell lysate samples had no detectable enzyme activity under these assay conditions using HHL as a substrate. Thus substitution of L395 to K557 for testis ACE L417 to Q579 does not abrogate ectodomain shedding and SomNdomNheI was still shed.

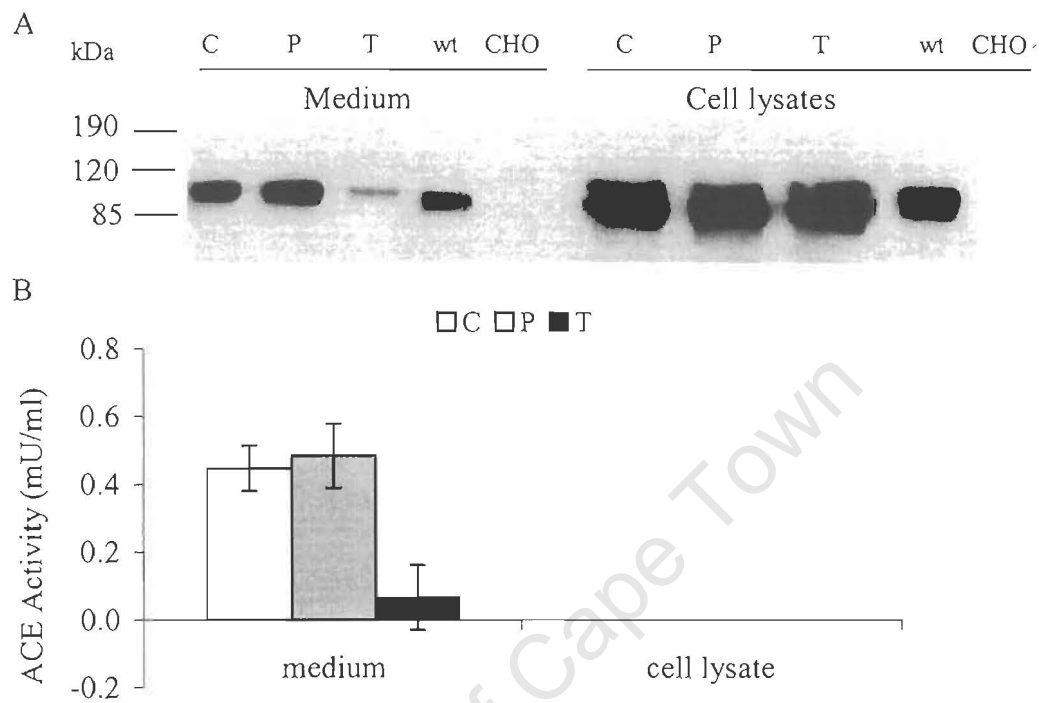


Figure 6.3.3 Determination of the shedding of SomNdomNhel from CHO-KI cells CHO-KI cells, expressing the chimeric cDNA, SomNdomNhel, were grown for 4 hrs in the absence (C) and presence of either 1μM PDBu (P) or 100 μM TAPI (T) before the culture medium and the cell lysate aliquots were separated on a 10% polyacrylamide SDS gel and analysed by western blot analysis with a rabbit anti-testis ACE polyclonal antibody (Figure A). CHO-KI cells, expressing testis ACE cDNA (wt) and, CHO-KI cells not expressing recombinant protein (CHO) were treated similarly and a cell lysate sample and a medium sample were included (Figure A). In Figure B, a 50μl aliquot was removed from the media and cell lysate samples of SomNdomNhel, and tested for ACE activity using HHL as a substrate.

The sequential replacement of testis ACE sequence with N domain sequence altered the enzymatic activity of the chimeric proteins and only the shedding of SomNdomBglII-NheI was affected (Table 6.3.1) (Figure 6.1). However, whether this is due to loss of the recognition motif or disruption of the native conformation is not certain.

Table 6.3.1 Summary of Chimeric proteins

Chimera	Testis ACE sequence replaced	ACE activity	Shed status
SomNdomBglII	M-31 to P163	Decreased activity	Shed
SomNdomBglII-NheI	D164 to V416	No activity	Not shed
SomNdomNheI	L417 to Q579	Decreased activity in the media and no cell-associated activity	Shed

6.3.2 **Cell-surface localisation of the testis ACE-N domain chimeric proteins**

The western blots of the cell culture media and cell lysates of the transfected CHO-KI cells confirmed that all the chimeric constructs were expressed. In the case of SomNdomBglII-NheI and SomNdomNheI, no cell-associated enzyme activity was detected. To confirm that these chimeric proteins were being correctly processed to the plasma membrane for shedding to occur, cell-surface labelling was performed using the polyclonal rabbit anti-testis ACE antibody and FITC-conjugated goat anti-rabbit secondary antibody. The expression of the chimeric proteins was detected by confocal microscopy.

The results of the confocal microcopy are shown in Figure 6.3.4. The CHO-KI cells, not transfected with cDNA, are shown in row A. The nuclei stained red with PI (column I) but there was no green fluorescence associated with the cell surface (column II) or cytoplasm (column III), verifying that there was no non-specific binding of the ACE antibody. Similarly, no green florescence was associated with the cells when the ACE antibody was not included in the experiment confirming that the secondary FITC-conjugated antibody did not bind non-specifically (data not shown). SomNdomBglII, depicted in row B, showed expression within the cytoplasm (column III) and on the cell surface (column II), which is in agreement with the western blot analysis.

SomNdomBglII-NheI was expressed intracellularly (row C, column III) but had no cell surface expression (row C, column II). The internal pattern of expression was also different from that of SomNdomBglII, as the green fluorescence extended into the nuclei, leaving only the nucleolus devoid of signal. This distinctive pattern of internal distribution might be due to the accumulation of the chimeric protein in the rough endoplasmic reticulum (RER), due to the incorrect folding of the enzyme. This accumulation could lead to high levels of protein in this nucleus-associated organelle and result in high fluorescence intensity that could be misleading in terms of the distribution of the chimeric protein. SomNdomBglII-NheI was not shed as indicated by western blot analysis and no enzyme activity was detected for this construct. The results obtained by confocal microscopy confirm that this protein was not shed, probably due to incorrect processing.

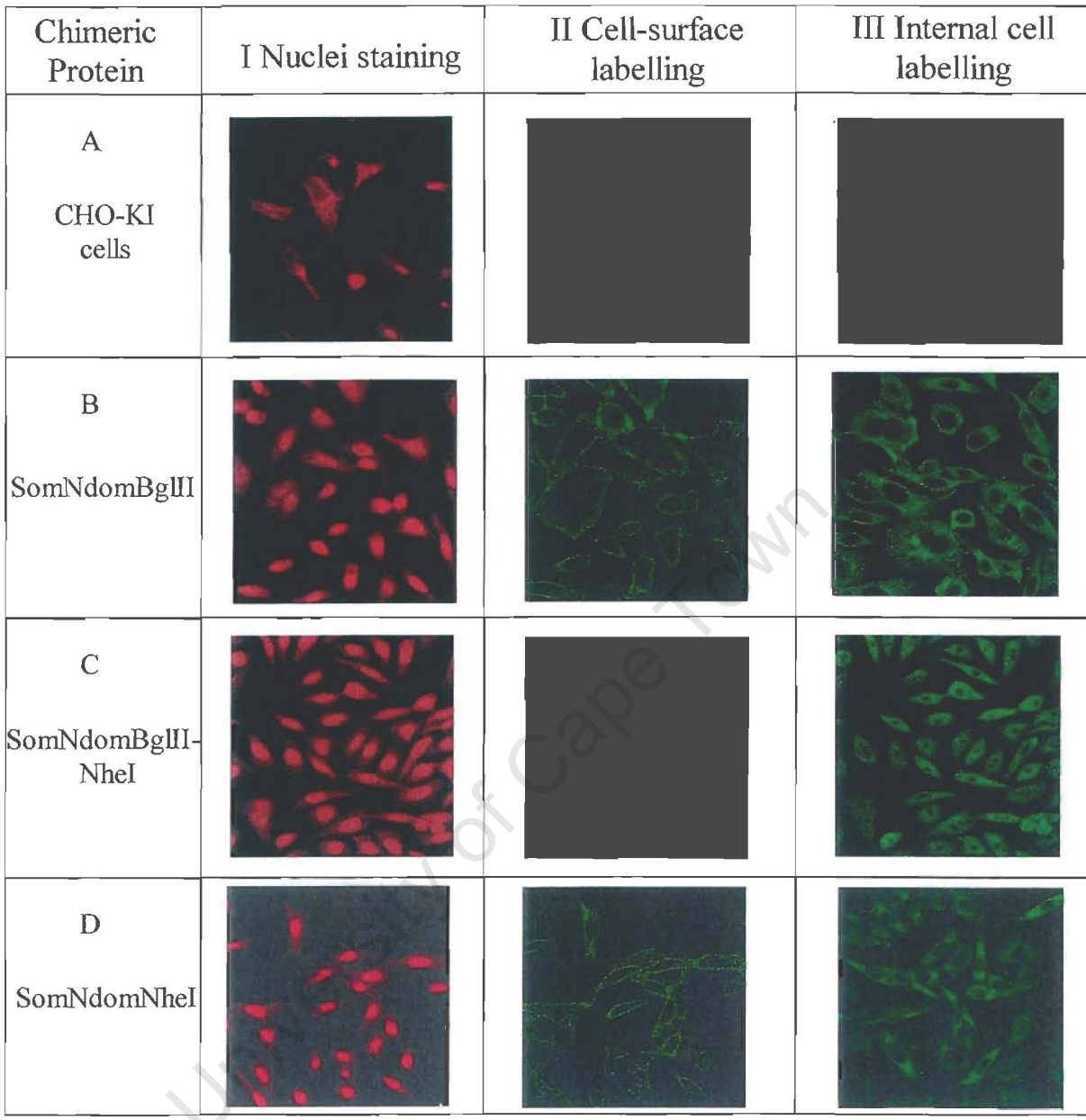


Figure 6.3.4 Confocal microscopy of CHO-KI cells expressing the chimeric proteins CHO-KI cells transfected with the cDNA of SomNdomBglII (row B), SomNdomBglII-NheI (row C) and SomNdomNheI (row D) were grown to confluence on coverslips and probed with a primary rabbit anti-ACE polyclonal antibody and secondary goat anti-rabbit FITC-labelled antibody either before (column II) or after (column III) membrane permeabilisation with methanol to obtain cell surface or internal cell labelling of the expressed recombinant proteins, respectively. In addition the cells were stained with propidium iodide (PI) after membrane permeabilisation for nuclei staining (column I) and the cells indicated, correspond to the cell-surface labelled cells in column II. To control for the specificity of the primary antibody, CHO-KI cells not transfected with cDNA, were treated similarly (row A).

Row D depicts the expression of SomNdomNheI at the cell surface in unpermeabilised cells (column II) and intracellularly in the membrane-permeabilised cells (column III). The cell-surface expression of this chimeric protein was unexpected as no enzyme activity was detected in the cell lysates of CHO-KI cells expressing the construct (Figure 6.3.3 B). The results depicted in row D, column II suggests that there were two populations of this chimeric protein: 1) an enzymatically active population that was immediately shed into the culture medium as determined by using the substrate HHL; and 2) a population that had no detectable enzyme activity but was processed to the cell surface.

6.3.3 **Mass spectral analysis of the testis ACE-N domain chimeras**

The chimeric proteins were purified from the culture medium as outlined in Chapter 2 and the purified proteins were subjected to mass spectrometric analysis to identify the peptide bond where cleavage by the ACE secretase occurred. The proteins were digested with endoproteinase Lys-C and each chimeric protein had its own specific set of peptides, as the protein sequence was altered in each case by the replacement of testis ACE sequence with that of the corresponding N domain sequence. In Appendix I.5, the protein sequences of the soluble chimeric proteins are shown with the substituted sequence indicated in bold.

In Table 6.3.3, the relevant peptides generated by the endoproteinase Lys-C of the chimeric proteins are shown. The amino acid residues, the calculated *m/z* of these peptides, and the *m/z* obtained by mass spectrometry are indicated.

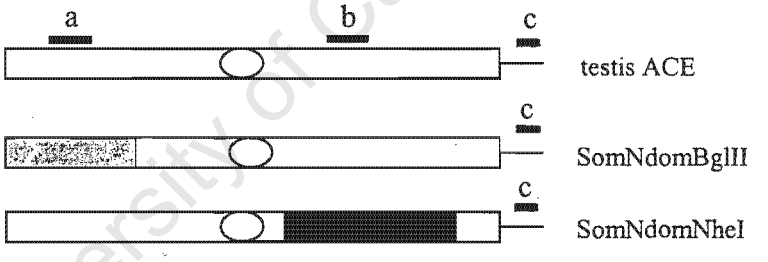


Figure 6.3.5 Schematic of the peptides generated by endoproteinase Lys-C digestion Testis ACE sequence is indicated by the open bar and the N domain sequence is shown as the grey bar for SomNdomBglII and the black bar for SomNdomNheI. Peptides a and b represents the testis ACE specific peptides, V119-K137 and D396-K425, respectively. Peptide c is the C-terminal peptide of the soluble form of the proteins. The active site is indicated by the clear oval.

In order to confirm the correct construction of the chimeric proteins, the *m/z* of the peptides found exclusively in the chimeric sequence were identified by mass spectrometry. The *m/z* of the peptide from V119 to K137 obtained from the digestion of testis ACE by endoproteinase Lys-C was not identified in the mass spectrum obtained for SomNdomBglII (Figure 6.3.5). This indicates that the chimeric protein was correctly constructed, as this peptide occurs within the protein sequence of testis

ACE replaced by the N domain sequence. Similarly, the inability to identify the peptide from D396 to K425 of testis ACE in the endoproteinase Lys-C digest of SomNdomNheI indicated that this chimeric protein was correctly constructed, as it was also derived from testis ACE sequence replaced with sequence from the N domain of somatic ACE (Figure 6.3.5). Thus, mass spectrometry confirmed the correct construction of SomNdomBglII and SomNdomNheI.

Table 6.3.3. Mass spectra of the endoproteinase Lys-C-digested chimeric proteins

Peptide residues	Calculated *m/z*	Observed *m/z*		
		Testis ACE	SomNdomBglII	SomNdomNheI
119-137	2217.4	2216.0		2217.7
396-425	3075.0	3074.3	3076.3	
598-613[a]	1951.2	1949.9	1951.9	1951.9
614-627[b]	1690.4	1690.1	1690.8	1690.8

The chimeric proteins comprise sections of protein sequence from the N domain and testis ACE. The N domain substitution in SomNdomBglII altered the numbering of the peptides generated by endoproteinase Lys-C digestion compared to wt testis ACE.
[a] corresponds to 576-591 of SomNdomBgll
[b] corresponds to 592-605 of SomNdomBgll

The cleavage site of ACE occurs within the juxtamembrane stalk region and this sequence is common to SomNdomBglII, SomNdomNheI, and testis ACE (Figure 6.3.5). The *m/z* (1690.4) of the C-terminal peptide of testis ACE, L614-R627, was observed in the mass spectrum of both chimeric proteins. This indicated that the cleavage site was not altered by the replacement of regions of testis ACE with that of the corresponding N domain sequence. The cleavage site for SomNdomBglII and SomNdomNheI occurred after R627 (R605, in the case of SomNdomBglII), the same as testis ACE.

6.3.4 **Enzyme kinetics of SomNdomBglII and SomNdomNheI**

The substrate specificity of the two domains of somatic ACE differs in that the ratio of the k_{cat} for the C domain to that of the N domain for z-PHL and HHL hydrolysis are 1.1 and 9.1, respectively. This indicates that the two domains hydrolyse z-PHL equally and that the C domain hydrolyses HHL 9 × more efficiently than the N domain (Danilov et al, 1994; Wei et al, 1991a). To determine whether the substrate specificity of the enzyme was altered by the replacement of testis ACE sequence with that of the corresponding N domain, the rate of enzyme hydrolysis using the two substrates, HHL and z-PHL, was determined for affinity-purified testis ACE, the N domain, and the chimeric proteins.

Table 6.3.4.1 Rate of enzyme hydrolysis of z-PHL and HHL by the chimeric proteins

Enzyme	z-PHL rate of hydrolysis (µmol/min/mg)	HHL rate of hydrolysis (µmol/min/mg)	z-PHL/HHL [a]ratio
Testis ACE	9.1	5.8	1.5 ± 0.17
SomNdom	3.1	0.3	11.6 ± 0.68
SomNdomBglII	1.8	0.07	23.5 ± 1.59
SomNdomNheI	0.98	0.04	24.7 ± 3.92

Substrate was 1 mM, enzyme concentration was 0.009 mg/ml and hydrolysis was tested within 5 min.
[a] Ratio = mean +/- SD (n=3)
SomNdom construct is the N domain directly fused to the TM and cytoplasmic tail of testis ACE (Pang et al, 2001).

In Table 6.3.4.1 the z-PHL/HHL ratio determined for testis ACE was 1.5, approximately 7- to 8-fold lower than obtained for SomNdom (N domain). Both chimeras exhibited ratios that were approximately 15 times greater than that for testis ACE. The altered ratios of hydrolysis are indicative of a change in enzyme activity of testis ACE with the replacement of regions of protein sequence distal to the active site. Essentially, these data indicate that the chimeric proteins have assumed N domain-like characteristics, even though the substituted regions did not include the active site.

Table 6.3.4.2 Ratio of z-PHL/HHL hydrolysis

	z-PHL/HHL ratio	Reference
Testis ACE	0.9 +/-0.2	Marcic et al, 2000b
C domain	0.67 +/- 0.007	Williams et al, 1996
N domain	4.5 +/- 0.35	Williams et al, 1996
SomNdomBglII	10.6 +/- 2.7	Marcic et al, 2000b

Ratio = mean +/- SEM

SomNdomBglII was constructed by Marcic et al and the ratio of z-PHL/HHL hydrolysis of this construct was 10.6, which is approximately half the ratio determined in this study (Table 6.3.4.2). However, the relative difference in the ratios between the N domain, testis ACE and SomNdomBglII is comparable: the ratio of hydrolysis of SomNdomBglII, constructed by Marcic et al, was approximately 12-fold higher than testis ACE and 2.5-fold higher than the N domain, similar to what was obtained here (Marcic et al, 2000b).

6.4 Discussion

Previous attempts to identify the recognition motif relied on the use of deletion mutants or fusion proteins (Sadhukhan et al, 1998). These techniques were unsuccessful, as deletions of certain regions of the ACE sequence rendered the protein inactive. The loss of enzyme activity suggests the loss of native protein structure and this made it difficult to ascertain whether shedding was inhibited by the deletion of the recognition motif for the ACE secretase or the disruption of the native conformation of the protein. This study aimed to circumvent these limitations by the sequential replacement of regions of the testis ACE protein with the corresponding sequences from the N domain. This approach was undergirded by the finding that the N domain, when directly linked to the stalk, transmembrane domain and cytoplasmic tail of testis ACE, was not shed (Pang et al, 2000). This suggested that the N domain, although ~60% identical to the C domain, lacked the sequence or structural motif that would identify it as an ACE secretase substrate. By replacing testis ACE sequence with corresponding N domain sequence, we hoped to remove the recognition motif and still maintain the native conformation of testis ACE.

Four chimeric proteins were constructed. The SomNdomBclI construct where the region from G583 to P623 of testis ACE was replaced with the corresponding G561 to P601 region from the N domain, was shed and enzymatically active (unpublished results) (Figure 6.4.1). This suggested that the putative recognition motif for the ACE secretase, did not reside within the C-terminal region of the ectodomain. The chimeric proteins discussed in this chapter, SomNdomBglII, SomNdomBglII-NheI, and SomNdomNheI, were all expressed in CHO-KI cells and this was confirmed by western blot analysis and confocal microscopy. SomNdomBglII and SomNdomNheI were shed into the culture medium, indicating that the testis ACE protein sequences that had been replaced with corresponding N domain fragments did not contain the recognition motif for the ACE secretase.

Enzyme kinetic data on SomNdomBglII has been published (Marcic at al, 2000b). Our data corroborated these findings and further indicated that the N-terminal region of testis ACE is integral to the enzyme activity of the protein (i.e., of the C domain), even though it is distal to the active site. In addition, SomNdomBglII was also

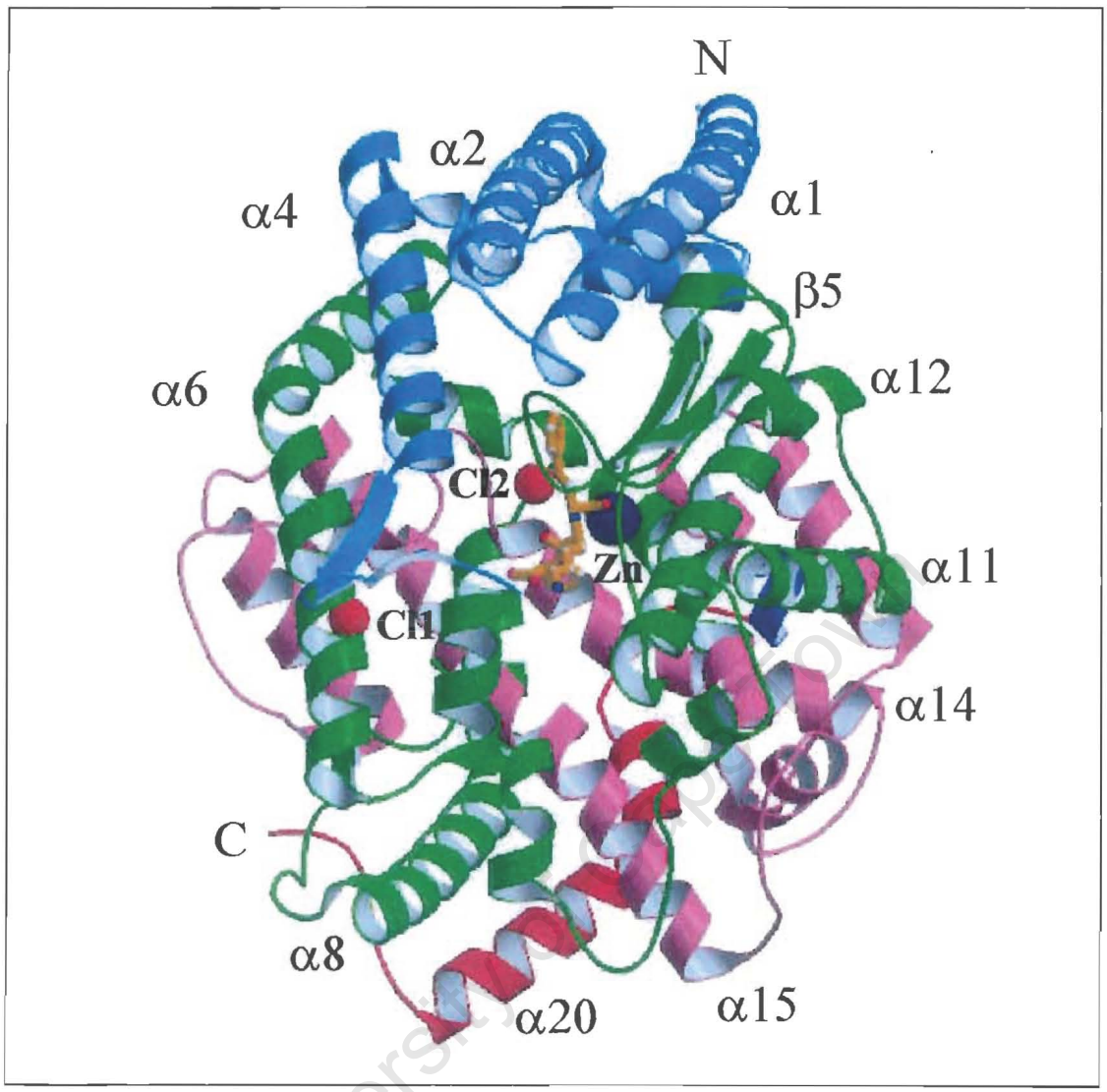


Figure 6.4.1 Position of chimeric sequences within the native conformation of testis ACE The ribbon representation of the crystal structure of testis ACE is shown above (Natesh et al, 2003) with the testis ACE sequences that were replaced in the construction of the testis ACE-N domain chimeras coloured differently: SomNdomBglII shown in cyan, SomNdomBglII-NheI shown in green, SomNdomNheI depicted in pink and SomNdomBclI shown in red. The chloride and zinc ions are shown as the red and black spheres, respectively. Lisinopril is shown in yellow. The blue ribbon indicates the sequence L580-T582, which encodes the *Bcl* I site and was not replaced in the construction of SomNdomNheI or SomNdomBclI, as the *Bcl* I site was used for sub-cloning of the constructs into pLEN.

reported to hydrolyse the N domain-specific substrate angiotensin 1-7 (Marcic et al, 2000b). Angiotensin 1-7 inhibits the C domain, but SomNdomBglII hydrolysed the peptide approximately 2-fold more efficiently than wild-type somatic ACE (Deddish et al, 1998; Marcic et al, 2000). This suggested that the C domain active site of SomNdomBglII had become more N domain-like and had an altered ability to hydrolyse HHL. The 3-dimensional crystal structure of testis ACE indicated that the region replaced in SomNdomBglII comprised α-helices, α1, α2, α3 and α4 and β-sheets, β1 and β2 (Figure 6.4.1). Helices α1, α2, and α3 formed a "lid" to the central groove formed by the two sub-domains of testis ACE comprising the active site. This "lid" structure could restrict access of large polypeptides to the active site (Natesh et al, 2003). The sequence identity between the N and C domains of the replaced region of SomNdomBglII is 25%. Moreover, the N domain sequence contains two additional putative *N*-glycosylation sites, which may alter the overall conformation of the N-terminal region of testis ACE (AI.5). Thus, the altered substrate specificity and enzymatic character of SomNdomBglII was likely caused by alterations in the "lid" structure of testis ACE.

The SomNdomNheI chimera had a region of protein sequence replaced 30 amino acid residues distal to the active site and the enzymes activity was altered similar to that of SomNdomBglII. The soluble form of this enzyme hydrolysed HHL at a much lower efficiency as indicated by its z-PHL/ HHL ratio, which was 12-fold and 3-fold higher than testis ACE and the N domain, respectively. Thus, the exchange of protein sequence distal to the active site between the two domains influenced the rate at which testis ACE hydrolysed HHL.

The western blot analysis, confocal microscopy, and enzyme kinetics of SomNdomNheI suggested there were two populations of the chimeric protein: (1) an enzymatically active population that was rapidly shed into the medium, and (2) an inactive population that was processed to the cell-surface. In addition, the western blot indicated a very intense cell-associated protein band. This protein band could comprise the mature and immature forms of the chimeric protein and its intensity could imply accumulation within the cell due to incorrect processing. The top band of the protein doublet detected by western blot analysis was most likely the mature form

of the processed protein, as it was of higher molecular weight and corresponded to the soluble form detected in the medium. The cell-associated mature population of chimeric protein, however, was enzymatically inactive. The testis ACE region replaced with N domain sequence has 62% identity with the N domain. The residues, found in this region, that associate with the chloride ions, zinc atom and lisinopril are all conserved including R522, which was identified as essential for chloride activation (Liu et al, 2001). The replacement of the testis ACE sequence with the N domain sequence introduced three additional putative *N*-glycosylation sites (AI.4 & AI.5). These sites, N438 (G438, testis ACE numbering), N502 (T502) and N516 (S516) (Appendix I.5), occur within or in close proximity to α-helices, α15, α16, α17 and the 3_{10} helix, H6 (Figure 6.4.1). The helices, α16, α17 and H6, contain the amino acid residues involved in chloride and lisinopril binding (Natesh et al, 2003). As these residues associate with lisinopril, the authors suggest that these helical structures might also interact with the substrate. The three-dimensional structure of testis ACE indicated that the carbohydrate moieties occurred at the surface of the molecule (Natesh et al, 2003). Thus, introduction of bulky molecules could disrupt the overall conformation of testis ACE and interfere with the integrity of the active site cleft. Moreover, the position of *N*-glycosylation sites affects the processing of proteins through the ER, such that different chaperones are recruited to interact with the nascent protein and alternative processing pathways are traversed (Davis et al, 2000). We can thus speculate that the introduction of additional *N*-glycosylation sites could affect the overall processing of testis ACE such that two populations of SomNdomNheI are produced.

The enzymatically active SomNdomNheI protein was rapidly shed into the culture medium, as indicated by the lack of cell-associated ACE activity (Figure 6.3.3 B). It is possible that the inactive population of chimeric protein was also shed, as this would not be detected by HHL hydrolysis. However, no concomitant decrease in cell-associated SomNdomNheI was observed in the presence of PDBu, making this unlikely (Figure 6.3.3 A). Speculation on the processing of inactive SomNdomNheI does not detract from the fact that this chimera was proteolytically cleaved within the stalk by the presumed ACE secretase. Thus, it is unlikely that the protein sequence

exchanged in the construction of this chimera comprised the recognition motif for the ACE secretase.

The region of protein sequence replaced in the chimeric protein, SomNdomBglII-NheI, included the zinc-binding site of testis ACE. This protein was inactive and not correctly processed to the cell surface, although confocal microscopy indicated expression of this protein intracellularly. As this chimeric protein was not correctly processed to the cell surface, no conclusions can be made about its ability to be shed. Thus, the region from D164 to V416 of testis ACE could potentially comprise the recognition motif for the ACE secretase (Figure 6.4.1).

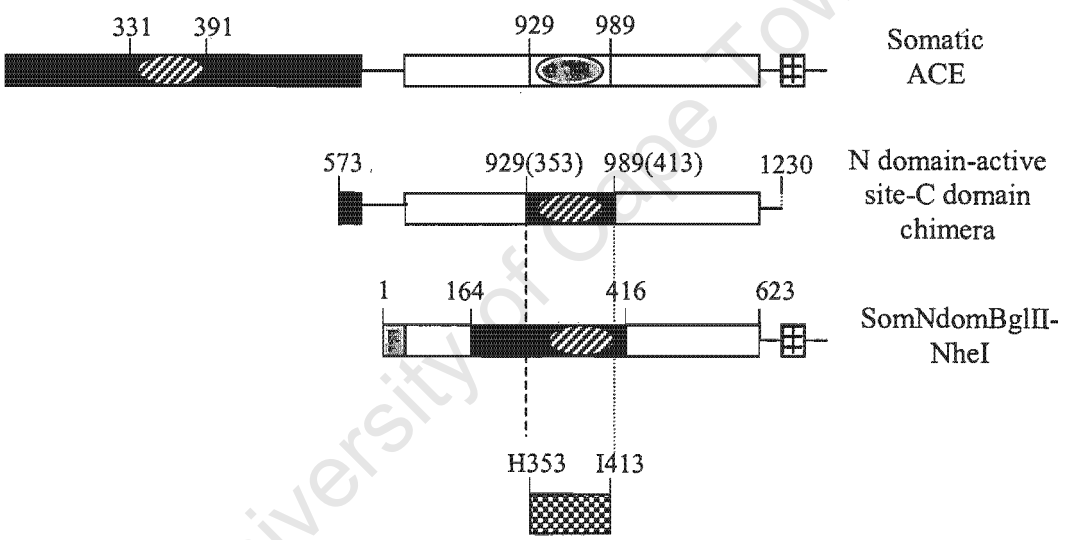


Figure 6.4.2 Schematic diagram of the region of testis ACE comprising the potential recognition motif Somatic ACE is indicated with the N domain (black bar) and the C domain (open bar) with the respective active sites of the two domains shown as the striped and grey oval. An N domain-active site-C domain chimera was constructed where 60 amino acid residues [(929-989) (353-413, according to testis ACE numbering)] of the C domain was replaced by the corresponding region from the N domain (331-391) (Williams et al, 1996). This chimera lacked the TM and included a region of the N domain at the N-terminus. SomNdomBglII-NheI is shown with the region from 164 to 416 (testis ACE numbering) replaced by N domain sequence (black bar). The region of testis ACE not essential for enzymatic activity is shown by the chequered bar from H353 to I1413.

Williams et al constructed a chimera where 60 amino acid residues from H929 to A989 (including the zinc-binding site) of the C domain were replaced with the corresponding region of the N domain (H331-I391) (Figure 6.4.2) (Williams et al, 1996). This chimera had k_{cat} and K_m values comparable to the C domain indicating that the zinc-binding site and the flanking residues did not play a role in the substrate specificity of the C domain. This is not surprising, as the N domain and C domain sequence alignments of the region extending from H353 to I413 (testis ACE numbering) exhibit 80% identity between the two domains (Figure 6.4.3). Therefore, the secondary structure of testis ACE i.e. β4, β5, α13, and H5 should not be disrupted (Figure 6.4.3) (Figure 6.4.1). SomNdomBglII-NheI was completely inactivated and not processed to the cell-surface when the region of testis ACE, from D164 to V416, was replaced with the corresponding region of the N domain from D142 to V393 (Figure 6.4.3). As the region from H353 to I413 was not involved in the substrate specificity or processing of the C domain, the corresponding N domain region substituted for D164-C352 of testis ACE in SomNdomBglII-NheI must differ significantly from the C domain, as it was not able to maintain the substrate specificity, cellular processing or structural integrity of testis ACE (Figure 6.4.2) (Williams et al, 1996).

The three-dimensional structure of testis ACE indicated that the C domain comprises two sub-domains, Thr39 – Val291 and Pro 292 to Pro 617 (Natesh et al, 2003). The region replaced in the construction of SomNdomBglII-NheI overlaps with the junction of these two sub-domains (Figure 6.4.3). The N domain sequence has introduced a putative *N*-glycosylation site at N311 (T311, testis ACE sequence) within helix α11 (A.I.5) and eliminated a *N*-glycosylation site at N337 within helix α12 (Figure 6.4.3). Helix α11 occurs at the N-terminus of the C-terminal sub-domain and could disrupt the overall integrity of the structure. Moreover, the construction of SomNdomBglII-NheI also eliminated R186, which binds to the first chloride ion. This chloride ion is bound by four ligands derived form both sub-domains of testis ACE (Natesh et al, 2003). The C domain active site is more strongly activated by chloride than the N domain (Jaspard et al, 1993). The actual mechanism of substrate-dependent chloride activation of ACE is unknown, but could involve structural changes, which affects the activity of the enzyme. Thus, the deletion of R186, a Cl^- ligand, might

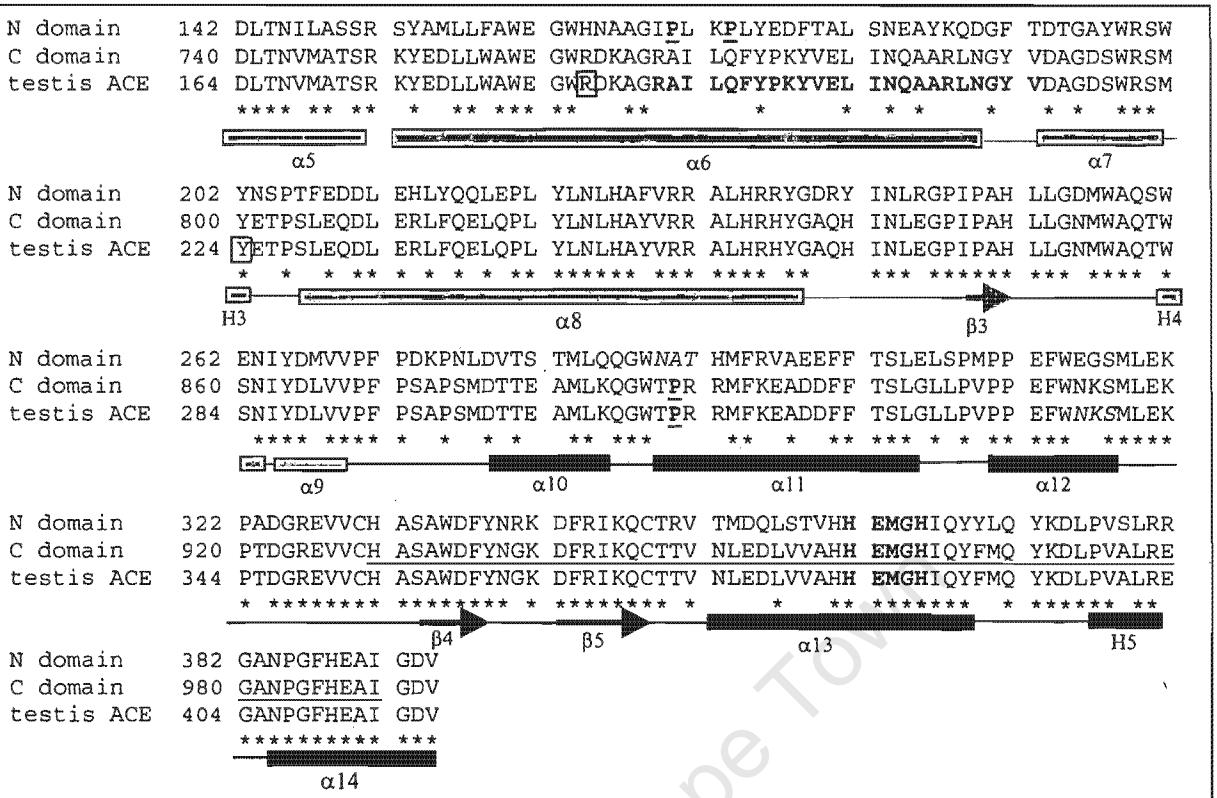


Figure 6.4.3 Sequence alignment of replaced region of SomNdomBglII-NheI Sequences of the N domain, C domain and testis ACE are aligned, numbered according to Soubrier et at and Ehlers et al, 1989. Sequence identity is indicated with a star below the amino acid residue identical in both domains and the secondary structure of testis ACE is illustrated below with the bars indicating α-helices (α) and 3_{10}-helices (H), the arrows representing β-sheets (β) and the lines indicating extended polypeptide structures. The grey and black bars and arrows represent the two sub-domains of testis ACE (Natesh et al, 2003). The underlined C domain sequence represents the region replaced by N domain sequence by William et al (Williams et al, 1996). The active site and the testis ACE region comprising the putative ACE secretase recognition motif is in bold. The amino acid residues involved in chloride binding are boxed: R186 (grey box) binds to Cl^-1 and Y224 (clear box) binds to Cl^- 2 (Natesh et al, 2003). The glycosylation sites are in italics. The proline residues not conserved are in bold and underlined.

affect the overall structure of ACE and result in the inactivation of the enzyme. The substitution mutagenesis of testis ACE in the construction of SomNdomBglII-NheI involved a large region of protein sequence and as indicated, these substitution mutations rendered testis ACE inactive. Thus, the region replaced in SomNdomBglII-NheI of the N domain, spanning various secondary structural motifs, was unable to maintain the three-dimensional structure of testis ACE. However, the substitution of

smaller regions of the N domain for testis ACE sequence might not have the same disruptive effect.

The C domain region, comprising the recognition motif of the ACE secretase is likely to have low sequence identity with the N domain while maintaining the native conformation. Due to the unavailability of a three-dimensional structure for the N domain, a direct comparison between the two domains is not possible. However, by comparing the sequence identity of the two domains and examining the three-dimensional structure of testis ACE, we have narrowed down the region that might comprise the recognition motif. The chimera constructed by Williams et al was truncated before the TM domain and thus its ability to be shed from the membrane could not be investigated (Williams et al, 1996). However, this construct plus the TM would, in all probability, be shed as the replaced region (H353 to I413, testis ACE numbering) has high sequence identity (80%) with the N domain and is thus, unlikely to comprise the recognition motif for the ACE secretase. There is approximately 62% identity between the two domains from D164 to C352 with the lowest similarity (21% identity) occurring between R191 and V214. The region R191-V214 occurs within helix α6, which is kinked due to the presence of P198 (Natesh et al, 2003). There are only three prolines not conserved in SomNdomBglII (Figure 6.4.3). The replacement of the testis ACE sequence with the corresponding N domain sequence introduced two prolines, P170 and P173 (N domain numbering) and deleted a third one, P312 (testis ACE numbering) (Figure 6.4.3). Residue P312 was replaced by a putative *N*-glycosylation site at N311 (AI.5) and this could disrupt the structure of testis ACE, as discussed previously. The region from R191 to V214 has the lowest sequence identity of the replaced region of SomNdomBglII and contains two of the three unconserved proline residues. Thus, the high conservation of the proline residues between the two domains and the affect these aromatic amino acid residues have on secondary structure suggests that the region R191-V214 might comprise the recognition motif for the ACE secretase. Moreover, helix α6 is located on the outside of the three-dimensional model of testis ACE and would thus be accessible to the ACE secretase. Therefore, replacement of these 24 amino acid residues with N domain sequence should not affect the native structure of testis ACE, but may be a good candidate for the region containing the ACE secretase recognition motif.

Chapter 7

The three-dimensional structures of alternate disulfide-bridged domains within the juxtamembrane region of testis ACE differentially affect shedding

7.1 Introduction

This chapter investigates the structural features of a juxtamembrane region that facilitate the proteolytic processing of membrane proteins. The juxtamembrane region or the stalk region is defined as that stretch of amino acid residues that connect the transmembrane domain to the extracellular domain of ectoproteins. Proteolysis occurs within this stalk region to release the active extracellular domain into the surrounding environment. As the stalk is the ultimate target for the secretase, this region has been investigated as a possible determinant for the release of membrane proteins. The nature of the stalk region could facilitate proteolysis by 1) comprising a sequence motif for the secretase, 2) providing a specific distance between the TM and the ectodomain for orientation of the secretase, or 3) consisting of a random structure that offered no obstruction to proteolysis as would other structurally distinct, folded domains.

Unaltered shedding of membrane proteins such as APP, L-selectin, TNF-α receptor (tumour necrosis factor), IL-6R and TNF-α whose residues proximal to their cleavage sites had been mutated suggests that sequence specificity does not play a significant role in determining the release of membrane proteins (Sisodia, 1992; Migaki et al, 1995; Brakebusch et al, 1994; Mullberg et al, 1994; Tang et al, 1996). In addition, the replacement of the stalk of ACE with the stalk region of the low-density lipoprotein receptor (LDL-R) maintained shedding (Ehlers et al, 1996). The effect of the length of the stalk on the shedding of ACE and pro-TNF-α was shown to be a limiting factor (Ehlers et al, 1996; Tang et al, 1996). The P- and E-selectins and the integrin β-subunits have short stalks ranging from five to eight residues and these proteins are

poorly shed (Kahn et al, 1994; Kishimoto et al, 1987), indicating that a stalk providing sufficient room between the ectodomain and the transmembrane domain is essential for shedding to occur. The limiting length of the stalk region hints at an alternative constraint on the shedding of membrane proteins. This constraint involves the steric hindrance imposed by the ectodomain when brought too close to the membrane thus not allowing the secretase access to the stalk region. In the case of L-, P-, and E-selectin the C-terminal domain comprises an EGF domain, and TNF-α-receptor has numerous cysteine residues in the proximal extracellular region thought to be highly disulfide bridged (Somers et al, 2000; Graves et al, 1994; Kahn et al, 1994; Nophar et al, 1990; Barclay et al, 1993). These disulfide bridges would create a highly structured folded domain which, if occurring near the transmembrane region, might cause steric hindrance. E- and P-selectin are not released from the membrane, whereas L-selectin is. The stalk length of L-selectin is longer than that of the other two selectins and deletion of regions of the stalk of L-selectin abolished shedding. This implied that once the disulfide-linked domain was brought nearer the transmembrane regions, the secretase was unable to cleave within the stalk region (Kahn et al, 1994; Brakebusch et al, 1994; Migaki et al, 1995).

To investigate whether the steric accessibility of the stalk region played a role in distinguishing shed proteins from those not targeted for release from the membrane, the stalk of testis ACE was replaced with the EGF domain from the LDL-R (Schwager et al, 1998). Surprisingly, this mutant, ACE-JMEGF, was shed from the membrane of CHO-KI cells. The proteolysis was shown to be upregulated in the presence of PDBu and inhibited by TAPI. Cleavage site analysis showed that this construct was cleaved within the third disulfide loop, which resulted in tethering of the ectodomain to the TM, by the disulfide bond after cleavage within the peptide backbone. This would account for the inefficient release of this molecule from the membrane (Schwager et al, 1998).

EGF domains consist of three disulfide bonds, the first two comprising a disulfide β-cross structure and the third loop containing two β-turns. Homology modelling of the EGF-like domain of ACE-JMEGF indicated that the third disulfide loop of the EGF-like domain of the LDL-R was sterically accessible allowing proteolysis to occur

(Schwager et al, 1998). The structure of the first two EGF-like domains of the LDL-R has been solved using NMR (Malby et al, 2001; Saha et al, 2001), and this structure correlates well with the homology model of the EGF-like domain of ACE-JMEGF (Schwager et al, 1998).

To investigate whether the three-dimensional structure of the third disulfide loop allowed for proteolysis, three additional ACE mutants were constructed. The ACE stalk was replaced with the EGF-like domain from the clotting agent, factor IX and the synthetic disulfide-linked peptide, min23, both of which have known crystal structures. To determine whether the number of residues in the loop of the LDL-R EGF domain affected shedding, the third ACE-stalk mutant was constructed. This ACE chimera was identical to ACE-JMEGF except for the deletion of three amino acid residues from the third disulfide loop of the EGF-like domain. The four constructs, ACE-JMEGF, ACE-JMfIX, ACE-JMmin23, and ACE-JMEGFΔ3 were expressed in CHO cells and their shedding was investigated. The cleavage sites were determined and the tertiary structure of the third disulfide loops of the constructs were compared to each other to elucidate the structural features required for proteolysis.

7.2 Methods

7.2.1 **Construction of the disulfide-linked testis ACE stalk mutants**

7.2.1.1 **ACE-JMfIX PCR**

ACE-JMfIX was constructed using a two-stage PCR procedure involving three independent PCR reactions. Firstly, the sequence encoding R651 to S701 of human

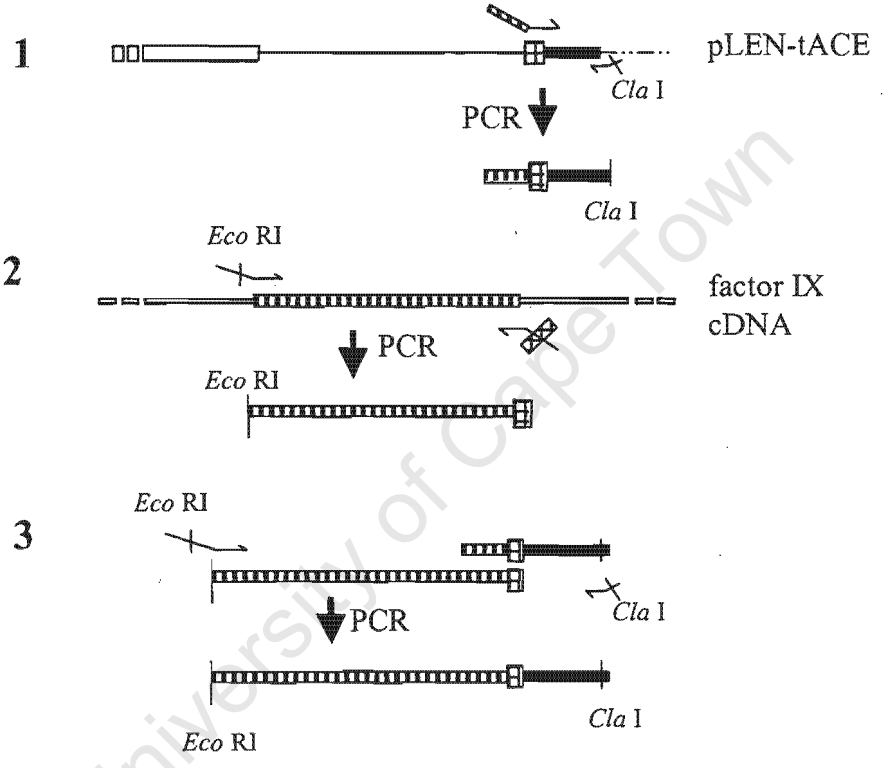


Figure 7.2.1 Substitution of the ACE stalk with the EGF-like homology domain of factor IX The two-stage construction of ACE-JMfIX is shown schematically above with the three independent PCR steps indicated. In step 1, the nucleotide sequence of pLEN-tACE spanning the TM (hatched box) and cytoplasmic tail (bold line) was amplified with the 5'-extension of the forward primer comprising complimentary sequence to the EGF-like domain (striped bar) of factor IX and the reverse ACE primer, MP2 introducing a *Cla* I site at nt 2222. In the second step the EGF-like domain from factor IX (striped bar) was amplified with the forward primer introducing an *Eco* RI site at its 5'end and the 5'-extension of the reverse primer comprising complementary sequence to the TM domain (hatched box) of testis ACE. The third step involved the annealing of the overlapping, complimentary sequences and PCR using the outer two primers. This PCR product was sub-cloned into pBS using *Eco* RI and *Cla* I and sequenced.

testis ACE (nt 2066-nt 2221) was amplified which comprised the transmembrane domain and cytoplasmic tail. In the second step, the cDNA of the human factor IX clotting factor was amplified from amino acid residue, D93 to E129, which encompassed the EGF-like domain with the introduction of an *Eco* RI site at the 5' end (Figure 7.2.1). The primers were chosen so that the reverse and forward primers of the two independent PCR reactions, consisted of overlapping, complimentary ends so that these two DNA fragments would anneal and allow for the amplification of the entire sequence with the two outside, flanking primers. In this way the EGF-like homology domain was directly fused to the transmembrane domain and cytoplasmic tail of testis ACE. The PCR product was cloned into pBS using the restriction enzyme sites *Eco* RI (introduced by silent mutation at the 5' end) and *Cla* I and sequenced using an ALFexpress DNA Automated Sequencer (APBiotech) to confirm the correct sequence of this ACE mutant.

7.2.1.2 **ACE-JMmin23 PCR**

Min23 is an autonomous folding unit derived from the squash trypsin inhibitor, EETI II, and comprises 23 amino acid residues with a cystine-stabilized β-sheet (CSB) structural motif comprising two disulfide bonds (Heitz et al, 1999). The ACE-JMmin23 construct was generated with the amplification of two regions of the testis ACE cDNA encoding: L580 to S625 and R651 to S701 (Figure 7.2.2). These two PCR products had overlapping, complimentary sequences encoding the sequence of Min23 that were generated by the 5'extensions of the reverse and forward primers of the two reactions. During the second stage of amplification, the overlapping Min23 sequence annealed and was amplified using the two testis ACE-derived, flanking primers, MP3 and MP2. ACE-JMmin23 has an *Eco* R1 site engineered at nt 1984 and a *Cla* 1 site at nt 2222. The final PCR product was sub-cloned into pBS using *Bam* H1 and *Cla* 1 and sequenced using the ALFexpress DNA Automated Sequencer (APBiotech) to confirm the correct sequence of the ACE mutant.

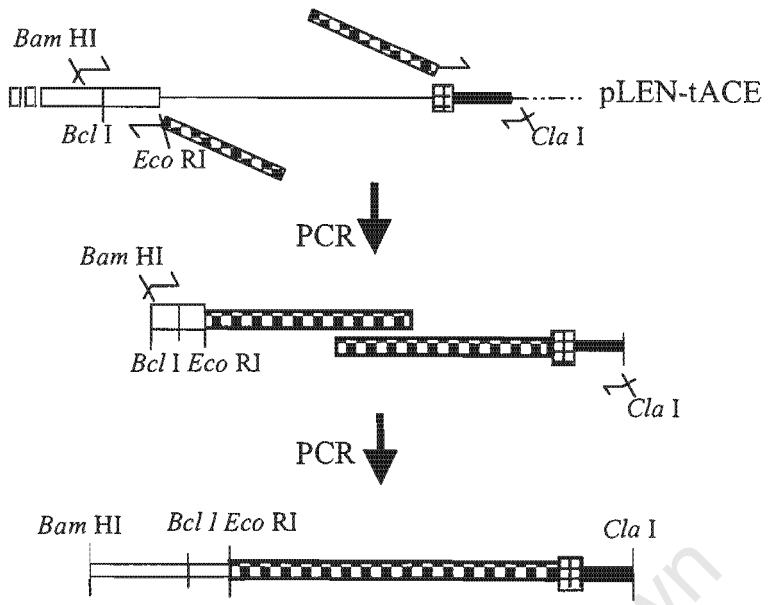


Figure 7.2.2 Substitution of Min23 for the ACE stalk Testis ACE is schematically depicted with the open bar indicating the ectodomain, the thin line indicating the stalk region, the hatched box representing the TM and the bold line indicating the cytoplasmic tail. Nucleotide sequence spanning the ectodomain (nt 1853 to nt 1990) and the TM and cytoplasmic tail (nt 2066 to nt 2221) was amplified to generate two DNA fragments with overlapping complementary sequence comprising the entire DNA sequence for min23 (chequered bar). The reverse primers of the independent PCR steps introduced an *Eco* RI site at nt 1984 and a *Cla* I site at nt 2222, respectively. The final PCR step involved the annealing of the complimentary min23 DNA sequence and amplification using the two outer primers, MP3 and MP2, which allowed the incorporation of a *Bam* HI site and a *Cla* I site at the 5' and 3'ends, respectively. These sites allowed the sub-cloning of the PCR product into pBS for sequencing.

7.2.1.3 **ACE- JMEGFΔ3 PCR**

In order to delete three amino acid residues from the ACE stalk mutant, ACE-JMEGF, two overlapping primers were designed which lacked the sequence encoding VAQ (656-658) of the EGF-like homology domain (Figure 7.3.8). ACE-JMEGFΔ3 was constructed using a two-stage PCR procedure whereby nucleotide sequence of ACE-JMEGF was amplified encoding L580 to L655 and R659 to S701 (Figure 7.2.3). Using this method, two PCR products of ACE-JMEGF were generated: the first comprised the ectodomain of testis ACE sequence fused to the EGF-like homology domain with an *Eco* R1 site engineered at nt 1984 and the second one had the EGF-

like homology domain directly fused to the transmembrane domain and cytoplasmic tail of testis ACE. The ACE-JMEGFΔ3 construct was generated by the combined amplification of the two PCR products using the flanking, testis ACE-derived primers, MP3 and MP2, introducing a *Bam* HI and a *Cla* 1 site at either end as shown. The final PCR product was subcloned into pBS using the *Bam* H1 and *Cla* 1 and sequenced using an ALFexpress DNA Automated Sequencer (APBiotech).

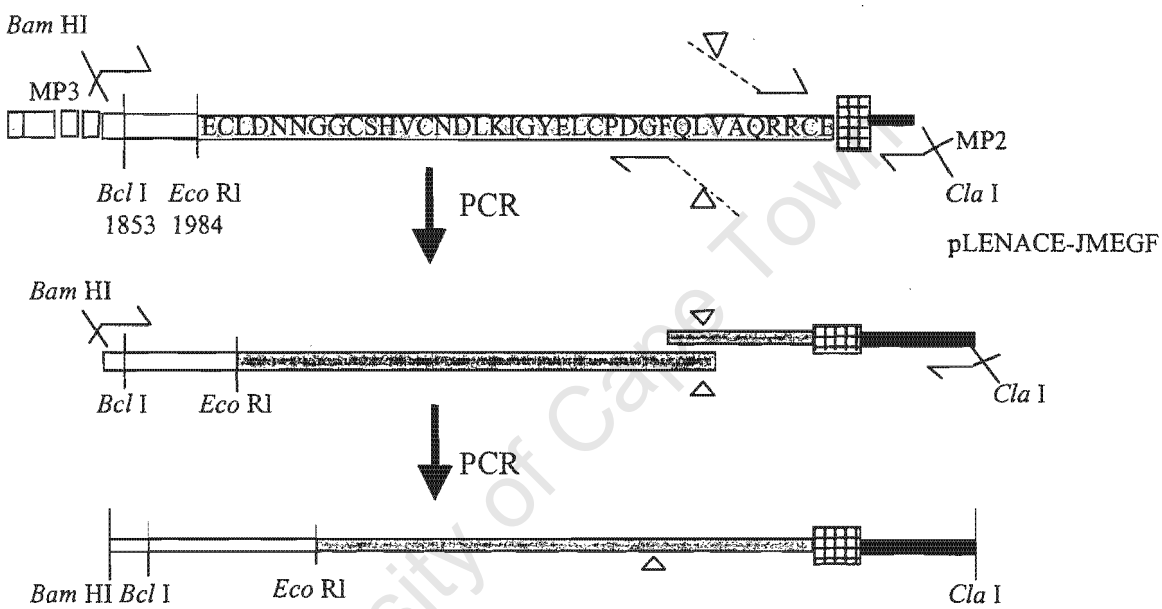


Figure 7.2.3 Deletion of 3 amino acid residues of the EGF-like homology domain of the LDL-R ACE-JMEGF is schematically represented with the open bar indicating the ectodomain (comprising an introduced *Eco* RI site at nt 1984, Schwager et al, 1998), the thin line indicating the stalk region, the hatched box representing the TM and the bold line indicating the cytoplasmic tail. The EGF domain, depicted as the grey bar, with the protein sequence within, was amplified using 4 primers and two independent PCR reactions. The two outer primers, MP2 and MP3, introduced *Bam* HI and *Cla* I sites at the 5'ends, respectively. The two inner primers were designed complimentary to the EGF nucleotide sequence so that the overlapping complimentary sequences at the 5'ends lacked the three amino acid residues, V656, A657 and Q658. The two stage amplification process resulted in two PCR products with overlapping complementary sequences as indicated and the final step involved the annealing of the overlapping sequences and amplification using the outer primers to generate the final PCR product which was digested with *Bam* HI and *Cla* I and sub-cloned into pBS for sequencing.

7.2.1.4 **Sub-cloning into pLEN**

Upon confirmation of the correct construction of the disulfide-linked domains by nucleotide sequencing, the PCR products were excised from pBS and sub-cloned into the mammalian expression vector pLEN. The PCR products were inserted into either pLEN-ACE-JMΔ24 or pLEN-tACE depending on the restriction enzyme sites used. pBSACE-JMflX was digested with *Eco* RI and *Cla* I and ligated to the similarly digested and gel-extracted (GenElute DNA extraction kit, SIGMA) pLEN-ACE-JMΔ24 as this construct has an engineered *Eco* RI site at nt 1984 which allowed the fusion of the EGF-like domain to the ectodomain of testis ACE while maintaining the correct open reading frame (Figure 7.2.4). pBS-ACE-JMmin23 and pBS-ACE-JMEGFΔ3 were digested with *Bcl* I and *Cla* I, gel-extracted (GenElute DNA extraction kit, SIGMA) and ligated to the similarly digested and treated pLEN-tACE (Figure 7.2.4).

These ligation reactions were transformed into either competent *E.coli* XL1-Blue cells or *E.coli* JM110 cells, a bacterial strain deficient in methylating enzymes depending on whether the *Bc*l I was used in the cloning procedure. The activity of *Bcl* I is sensitive to methylation. The ampicillin resistant cfus were cultured in 5 ml LB aliquots supplemented with ampicillin (100 μg/ml) and the DNA extracted using the phenol-based DNA mini-preparation method before digested with *Eco* RI and *Cla* I and the DNA fragments were compared to that obtained of the similarly digested pLEN-tACE. The cfu with the correct DNA fragment sizes was inoculated into 50 ml LB supplemented with ampicillin (100 μg/ml), cultured O/N and the DNA extracted using the QIAGEN midi kit. The DNA extracted from the 50 ml LB bacterial cultures were digested with *Eco* RI and *Not* I to test whether the nucleotide sequence encoding the EGF-like homology domains were successfully inserted in frame with the ectodomain of testis ACE. The introduction of an *Eco* RI site at nt 1984 and the loss of the *Not* I site at nt 2020 confirmed the correct construction of the disulfide-linked ACE stalk mutants (AII.4).

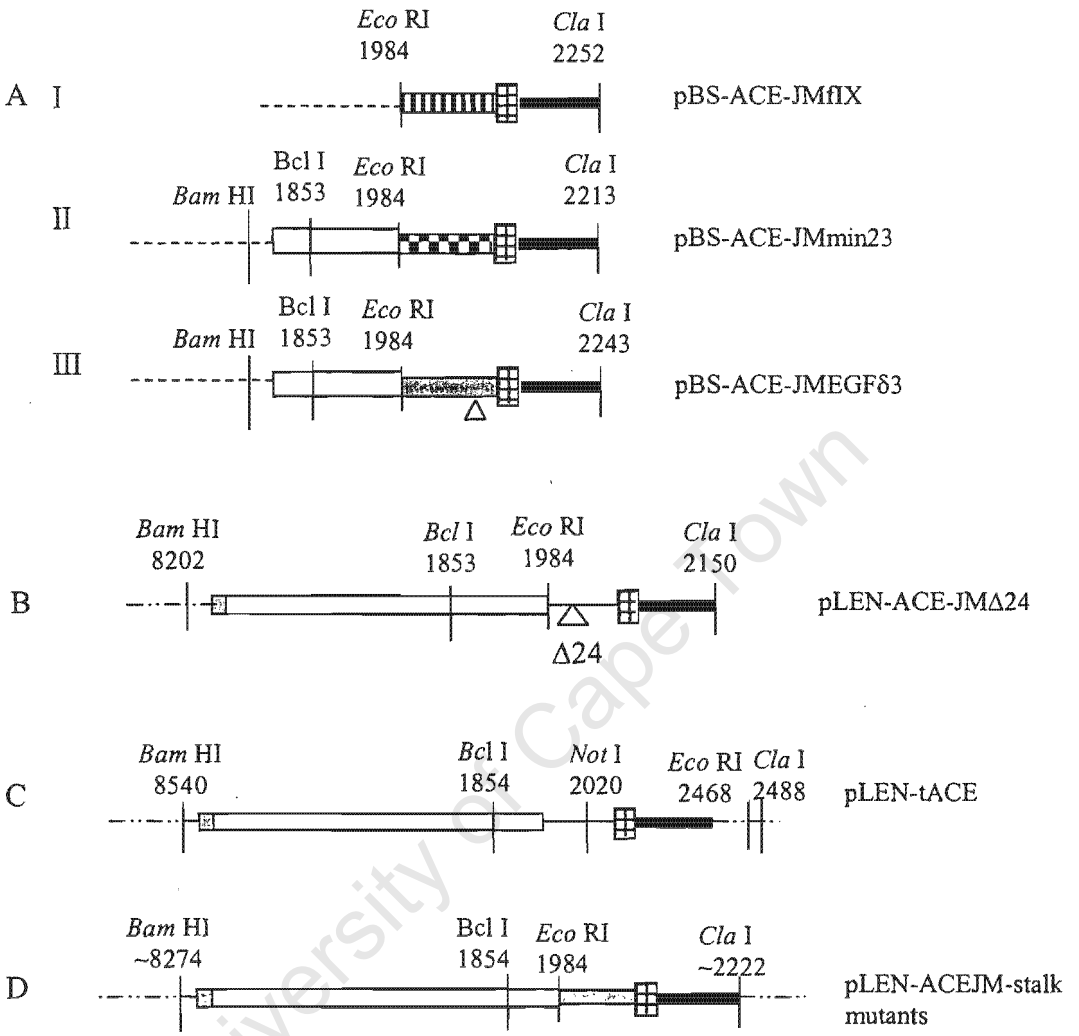


Figure 7.2.4 Cloning of the disulfide-linked ACE stalk constructs into the mammalian expression vector, pLEN The PCR products sub-cloned into pBS are shown in Figure A: ACE-JMfIX (I), ACE-JMmin23 (II) and ACE-JMEGFΔ3 (III) The PCR products were excised from pBS and sub-cloned into pLEN-ACE-JMΔ24 which has an introduced *Eco* RI site at nt 1984 (Ehlers et al, 1996) and pLEN-tACE (Figure C) to obtain the final construct whereby the stalk of testis ACE was replaced by the disulfide-linked domains in the mammalian expression vector, pLEN (Figure D). The open bar represents the ectodomain of testis ACE, the hatched box represents the TM and a bold line indicates the cytoplasmic tail. The stalk region is represented by a thin line in the case of ACE-JMΔ24 (Figure B) and wild type testis ACE (Figure C) whereas the disulfide-linked domains are indicated with a striped (ACE-JMfIX), chequered (ACE-JMmin23) or dark grey (ACE-JMEFGΔ3) bar in Figure A and a light grey bar in Figure D represents all of the stalk constructs cloned into the expression vector, pLEN.

7.2.2 **Expression of ACE constructs in CHO-KI cells, shedding kinetics and cleavage site analysis**

The three ACE mutants, ACE-JMfIX, ACE-JMmin23 and ACE-JMEGFΔ3 as well as ACE-JMEGF (Schwager et al, 1998), were stably expressed in CHO-KI cells and the shedding kinetics, cell surface localisation, protein purification and cleavage site analysis was determined as outlined in Chapter 2, section 2.3. The shedding from isolated membrane was performed as discussed in detail in Chapter 3, section 3.2 and outlined in Chapter 2, section 2.5.

7.2.3 **Determination of the intracellular proteolysis of ACE-JMfIX**

CHO-KI cells stably transfected with ACE-JMfIX cDNA were grown to confluence and induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM) before the cells were scraped into fresh 2% FCS complete medium and incubated at 15°C for 3 hrs. Incubation at this temperature allowed proteolysis but inhibited all intracellular membrane trafficking (Alfalah et al, 2001). The cells were then centrifuged at 4°C at 11,000 x g for 2 min and the cell pellet was resuspended in 1% Triton X-100 lysis buffer before the cellular proteins were separated into aqueous and detergent phases using Triton X-114. The aqueous and detergent phases were tested for ACE activity using the ACE substrate HHL. In addition, CHO-KI cells expressing ACE-JMfIX were incubated at either 4°C or 37°C for 5 min before centrifugation, cell lysis and phase separation to determine the level of soluble ACE activity associated with the cell lysate if shedding was inhibited (4°C) or allowed to continue normally (37°C). Similarly, CHO-KI cells expressing wild type testis ACE were also incubated at 4°C for 5min before centrifugation, cell lysis and phase separation. To control for the effect of Triton X-100 on the Triton X-114 phase separation technique, purified, soluble testis ACE was resuspended in either 1% Triton-X100 lysis buffer or Hepes buffer (20 mM) and subjected to Triton X-114 phase separation.

7.3 Results

7.3.1 **Shedding kinetics of the disulfide-linked testis ACE stalk mutants**

7.3.1.1 **Shedding from intact CHO-KI cells**

CHO-KI cells stably expressing testis ACE and the disulfide-linked ACE stalk mutants were grown in 35 mm dishes till confluent. To investigate the affect of disulfide-linked stalks on the shedding of ACE, ACE activity was determined in the culture medium and the cell lysates at increasing time points using the ACE substrate, HHL. The shedding of testis ACE and ACE-JMEGF was previously investigated (Schwager et al, 1998). The average level of ACE activity in the medium of the ACE mutants was expressed as the percentage soluble ACE activity of total ACE present in the cell lysates and culture media (Figure 7.3.1).

The shedding of testis ACE increased 4- to 5-fold with a concomitant decrease in cell-associated ACE activity in the presence of PDBu (Figure 7.3.1) (Schwager et al, 1998). The shedding of ACE-JMEGF was reported to be approximately 2- to 3-fold less than that of testis ACE in the absence of PDBu. Upon addition of PDBu, ACE-JMEGF shedding increased 3-fold with no concomitant significant decrease in cell-associated activity (Figure 7.3.1) (Schwager et al, 1998). In the presence of PDBu, the increase in shedding of ACE-JMEGF was 5-fold lower than that observed for testis ACE. These results indicated that this ACE mutant was shed less efficiently than testis ACE (Figure 7.3.1) (Schwager et al, 1998).

The constitutive shedding of ACE-JMflX and ACE-JMmin23 in the absence of PDBu was approximately 2-fold lower than that of testis ACE similar to that of ACE-JMEGF (Figure 7.3.2). The up-regulation of ACE-JMmin23 shedding in the presence of PDBu was identical to that of ACE-JMEGF with a 2- to 3-fold increase (Figure 7.3.2 B). However, unlike ACE-JMEGF and ACE-JMmin23, ACE-JMflX exhibited no stimulation in the presence of PDBu (Figure 7.3.2 A). Therefore, the introduction of a disulfide-linked stalk within testis ACE lowered the efficiency at which these mutants were shed from the membrane and in the case of ACE-JMflX, eliminated the stimulatory response to PDBu. ACE-JMEGFΔ3 comprises a disulfide-linked domain identical to ACE-JMEGF except for the third disulfide loop that has 3 amino acids

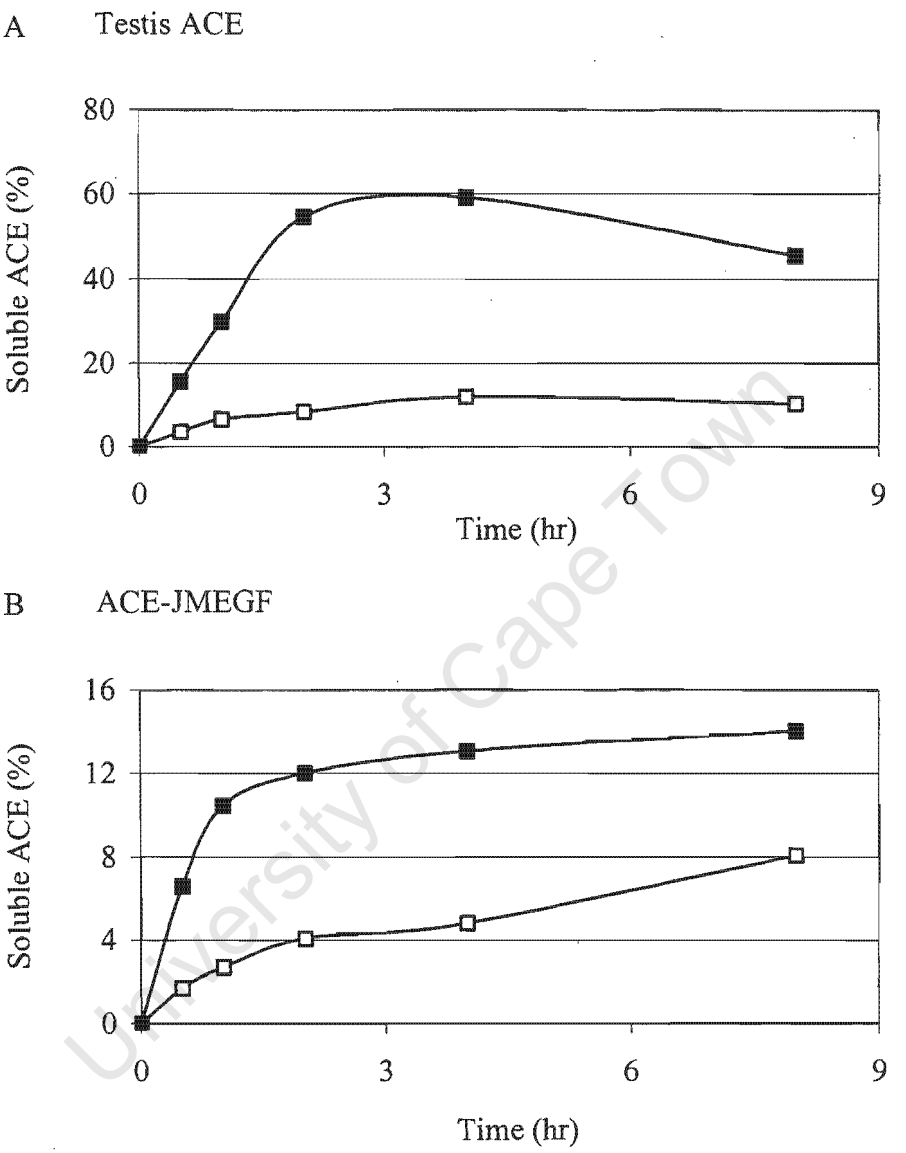


Figure 7.3.1 Shedding of testis ACE and ACE-JMEGF The level of soluble ACE is indicated as the percentage of ACE activity in both the culture media and the cell lysates (Schwager et al, 1996). Shedding in the presence (black squares) and absence (open squares) of PDBu was investigated. (n=3)

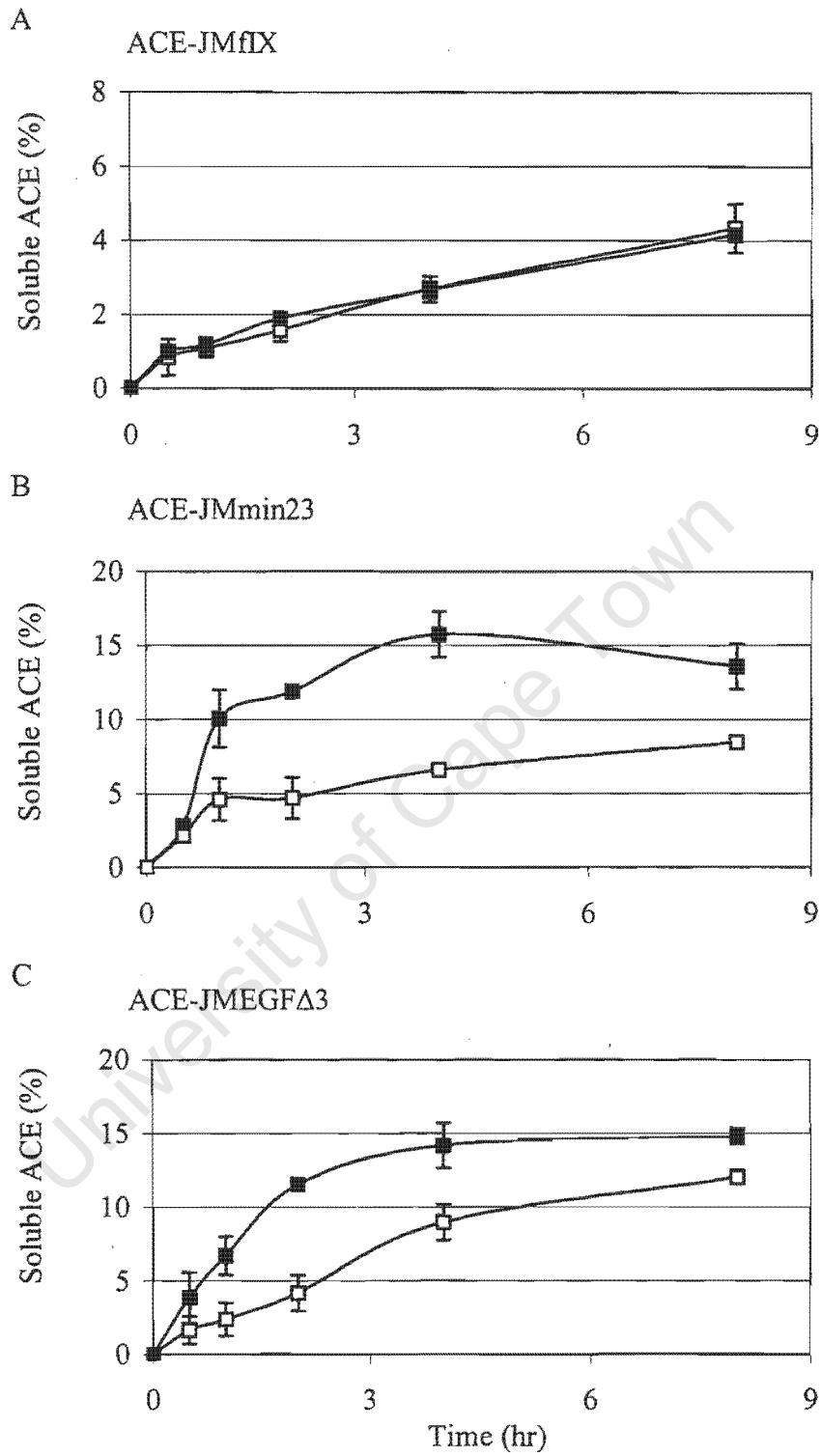


Figure 7.3.2 Shedding of ACE-JMfIX, ACE-JMmin23 and ACE-JMEGFΔ3 The level of soluble ACE is indicated as the percentage of ACE activity in both the culture media and the cell lysates. Black squares indicate shedding in the presence of PDBu and open squares indicate shedding in the absence. (n=3 +/- SD)

deleted. The percentage of soluble ACE-JMEGFΔ3 in the absence of PDBu was similar to testis ACE and higher than ACE-JMEGF indicating that the shortening of the third disulfide loop enhanced constitutive shedding (Figure 7.3.2 C). However, ACE-JMEGFΔ3 shedding was poorly upregulated in the presence of PDBu (1.5-fold). Thus, ACE-JMEGFΔ3 was shed as efficiently as testis ACE in the absence of PDBu but had a 3-fold lowered response to PDBu.

The replacement of the ACE stalk with the disulfide-linked domains of factor IX and min 23 lowered the efficiency of constitutive shedding although, ACE-JMEGFΔ3 shedding did not differ from that of testis ACE. However, the regulated shedding of the disulfide-linked ACE stalk constructs exhibited low or no stimulation in the presence of PDBu. Thus, the introduction of different disulfide-linked domains in the juxtamembrane region of ACE affected the shedding of ACE differently. This suggests that the accessibility of the stalk region is not only defined by random peptide stretches but is also dependent on either the nature of its primary sequence or its three-dimensional structure.

7.3.1.2 **Shedding from isolated membranes of CHO-KI cells**

The release of membrane proteins is a regulated event and thus mutations introduced to investigate the effect on shedding could also affect cellular processing of the native protein. To eliminate the effect disulfide-linked domains could have on the cellular processing of ACE, membranes were isolated from CHO-KI cells, stably expressing the different ACE stalk constructs. The membranes were incubated at 4°C, 15°C, and 37°C and shedding was compared to the release of testis ACE previously determined in Chapter 3, section 3.3.4.3. The shedding of ACE-JMmin23 from intact CHO-KI cells did not differ from that of ACE-JMEGF (Figure 7.3.2 B) and this stalk construct was not further investigated using isolated membrane.

There was no change in soluble ACE activity over a 2 hr period when the membranes of CHO-KI cells expressing ACE-JMEGF, ACE-JMfIX, and ACE-JMEGFΔ3 were incubated at 4°C, indicating that no shedding occurred at this temperature (Figure 7.3.3). However, soluble ACE activity increased when membranes were incubated at

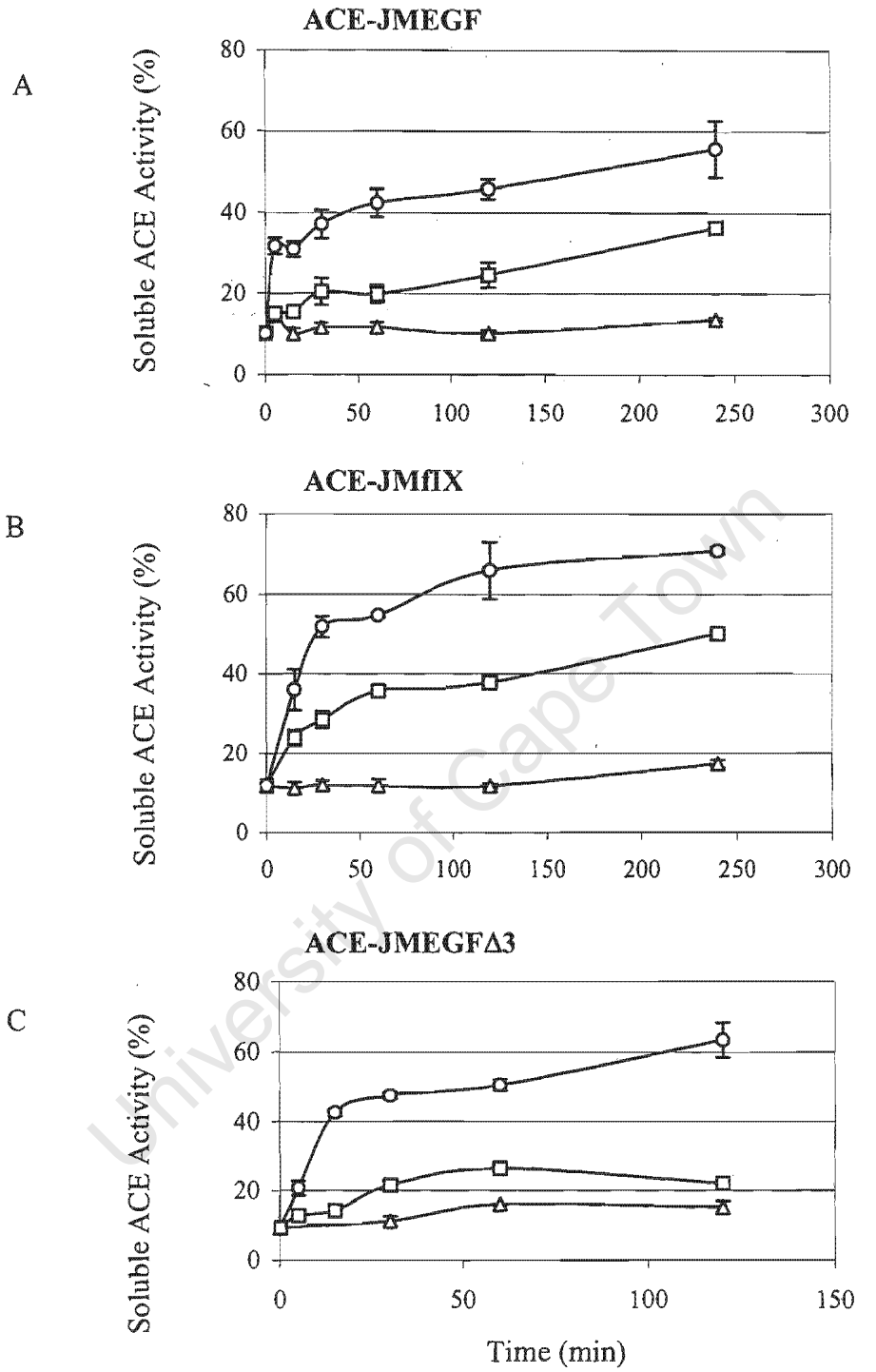


Figure 7.3.3 Shedding of the disulfide-linked ACE stalk constructs Membranes were purified from CHO-KI cells expressing ACE-JMEGF (A), ACE-JMflX (B) and ACE-JMEGFΔ3 (C) using the method outlined in Chapter 2: Total Membrane Isolation Protocol II. The membranes were incubated at 37°C (open circles), 15 °C (open squares) and 4 °C (open triangles) for the times indicated on the x-axis before the soluble ACE constructs were separated by centrifugation from its membrane-bound counterparts. The level of shedding is indicated by the fraction of ACE activity associated with the supernatant as a percentage of the total ACE activity in the membrane pellet and the supernatant. (n=3 +/- SD)

37°C and 15 °C in a time- and temperature-dependent fashion indicating that the chimeric proteins were shed from the membrane and that shedding was susceptible to changes in temperature (Figure 7.3.3). Maximum solubilisation (~70%) of testis ACE (Figure 3.3.6) from isolated membrane occurred within the first 5 to 10 min of incubation at 37°C, indicating efficient shedding whereas only ~35% somatic ACE was solubilised in the same time period (Figure 4.3.4). The shedding of the disulfide-linked ACE stalk constructs at 37°C showed strong similarity to the shedding of somatic ACE at 37°C after 5 to 10 min of incubation (Figure 7.3.3). This indicated that the substitution of the stalk sequence with a disulfide-linked domain lowered the efficiency of shedding of testis ACE. The constitutive shedding of ACE-JMEGFΔ3 from intact cells was very similar to testis ACE. However, the rapid shedding of testis ACE from isolated membrane was not observed for ACE-JMEGFΔ3 (Figure 3.3.6). In fact its shedding from isolated membrane was as inefficient as ACE-JMEGF (Figure 7.3.3). Thus, when shedding was investigated at the membrane level and cellular integrity was disrupted, ACE-JMEGFΔ3 was inefficiently shed, but with intact cellular machinery, the shedding of this construct was similar to wild type ACE. This suggests that alterations of primary sequence and the structure of the disulfide-linked domain could affect the cellular processing of ACE differently.

The cleavage site of ACE-JMEGF was identified as occurring within the third disulfide loop and the cleavage product remained tethered to the membrane by the disulfide bond (Schwager et al, 1998). This stalk mutant was shed in a time-dependent manner from isolated CHO-KI membranes, indicating that the release of the tethered, proteolytically cleaved product could be due to the presence of a reducing agent in the membrane fractions isolated. Alternatively, it could indicate that there are two populations of ACE-JMEGF: one with and one without the third disulfide bond.

7.3.2 **Specificity of shedding determined by protease inhibitors and phorbol esters**

The shedding of membrane proteins has been proposed to be the function of two classes of secretases (Ehlers et al, 1996): the first class is a family of metalloproteases responsive to hydroxamate inhibitors implicated in the shedding of TNF-α (Mohler et

al, 1994; Gearing et al, 1994; McGeehan et al, 1994), APP (Lammich et al, 1999), Notch receptor (Pan et al, 1997), p75 TNF-α (Crowe et al, 1995) and ACE (Ramchandran & Sen, 1995; Schwager et al, 1998); and the second class consists of elastase-like serine proteases involved in the release of TGF-α and the kit ligands, KL-1 and KL-2 (Pandiella et al, 1992; Cappelluti et al,1993). Recently, it was shown that the introduction of a point mutation within the stalk region of testis ACE resulted in the recruitment of a serine protease to release ACE, not at the cell surface as expected but in the endoplasmic reticulum (ER) (Alfalah et al, 2001). To test whether the differences observed in the shedding of the ACE stalk disulfide-linked constructs discussed in this chapter were due to processing by alternative secretases, CHO-KI cells expressing these constructs were grown in the presence of protease inhibitors to test the specificity of action of the ACE secretase(s) involved in each case.

7.3.2.1 **Specificity of shedding from intact CHO-KI cells**

CHO-KI cells stably expressing the disulfide-linked stalk ACE constructs were grown to confluence and induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (40 μM) before the addition of fresh 2% FCS complete medium containing either PDBu (Figure 7.3.4 A), the hydroxamate inhibitor TAPI (Figure 7.3.4 B), or the serine protease inhibitor DCI (Figure 7.3.4 C) and grown for 4 hrs. Testis ACE, ACE-JMEGF and ACE-JMmin23 shedding were stimulated in the presence of PDBu. However, there was no phorbol ester stimulation of ACE-JMfIX shedding and ACE-JMEGFΔ3 shedding increased only slightly (Figure 7.3.4 A).

The shedding of ACE-JMEGF, ACE-JMmin23 and ACE-JMEGFΔ3 was inhibited by TAPI, indicating that these constructs were shed by a metalloprotease. Only, the shedding of ACE-JMfIX was not inhibited by TAPI suggesting that the secretase responsible for the shedding of this construct was not a member of the metalloprotease family implicated in ACE shedding. DCI, was shown to inhibit the shedding of ACE stalk mutants (Schwager et al, 1999; Alfalah et al, 2001). To test the effect of DCI on the shedding of ACE-JMfIX, CHO-KI cells expressing this construct were grown in the presence of DCI. The shedding of this construct was inhibited by ~60% whereas wild-type testis ACE shedding was increased 2- to 3-fold. As ACE-JMEGFΔ3 shedding was only slightly stimulated by PDBu, DCI was tested as a

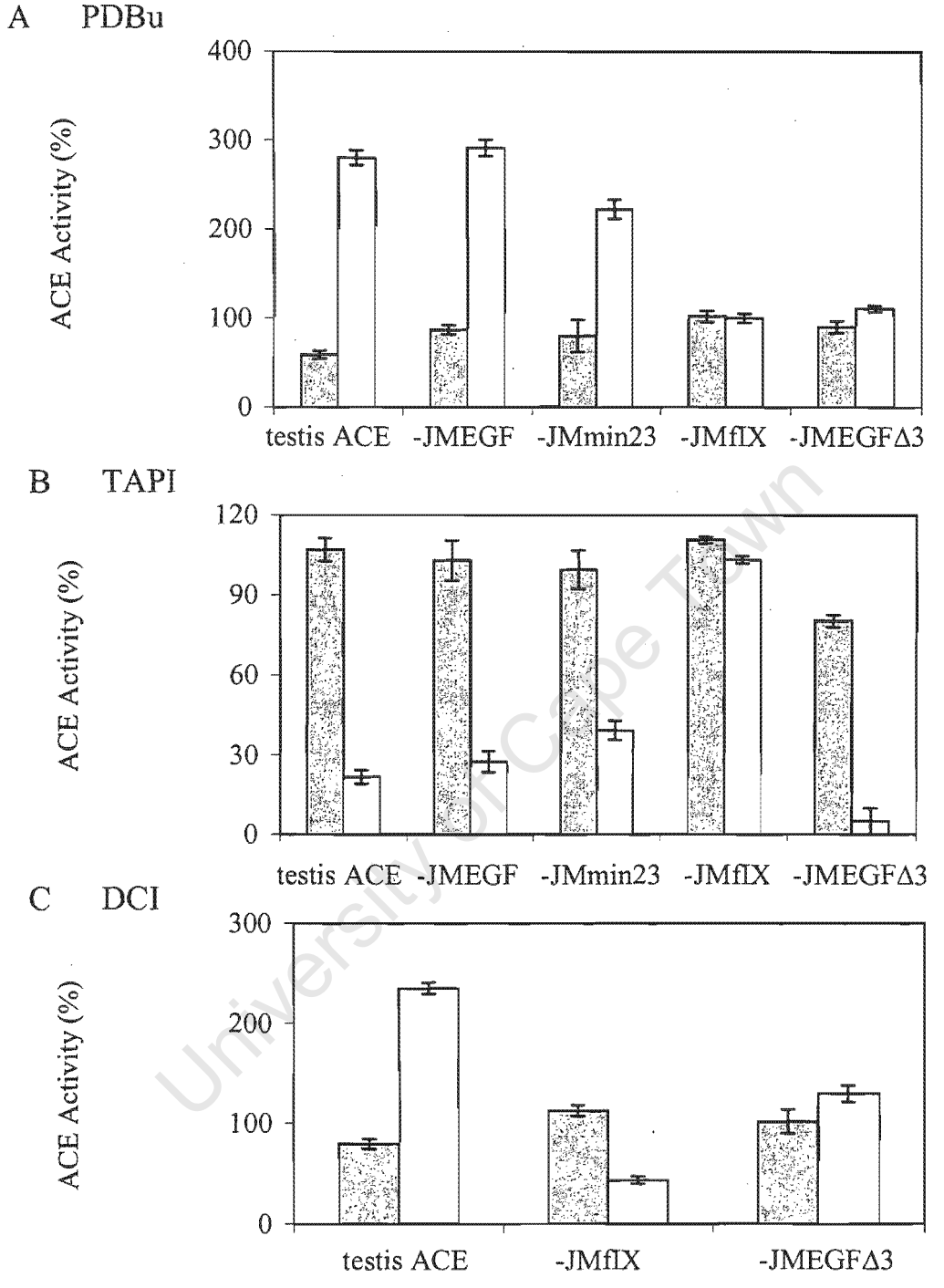


Figure 7.3.4 Determination of the specificity of proteolysis by the addition of stimulators and inhibitors CHO-KI cells expressing the cDNAs of testis ACE and the disulfide-linked ACE stalk constructs were grown in the presence of PDBu (1 μM) (A), TAPI (100 μM) (B) and DCI (200 μM) (C) for 4 hours before the medium (white bars) and the lysed cells (grey bars) were assayed using the ACE substrate HHL. Results are expressed as a percentage relative to the control (no treatment) which were taken as 100% in each case. (n=3 +/- SD)

possible shedding inhibitor of this construct. ACE-JMEGFΔ3 was not inhibited by DCI and furthermore the sharp increase in shedding seen for wild type testis ACE was not observed (Figure 7.3.4 C). This suggested that the shedding of ACE-JMEGFΔ3 was not due to the action of a serine protease although the upregulation of shedding in response to phorbol esters and DCI was less marked than wild-type testis ACE.

7.3.2.2 **Specificity of shedding from isolated membranes of CHO-KI cells**

The proteolysis of ACE occurs predominantly at the cell surface and thus shedding inhibitors do not have to cross the cell membrane to be effective (Beldent et al, 1995). However, differences were observed between the inhibition profiles of rabbit ACE shedding from intact mouse C127 cells compared to when shedding was tested in a cell free membrane assay system (Ramchandran et al, 1994; Oppong & Hooper, 1993; Ramchandran & Sen, 1995). To test whether this was true for the ACE stalk constructs, discussed in this chapter, membranes were isolated from CHO-KI cells stably expressing the cDNA of these constructs and shedding was determined in the presence of inhibitors

ACE-JMEGF shedding was inhibited by TAPI (Figure 7.3.5). ACE-JMEGF remains tethered to the membrane via a disulfide linkage (Schwager et al, 1998). To determine whether the reduction of this covalent bond by a disulfide isomerase was necessary for its release from the membrane, ACE-JMEGF shedding was tested in the presence of bacitracin. The effect of bacitracin was previously tested on the shedding of wild type testis ACE, ACE-JMEGF, and ACE-JMfIX from intact CHO-KI cells (results not shown), and it inhibited the shedding of all these ACE molecules in a dose-dependent manner, suggesting that the disulfide isomerase could be involved in the processing of ACE. However, bacitracin did not inhibit the shedding of somatic or testis ACE from membranes purified from stably transfected CHO-KI cells, indicating that the actual release of the ACE molecule from the membrane was not dependent on the action of the disulfide isomerase (Chapter 4, Figure 4.3.5). ACE-JMEGF shedding from isolated membrane, however, was inhibited in the presence of bacitracin (3 mM) (Figure 7.3.5 A), indicating that the reduction of the tethering disulfide bond was required for the release of this stalk construct from the membrane. This suggests that there is only one population of mature ACE-JMEGF with an intact third disulfide

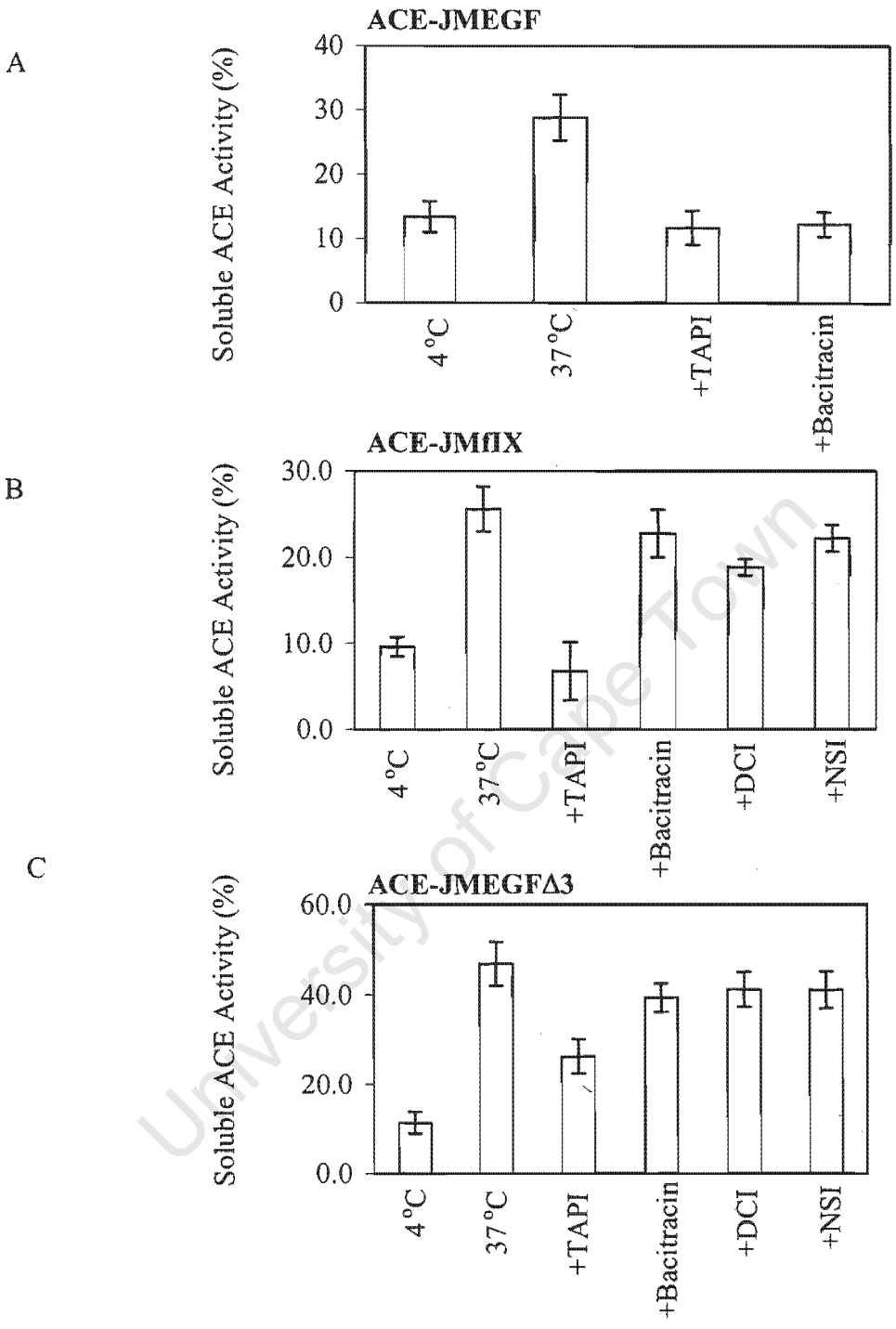


Figure 7.3.5 Effect of inhibitors on the release of the disulfide-linked ACE stalk mutants from isolated membranes of CHO-KI cells CHO-KI cells expressing ACE-JMEGF (A),ACE-JMfIX (B) and ACEJMEGFΔ3 (C) were grown to confluence and membranes were isolated using the Total Membrane Isolation Protocol II as outlined in Chapter 3. The membrane preparations were incubated at 4°C and 37°C in the absence of, and at 37°C in the presence of, TAPI, bacitracin, DCI and non-specific serine protease inhibitors (NSI) for 1 hr before the solubilised ACE was separated from its membrane-bound form by centrifugation at 12,000 xg for 5 min before the supernatant and membrane pellet were assayed for ACE activity using the ACE substrate HHL. (n=3 +/- SD)

loop and not two as was previously suggested (Schwager et al 1998). The shedding of ACE-JMfIX and ACE-JMEGFΔ3 was not inhibited in the presence of bacitracin. Thus, the release of ACE-JMEGFΔ3, although identical to ACE-JMEGF except for the shortened third disulfide loop, did not seem to be dependent on the action of the disulfide isomerase. This suggests that the cleavage site of ACE-JMEGFΔ3 may not occur within the third disulfide loop.

The effect of TAPI on the shedding of ACE-JMEGFΔ3 was equivocal, as only ~50% inhibition occurred compared to the ~100% inhibition of shedding of ACE-JMEGF (Figure 7.3.5 C). This suggests the action of two secretases with only one falling into the metalloprotease family. ACE-JMEGFΔ3 shedding was not inhibited by DCI or aprotinin and PMSF (NSI) (inhibitors of serine proteases not implicated in shedding), precluding cleavage by a serine protease (Figure 7.3.5). However, TAPI inhibited the shedding of ACE-JMEGFΔ3 from intact cells (Figure 7.3.4 B). This suggests that an alternate protease is involved in the release of ACE-JMEGFΔ3 from isolated membrane, but not from intact cells. Similarly, the inhibition of ACE-JMfIX shedding from isolated membranes differed to that determined for intact CHO-KI cells. Shedding of ACE-JMfIX was inhibited by TAPI and not DCI (Figure 7.3.5 B). This suggested that the presence of the EGF domain from factor IX within the stalk region of ACE altered the processing of ACE so that ACE-JMfIX was shed by a serine protease from intact CHO-KI cells and by a metalloprotease from membrane preparations of that same cell line. Aprotinin and PMSF did not inhibit the shedding of ACE-JMfIX from isolated membranes indicating the lack of involvement of serine proteases in this process.

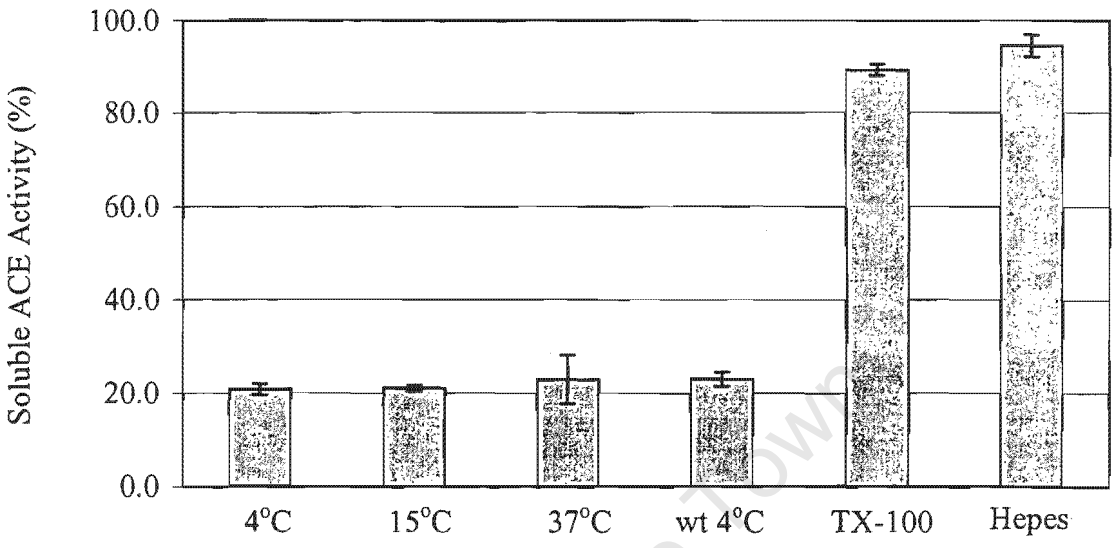


Figure 7.3.6 Intracellular shedding of ACE-JMfIX CHO-K1 cells expressing stably expressing ACE-JMfIX were grown to confluence in 100 mm dishes and ACE expression induced O/N with 2% FCS complete medium supplemented with $ZnCl_2$ (0.1μM). The cells were scraped into fresh medium and incubated at either 4°C, or 37 °C for 5 min or 15 °C for 3 hrs before the cells were centrifuged at 12 000 xg and the cells were lysed in 1% Triton X-100 lysis buffer. These cell lysates were then subjected to Triton X-114 phase separation and the ACE activity was determined for the aqueous and detergent phase using the substrate HHL. CHO-KI cells stably expressing testis ACE were treated similarly but the cells were only incubated at 4 °C for 5 min (wt 4 °C). Purified soluble testis ACE was resuspended in either 1% Triton X-100 lysis buffer (TX-100) or Hepes buffer (20 mM, pH 7.5) (Hepes) and subjected to Triton X-114 phase separation. The level of soluble ACE activity is depicted as the fraction of ACE activity in the aqueous phase as a percentage of the total ACE activity in both phases. (n=3 +/- SD)

7.3.3 **Determination of the intracellular cleavage of ACE-JMfIX**

To test whether ACE-JMfIX was cleaved intracellularly, CHO-K1 cells were incubated at 15°C to inhibit vesicle trafficking and the transport of ACE-JMfIX to the cell membrane. This did not result in an increase in soluble ACE activity associated with the cell lysates compared to the cell lysates of cells incubated at 4°C or 37°C for 5 min (Figure 7.3.6). In addition, the ACE activity detected within the detergent-poor fraction of the cell lysates of CHO-K1 cells expressing ACE-JMfIX was identical to

those of CHO-KI cells expressing wild type testis ACE. This indicated that the proteolysis of ACE-JMfIX by the serine protease did not occur within the cell, as incubation at 15°C would have inhibited all intracellular membrane trafficking and thus allow for the accumulation of soluble ACE within the cell. To control for the effectiveness of Triton X-114 for the separation of soluble ACE from membrane-bound ACE, purified testis ACE was resuspended in either Triton X-100 (to control for the effect of additional detergent on Triton X-114 phase separation) or Hepes buffer (20mM) before carrying out Triton X-114 phase separation. Nearly 100% soluble wild-type testis ACE activity was detected in the aqueous phase, indicating that the phase separation technique was successful and that the presence of Triton X-100 did not affect the phase separation of the cell lysates of CHO-KI cells.

7.3.4 **Cell-surface localisation of ACE-JMfIX**

To confirm the cell-surface localisation of ACE-JMfIX, CHO-KI cells expressing testis ACE or ACE-JMfIX were grown to confluence on coverslips and probed with a polyclonal rabbit testis ACE antibody. The cells were thoroughly washed and then re-probed with a secondary anti-rabbit FITC-conjugated antibody. Confocal microscopy was used to view the presence of ACE molecules at the cell surface of unpermeabilised cells and intracellularly for methanol-permeabilised cells. ACE-JMfIX was present intracellularly and on the cell-surface as indicated by the presence of the green fluorescent signal (Figure 7.3.7). ACE-JMfIX expression showed no obvious differences in cell-surface and intracellular expression compared to wild type testis ACE.

7.3.5 **Cleavage site determination by mass spectrometry**

ACE-JMfIX and ACE-JMmin23 were purified by affinity chromatography as outlined in Chapter 2, section 2.3.4 and the purified proteins were denatured, reduced, and the sulfhydryl groups protected using vinylpyridine. The modified ACE constructs were then digested with endoproteinase Lys-C and subjected to HPLC before the mass of the peptides was determined by MALDI-TOF mass spectrometry.

The mass of the penultimate peptide, *m/z* 1951.2, from amino acid residue P598 to the lysine residue in the stalk region, K613, was identified in the digests of ACE-JMfIX

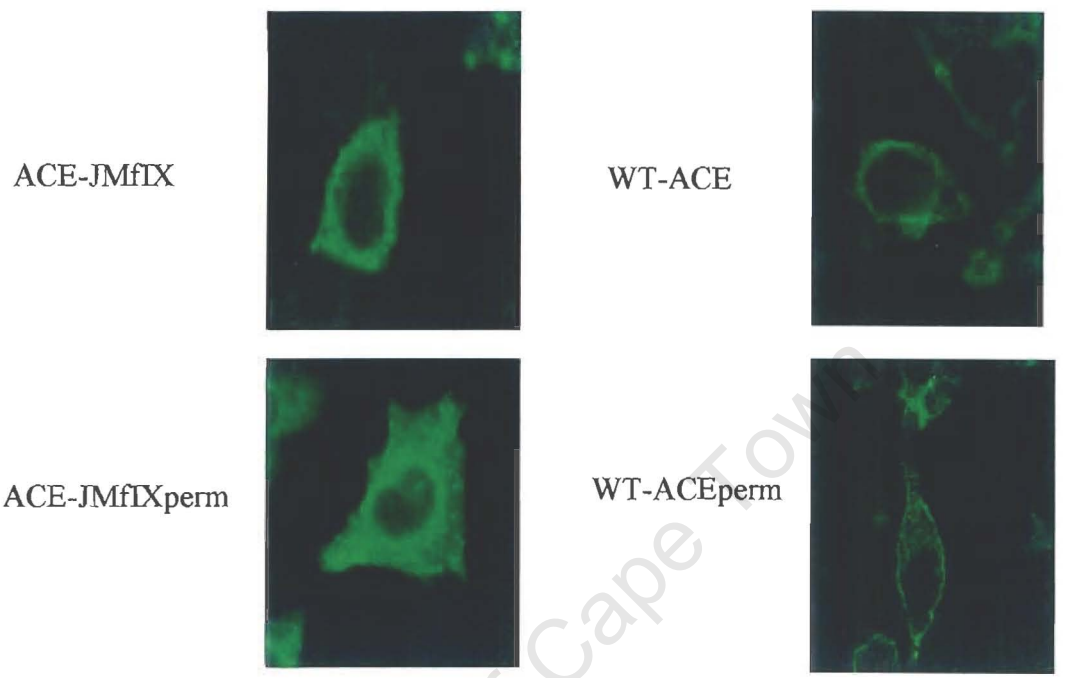


Figure 7.3.7 Confocal microscopy of CHO-KI cells expressing ACE-JMfIX CHO-KI cells transfected with the cDNA of wild-type testis ACE (WT-ACE) and ACE-JMfIX were grown to confluence on coverslips and probed with a primary rabbit anti-ACE polyclonal antibody and secondary goat anti-rabbit FITC-labelled antibody either before or after (ACE-JMfIXperm and WT-ACEperm) membrane permeabilisation with methanol to obtain cell surface or internal cell labelling of the expressed recombinant proteins, respectively.

and ACE-JMmin23 (Table 7.3.1). The C-terminal peptide of wild type testis ACE, *m/z* of 1690, was not identified in the spectra of peptides generated for the stalk constructs, as the ACE stalk sequence has been replaced with the alternative disulfide-linked domains. The *m/z* ions determined for the C-terminal peptide for ACE-JMfIX and ACE-JMmin23 were 1261.3 and 1576.7, respectively, which corresponded to the calculated masses of the peptides of L614-P623 and L614–L626. In addition, the peptide L614-P623 from ACE-JMfIX was isolated by HPLC and N-terminal sequencing confirmed the identity of this peptide. The identification of the cleavage sites, P623 and L626 for ACE-JMfIX and ACE-JMmin23, respectively, indicated that proteolysis occurred outside of the disulfide-linked domain (Figure 7.3.8).

The identity of the cleavage site of ACE-JMEGF, G652-F653, led us to conclude that proteolysis occurred within the third disulfide loop of the EGF-like homology domain. The three-dimensional structures of the disulfide-linked domains in ACE-JMfIX and ACE-JMEGF are very similar. To determine whether the shortening of the third disulfide loop of ACE-JMEGF by three amino acid residues to resemble that of the third disulfide loop of ACE-JMfIX, would alter the cleavage site, ACE-JMEGFΔ3 was constructed. This construct was purified from the culture medium of ACE-JMEGFΔ3-CHO cells and treated as outlined above, before the peptides generated by Lys-C hydrolysis were subjected to mass spectrometry. The *m/z* 1951.7 of the penultimate peptide, P598-K613, characteristic of endoproteinase Lys-C digestion of testis ACE and testis ACE mutants, was observed (Schwager et al, 1999). In addition, the peptide from L614 to K642 was also observed with an *m/z* of 3582.3 (Table 7.3.1). Only peptides with a C-terminal lysine residue were identified. Thus, to determine whether cleavage was occurring downstream from K642, ACE-JMEGFΔ3 was hydrolysed with CNBr (cyanogen bromide) to generate a different set of peptides. The *m/z* of the peptide L593-C647 indicated that cleavage occurred at the C647-L648 bond (Table 7.3.1), between the second and third disulfide loops and not within the third disulfide loop as for ACE-JMEGF (Figure 7.3.8).

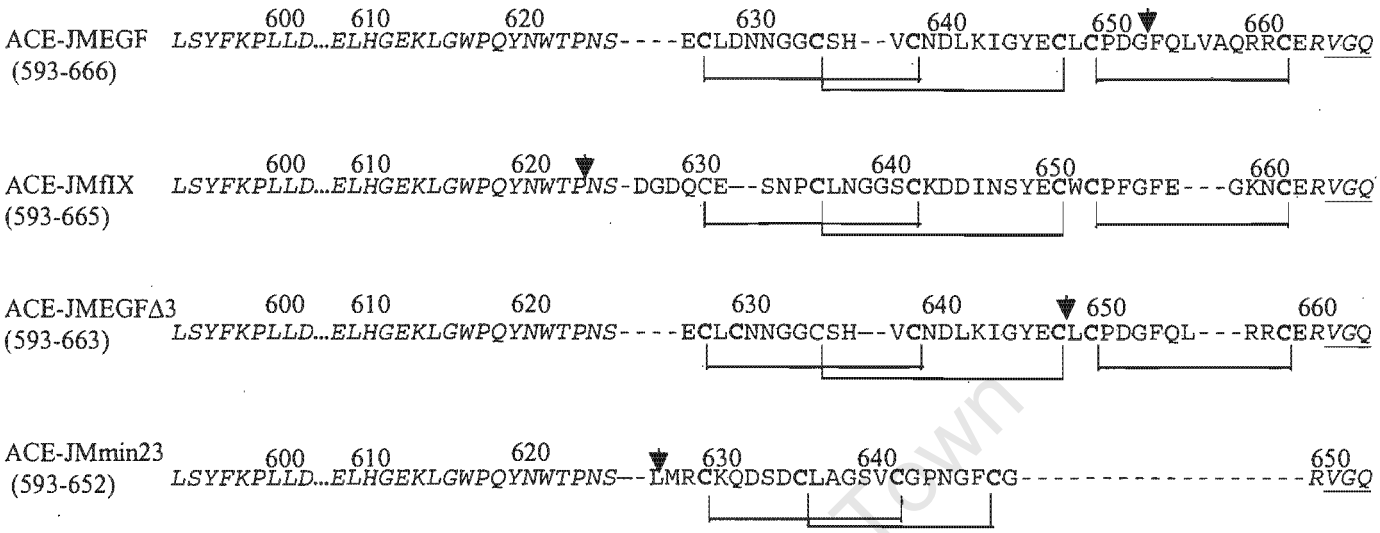


Figure 7.3.8 Sequences of the disulfide-linked ACE stalk constructs In the ACE stalk constructs, numbering is in accordance with the flanking testis ACE sequence. The flanking testis ACE sequence is in italics and the TM is underlined. The conserved cysteine residues are in bold and the disulfide bonds are indicated. The position of the inserted disulfide linked domains in the ACE stalk chimeras, ACE-JMEGF (E626-E662), ACE-JMfIX (D626-E662), ACE-JMEGFΔ3 (E626-E659) and ACE-JMmin23 (L626-G648) correspond to E296-E332, D4-E38, E296-E332 and L1-G23 for LDL-R EGF, Factor IX EGF, LDL-R EGF and min23, respectively. The arrows indicate the cleavage sites.

Table 7.3.1 Observed $[M+H]^+$ Ions of the peptides generated by endoproteinase Lys-C digestion of soluble ACE-JMfIX, ACE-JMmin23 and ACE-JMEGFΔ3 purified by affinity chromatography

		Observed *m/z*		
Peptide residues	Calculated *m/z*	ACE-JMfIX	ACE-JMmin23	ACE-JMEGFΔ3
598-613	1951.2	1952.2	1951.0	1951.7
614-642	3582.0			3582.3
614-623	1262.4	1261.3[a]		
614-626	1576.6		1576.7	

[a] The identity of the peptide was confirmed by N-terminal sequencing.

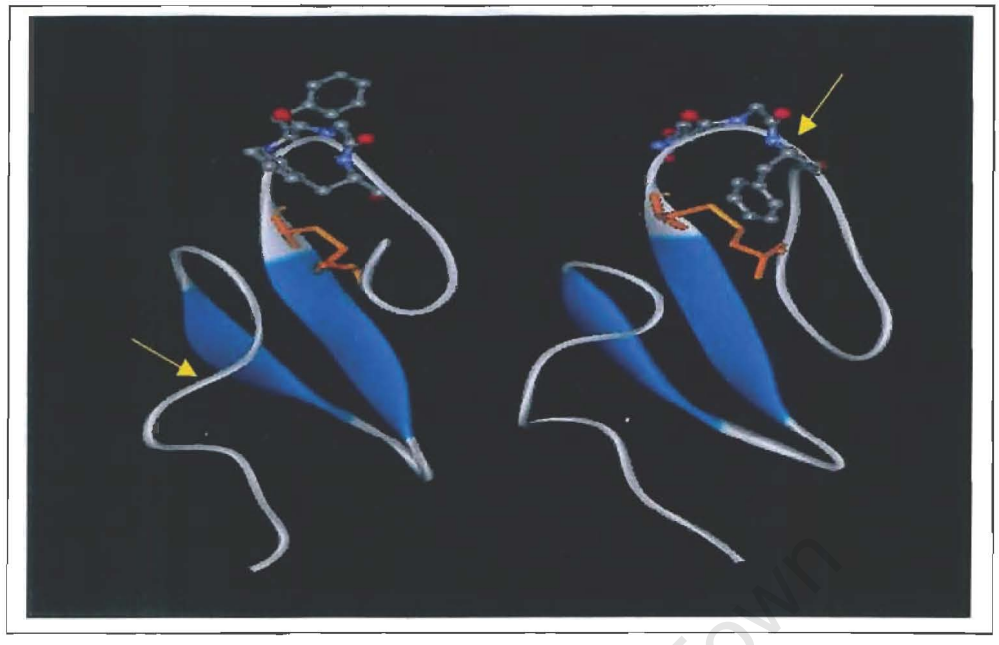
Table 7.3.2 Observed $[M+H]^+$ Ions of the peptides generated by the CNBr hydrolysis of soluble ACE-JMEGFΔ3 purified by affinity chromatography

Peptide residues	Calculated *m/z*	Observed *m/z*
341-385	5367.1	5366.9
393-448	5994.7	5994
593-647	6823.7	6823.4

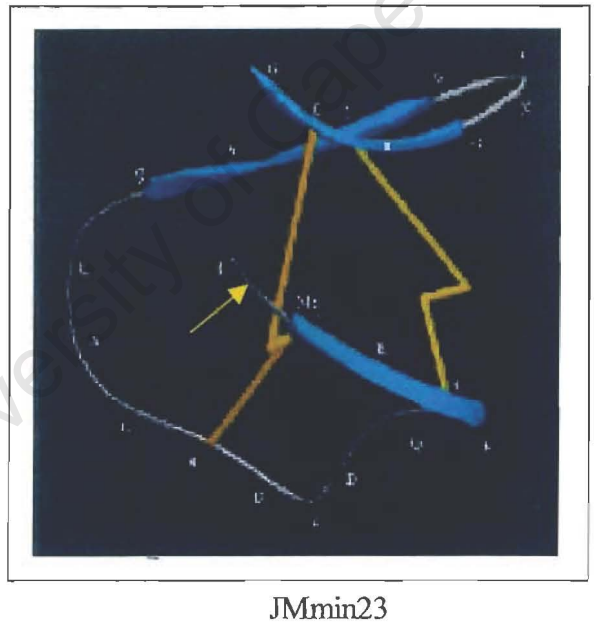
Comparison of the three dimensional structures of the disulfide-bridged domains

The NMR structure of the first two EGF-like domains of the LDL-R was solved by Malby et al (Malby et al, 2001; Saha et al, 2001) and the structure of the first EGF domain correlated well with the homology model of the EGF-like domain used in the construction of ACE-JMEGF. The homology-modelled structure of the EGF domain of the LDL-R and the crystal structure of the EGF-like domain of factor IX exhibit the β-cross motif characteristic of EGF-like domains whereas the structure of min23 shows the cystine-stabilised β-sheet (CSB) motif characteristic of the autonomous folding unit of the cystine-knot structure of the squash inhibitor, EETI (Heitz at al, 1999) (Figure 7.3.9). Min23 comprises 23 amino acid residues tightly folded to form three β-sheets stabilised by two disulfide bonds.

The cleavage site of ACE-JMEGF occurs within the third disulfide loop and the structure of this region was compared to that of the corresponding region of ACE-JMfIX and ACE-JMEGFΔ3 (Figure 7.3.10). The C-terminal loop of the EGF-like domain of ACE-JMEGF consists of two β-turns, one a type II turn and the other a type I turn. D651 to F653 comprised the first turn with the cleavage site occurring at the G652-F653 bond. The two residues at the cleavage site of ACE-JMEGF, (G652 and F653), and ACE-JMfIX (F654 and F656) are depicted in a ball-and-stick representation (Figure 7.3.9). The third loop of ACE-JMfIX and of ACE-JMEGFΔ3 is three amino acids shorter than that of ACE-JMEGF (Figure 7.3.8). The longer third disulfide loop of ACE-JMEGF seems to allow for the accommodation of the aromatic ring of F653 within the loop, whereas the aromatic rings of F654 and F656 of factor IX face outwards (Figure 7.3.10 A). This is corroborated by the crystal structure of fibrillin (1EMN), which is 32% identical to the EGF domain of the LDL-R. Fibrillin has a third disulfide loop of identical length and its phenyl ring was accommodated within the loop. Homology modelling of factor IX could not generate low energy conformers with the aromatic ring of F654 positioned inside the loop. This is most likely because the loop was shorter and more constrained. In addition, the homology model of EGFΔ3 showed that F653 also faced outside the loop once three amino acid residues were deleted from the EGF sequence (Figure 7.3.10 A) (Modeler 5.0 and CHARMm Programme). It is thus likely that ACE-JMEGFΔ3 was cleaved at the



JMflX JMEGF



JMmin23

Figure 7.3.9 3-dimensional structures of the disulfide-linked domains The similarity between the structures of the EGF-like domains of factor IX (JMflX) and the LDL-R (JMEGF), as well as the compact structure of the synthetic domain, min23, are indicated. F653 and G652 of the third disulfide loop of JM-EGF and F654 and F656 of JMflX are shown as a ball-and-stick representation. The blue ribbon and blue arrows represent the β-sheet structures and the orange lines represent the disulfide bonds. The yellow arrows indicate the positions of the cleavage sites.

C647-L648 bond instead of the G652-F653 because of the steric hindrance created by the orientation of the aromatic ring of F653. Therefore, the G652-F653 bond is sterically more accessible in ACE-JMEGF than in ACE-JMfIX and ACE-JMEFGΔ3 due to the orientation of the aromatic side chains of the residues proximal to the scissile bond.

The orientation of the side chains of F654 and F656 could explain why the cleavage site of ACE-JMfIX does not occur at the G652-F653 bond. However, the cleavage site was not shifted to the C650-W651 bond as in the case of ACE-JMEGFΔ3 when the third disulfide loop became more constrained. Comparison of the three-dimensional structure of the cleavage site of ACE-JMEGFΔ3 with the homologous region of ACE-JMfIX indicated that the aromatic ring of W651 of ACE-JMfIX could sterically interfere with the ACE secretase making the C650-W651 bond less accessible for proteolysis (Figure 7.3.10 B). In ACE-JMEGFΔ3, the tryptophan is replaced by a leucine, a less bulky amino acid residue. Thus, the steric accessibility of cleavage sites seems to determine whether these scissile bonds are susceptible to proteolysis.

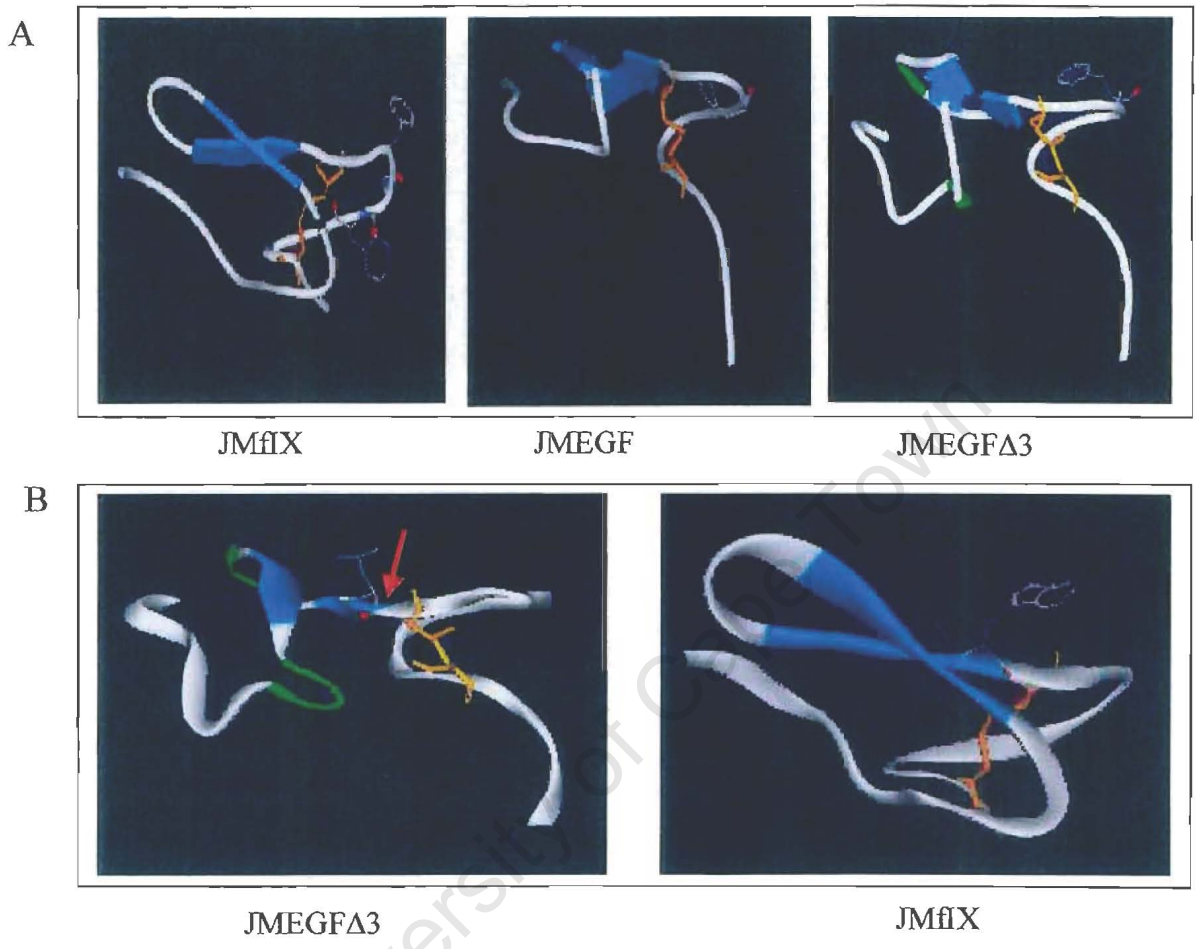


Figure 7.3.10 Comparison of the orientation of the amino acid residues proximal to the cleavage site Figure A demonstrates the orientation of the aromatic side chain of F653 of the third disulfide loop of JMEGF and JMEGFΔ3 and the orientation of F654 and F656 of JMflX. Figure B indicates the cleavage site of JMEGFΔ3 (red arrow) at the C647-L648 bond and the corresponding region of JMflX, showing the orientation of the aromatic side chain of W651. The blue ribbon and blue arrows represent the β-sheet structures and the orange lines represent the third disulfide bonds.

7.4 Discussion

As only a small fraction of membrane proteins are shed from the membrane, proteins susceptible to proteolysis must have certain characteristic features enabling their release from the membrane. The stalk regions of shed proteins are thought not to comprise folded domains, allowing easy access to the scissile bonds. Proteins with EGF-like domains adjacent to the TM are not shed from the membrane (Kahn et al, 1994; Kishimoto et al, 1987; Chen et al, 1995). This prompted the substitution of the ACE stalk with the N-terminal EGF domain of the LDL-R to determine whether the presence of a disulfide-linked domain within the stalk region would inhibit the shedding of ACE (Schwager et al, 1998). This chimeric protein continued to be shed with lowered efficiency and the position of the cleavage site resulted in this protein remaining attached to the membrane via a disulfide bond. It was suggested that membrane proteins with juxtamembrane disulfide-linked domains may resist release by disulfide linkages tethering the membrane proteins to the membrane after proteolysis. To further investigate whether juxtamembrane disulfide-linked domains resisted proteolysis because of secondary structure or disulfide-bond tethering, the stalk of ACE was substituted with the EGF-like domain from factor IX and with a compact cystine knot domain, min23.

The proteolysis of ACE-JMEGF occurred within the third disulfide loop and it was proposed that this loop formed an extended, accessible structure (Schwager et al, 1998). The homology modelling of the N-terminal EFG domain of the LDL-R, using 1EMN as a template, is in close agreement with the NMR structure and these structures are very similar to the known crystal structure of the EGF domain of factor IX. The three-dimensional structure of the EGF domain of factor IX indicated that the C-terminal third disulfide loop consisted of two β-turns and was more exposed than the more compact β-cross comprising the first and second disulfide loops. This made the EGF domain from factor IX an ideal choice to investigate whether the extended, exposed third disulfide loop is cleaved due to accessibility.

The ACE-JMfIX chimera was shed from the membrane but with lowered efficiency, as expected. These data corroborated our hypothesis that a stalk with a compact

disulfide-linked domain reduces but does not totally inhibit the shedding of ectodomains. This chimera, however, was not cleaved within the third disulfide loop but instead at the P623-N624 bond within the remaining stalk sequence of testis ACE. A comparison of the EGF domains of the LDL-R and factor IX (Figure 7.3.9 and Figure 7.3.10 A) indicated that the third disulfide loop in ACE-JMfIX was more physically constrained, as it comprised a shorter span of amino acid residues than ACE-JMEGF and had an additional aromatic amino acid residue, F654. In addition, the aromatic rings of F654 and F656 were orientated to face out of the loop whereas the phenyl ring of F653 of ACE-JMEGF was accommodated within the loop. Thus, the third disulfide loop of ACE-JMEGF appeared to be more sterically accessible, allowing proteolysis to occur at the G652-F653 bond. The accessibility of the C-terminal loop of ACE-JMfIX was greatly diminished, in comparison, shifting the cleavage site to outside the disulfide-linked domain.

Comparing the third disulfide loop of the EGF domains of the LDL-R and factor IX, it was difficult to identify precisely the source of the steric hindrance; i.e. did it result from (a) the difference in length of the third disulfide loop, (b) the presence of an additional aromatic amino acid residue (F654) in ACE-JMfIX, or (c) the orientation of the aromatic side chains to the outside of the loop? To further investigate these possibilities, a third ACE mutant was constructed in which the ACE stalk was replaced with the 23 amino acid residues from the squash inhibitor, EETI II. This compact domain is comprised of a triple-stranded β-sheet stabilised by two disulfide bonds called the cystine-stabilised β-sheet (CSB) motif (Heitz et al, 1999). ACE-JMmin23 was shed with lowered efficiency, as expected with a juxtamembrane-proximal disulfide-linked domain. Mass spectrometry identified the cleavage site as the L626-M627 bond, upstream of the disulfide-linked domain.

The CSB motif consists of two disulfide loops termed the N- and C-terminal loops (Figure 7.3.9). The C-terminal loop comprises 11 amino acid residues similar to the C-terminal loop of the EGF domain of ACE-JMEGF and has the sequence CGPNGF, similar to that of ACE-JMEGF (C-PDGF). However, the compact structure of min23 results in the orientation of the aromatic ring of F646 to the outside of the loop similar to F656 of ACE-JMfIX. The orientation of F646 is likely to create steric interference

making the G645-F646 bond inaccessible to the secretase. Thus, it is likely that the G655-F656 bond of ACE-JMfIX remained uncleaved not due to the presence of F654 but due to the tighter turns and outward orientation of F656.

To confirm this hypothesis, three amino acid residues from V656 to Q658 were deleted from the third disulfide loop of ACE-JMEGF to construct ACE-JMEGFΔ3. Three dimensional modelling of this mutant confirmed that the aromatic ring of F656 was now orientated to the outside of the loop, as in ACE-JMfIX. Thus, the tighter turn of the shortened loop resulted in reorientation of the aromatic side chain, as predicted. Moreover, the cleavage site of this construct no longer occurred at the G655-F656 bond within the third disulfide loop, but at the C647-L648 bond, between the second and third disulfide loops. Comparison of this cleavage site to the analogous region of ACE-JMfIX indicated that L648 corresponded to W651 in the latter and that the aromatic side chain of this amino acid residue was orientated to the outside of the disulfide-linked domain. Therefore, the orientation of the bulky side chain of W651 could make the C650-W651 bond of ACE-JMfIX inaccessible to the secretase.

These results suggest that the third disulfide loop of ACE-JMfIX is sterically constrained by the limited length of the loop and the orientation of the aromatic side chains. As is the case for the compact min23 domain, this resulted in proteolysis occurring distal to the disulfide-linked domain. In the case of ACE-JMEGF, in contrast, the third disulfide loop offers less steric interference due to the accommodation of bulky side chains within the longer loop and cleavage was not inhibited in this region. In addition, the results also imply that ACE-JMEGF could also be cleaved at its C647-L648 bond but that the G655-F656 bond was presumably more favourable for cleavage. By shortening the third disulfide loop in ACEJM-EGFΔ3, proteolysis occured at an alternative cleavage site, C647-L648, because the G655-F656 bond became inaccessible. The shedding of ACEJM-EGFΔ3 showed poor upregulation in the presence of PDBu, indicating that the C647-L648 bond was less favourable for cleavage. These data confirm that the ACE secretase is not constrained by a specific primary sequence but by the accessibility of the scissile bond.

It is reasonable to propose that the secretase is able to cleave within a defined stalk region or disulfide-linked domain depending on the steric accessibility provided by the three dimensional structure of the region. A point mutation in the stalk of somatic ACE (P119 to L119, 5 residues N-terminal to the cleavage site at R1203-S1204) enhanced the level at which somatic ACE was shed from the membrane. It was proposed that L1119 improved the degree of accessibility of the region, thus facilitating shedding (Eyries et al, 2001). Similarly, Althoff et al proposed that disorder of the juxtamembrane region influenced shedding (Althoff et al, 2001). Thus, proteolysis of membrane proteins is dependent on the availability of a sterically accessible or flexible loop of sufficient length within the juxtamembrane region.

In addition to clarifying the structural requirements for the shedding of membrane proteins, this study has also raised other questions; e.g. (1) is more than one secretase involved in the shedding of ACE, and (2) does the introduction of alternate sequences differentially affect the processing of ACE? More than one secretase has been implicated in the shedding of ACE (Schwager et al, 1999, Alfalah et al, 2001), but the primary secretase has been shown to be a member of the of the hydroxamate-inhibitable ADAMs family (Parvathy et al, 1997). Nevertheless, the release of ACE-JMfIX from intact CHO-KI cells was inhibited by DCI. This suggested that the secretase involved in this process was a serine protease distinct from the protease implicated in the constitutive shedding of wild-type testis ACE, ACE-JMEGF, ACE-JMmin23, and ACE-JMEGFΔ3. DCI was reported to inhibit the shedding of a stalk glycosylation mutant, ACE-JGL, and an ACE mutant with a point mutation in the stalk (N631Q) (Schwager et al, 1999, Alfalah et al, 2001). However, whereas the N631Q mutant was shed intracellularly, the shedding of ACE-JMfIX is likely to occur at the cell surface. These data imply that the three-dimensional structure of the EGF domain of ACE-JMfIX was sterically inaccessible for cleavage by the primary ACE secretase. This may result in recruitment of an alternative secretase from the serine protease family. When ACE-JMfIX shedding from isolated membrane was investigated, conflicting results were obtained. The shedding of ACE-JMfIX from membranes isolated from CHO-KI cells was inhibited by TAPI and not DCI. This pattern of inhibition was identical to wild-type testis ACE. This result suggested that in intact cells, ACE-JMfIX was shed by a serine protease but once these cells were

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Column Wash Buffer
[0.5 M NaCl, 20 mM Hepes, pH 7,5]
29.22 g NaCl was dissolved in 980 ml sH_2O, to which 20 ml 1 M Hepes (pH 7.5) was added, and then sterilised by autoclaving.

10% FCS Complete medium
[50% Ham's F-12/50% DMEM supplemented with 20 mM Hepes, pH 7.5, 20 mM L-glutamine and 10 % heat-inactivated foetal calf serum (FCS)]
22 ml Ham's F-12, 22 ml DMEM, 5 ml heat inactivated FCS (56°C for 30 minutes) and 1 ml 1 M Hepes, pH 7.5, was mixed in a 50 ml tube and filter sterilised.

2% FCS Complete medium
[Ham's F-12/DMEM supplemented with 20 mM Hepes, pH 7.5, 2 % FCS (70°C, 15 min heat-inactivated) and 40µM $ZnCl_2$]
24 ml Ham's F-12, 24 ml DMEM, 1 ml heat inactivated FCS (70°C for 15 minutes), 1 ml 1 M Hepes, pH 7.5, and 20 µl 0.1 M $ZnCl_2$ was mixed in a 50 ml tube and filter sterilised. Ham's F-12 and DMEM were obtained from laboratory stocks, while FCS from Gibco BRL, Life Technologies.

DMEM Tissue Culture Medium
Laboratory stocks were used for growing CHO cells. DMEM was bought from Highveld Biological [P.O. Box 488, Kelvin, 2054, South Africa] in a powder form which was hydrated and filter sterilised.
References: Dulbecco R., G. Freeman, Virology 8, 395, (1959); Smith, J. D., et al., Virology 12, 185 (1960). Composition (amounts in mg/l): NaCl (6400), KCl (400), $CaCl_2$ (200), $MgSO_4.7H_2O$ (200), NaH_2PO_4 (124), D-glucose (1000), $Fe(NO_3)_3.9H_2O$ (0.1), Na-Pyruvate (110), Phenol Red (15), NaHCO3 (3700), L-Arginine.HCl (84), L-Cysteine (48), L-Glutamine (580), L-Histidine.HCl.H_2O (42), L-Isoleucine (105), L-Leucine (105), L-Lysine.HCl (146), L-Methionine (30), L-Phenylalanine (66), L-Threonine (95), L-Tryptophan (16), L-Tyrosine (72), L-Valine (94), Glycine (30), L-Serine (42), Choline Chloride (4), Folic Acid (4), I-Inositol (7.2), Nicotinamide (4), D-Ca-Pantothenate (4), Pyridoxal.HCl (4), Riboflavin (0.4), Thiamine.HCl (4).

DNA Loading Buffer (20 ml)
[0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 30% Glycerol]
Dissolve 50 mg Bromophenol Blue, 50 mg Xylene Cyanol FF, and 6.0g Glycerol in sdH_2O. Adjust to 20ml. Filter sterilise and store at 4°C.

0.5 M EDTA (200 ml, pH 8.0)
Dissolve 37.2 g EDTA in 150 ml H_2O. Adjust pH to 8.0 with 10 M NaOH, then the volume to 200ml with H_2O. Autoclave and store at RT.

5 mM EDTA/PBS (50 ml, pH 8.0)
Add 500 µl of 0.5 M EDTA to 50 ml PBS. Filter sterilise and store at 4°C.

Ethidium Bromide Stock [EtBr] (10 ml)
(1000× stock solution, 0.5 mg/ml)
Dissolve 5 mg of ethidium bromide [Sigma, E8751] in 10 ml H_2O. Cover with foil and store at RT.

FCS
Foetal calf serum (Amersham) was heat inactivated at 56°C for 30 min. prior to use in complete medium. For use in induction medium, the FCS was further heat inactivated to 70 °C for 15 min.

6 M GnCl buffer
[6 M GnCl, 0.1 M Tris, pH 8.5]

200 mM L-glutamine
Dissolve 292 mg L-glutamine [GibcoBRL; 146 g/mol] in 10 ml H_2O, filter sterilise.

Hams F-12 Tissue Culture Medium

Laboratory stocks were used for growing CHO cells. Hams F-12 was bought from Highveld Biological [P.O. Box 488, Kelvin, 2054, South Africa] in a powder form which was hydrated and filter sterilised. References: Ham, R. G., PNAS, 53, 288 (1965). Composition (amounts in mg/l): NaCl (7599), KCl (223.6), Na_2HPO_4 (142), $CaCl_2.2H_2O$ (44), $MgCl_2.6H_2O$ (122), $FeSO_4.7H_2O$ (0.834), $CuSO_4.5H_2O$ (0.00249), $ZnSO_4.7H_2O$ (0.863), D-Glucose (1802), Na-Pyruvate (110), Phenol Red (1.2) [10 mg/l final], $NaHCO_3$ (1176), L-Alanine (9), L-Arginine.HCl (211), L-Asparagine (13.2), L-Aspartic Acid (13.3), L-Cysteine.HCl (31.5), L-Glutamine (146), L-Glutamic acid (14.7), Glycine (7.5), L-Histidine.HCl.H_2O (21), L-Isoleucine (4), L-Leucine (13), L-Lysine.HCl (36.5), L-Methionine (4.47), L-Phenylalanine (5), L-Proline (34.5), L-Serine (10.5), L-Threonine (12), L-Tryptophan (2), L-Tyrosine (5.4), L-Valine (11.7), Biotin (0.0073), D-Ca-Pantothenate (0.48), Folic acid (1.3), I-Inositol (18), Nicotinic acid amide (0.037), Pyridoxine.HCl (0.062), Riboflavin (0.038), Thiamine.HCl (0.34), Vitamin B12 (1.36), Hypoxanthine (4.1), Thymidine (0.73), Lipoic acid (0.21), Linoleic acid (0.084), Putrescine.$2H_2O$ (0.161).

1M Hepes (500 ml, pH 7.5)

Dissolve 119.15g N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (Hepes) [Sigma: H3375] in 400ml H_2O, adjust to pH 7.5 with 10M NaOH and 500ml with sH_2O.

HHL assay solution (24 ml)

58.2 mg Hippuryl-His-Leu [Sigma: H1635] was dissolved in 5 ml 0.025 M NaOH, with heating. Once dissolved, 1250 µl 1 M Hepes, pH 7.5, 3750 µl 2 M NaCl and 14 ml dsH_2O were added. Stored at 4 °C.

Homogenizing buffer

Hepes, 20 mM, pH 7.5; PMSF, 1 mM

Sepharose-[28Å]-lisinopril column wash buffer (1 L)

[0.5 M NaCl, 20 mM Hepes, pH 7,5]
29.22 g NaCl was dissolved in 980 ml H_2O, to which 20 ml 1 M Hepes (pH 7.5) was added, and then sterilised by autoclaving. When equilibrating the Lisinopril-sepharose column for cell extract samples, the wash buffer was further supplemented with 10 ml Triton X-100 (1% final) and 10 ml 100 mM PMSF (1 mM final). The columns are stored in wash buffer with the addition of 0.02% Sodium azide.

Luria Agar Plates (LA) (400 ml, 20 plates)

Luria agar plates were made by adding Tryptone (4 g), Yeast Extract (2 g), NaCl (4 g) and Bacteriological Agar (6g) to 400 ml H_2O in a 500 ml blue-capped bottle. This was autoclaved at 121°C for 30 min, and allowed to cool to 50°C in a water bath. Ampicillin (40 mg) was added and the solution was mixed thoroughly, but gently. The liquid agar was poured into 20 sterile Petri dishes. After the agar had set, the plates were dried in a tissue culture hood, wrapped in cling-wrap and stored at 4°C.

Luria Broth (LB) (500 ml)

Tryptone (5 g), Yeast Extract (2.5 g) and NaCl (5 g) was dissolved in 500 ml H_2O. This was allocated into 20 ml Universals (5 ml), or foil wrapped 500 ml flasks (50 ml), and autoclaved at 121°C for 30 minutes. 1.0 µl/ml of the Ampicillin stock (100 mg/ml) was added when inoculating the broth. Bacteria were grown in inoculated LB at 37°C overnight.

MOWIOL mounting solution

Add 2 ml glycerol to 2 g MOWIOL (CalBiochem), and mix. Add 4 ml H_2O, mix, and leave for 4 hours at RT. Add 8ml 0.2 M Tris, pH 8.5, and incubate at 50°C with intermittent shaking until MOWIOL is completely dissolved (~1.5 hrs). Centrifuge for 15 minutes at 5000 × g, collect supernatant, and add 1,4-diazobicyclo-[2,2,2]-octane (DABCO) to a final concentration of 2.5% (0.25 g in 10 ml). Store 2 ml aliquots at -20°C (storage), else 4°C (working).

1 M Na_2HPO_4 (10 ml, pH ~8.0)

(Disodium phosphate)
Dissolve 1.42 g of Na_2HPO_4 [Sigma, S7907] in 10 ml H_2O.

1 mM Na_2HPO_4 (10 ml, pH >7.5)
Add 10 µl of 1 M Na_2HPO_4 to 10 ml H_2O. Use for RNA absorption reading.

***o*-phthaldiadehyde (20 mg/ml in methanol)**
Dissolve 100 mg o-phthaldiadehyde in 5 ml methanol.

10× PBS (1 L)
(Phosphate buffered saline)
[1,37 M NaCl, 27 mM KCl, 43 mM $Na_2HPO_4.2H_2O$, 14 mM KH_2PO_4]
Dissolve 80 g NaCl, 2 g KCl, 7.7 g $Na_2HPO_4.2H_2O$ and 2 g KH_2PO_4 in ~800 ml H_2O. Adjust the pH to 7.4, and make up to 1 L. Autoclave.

PBS^{++} (1 L)
[PBS + 1 mM $CaCl_2$ + 1mM $MgCl_2$]
Dilute 100 ml 10× PBS in 800 ml of H_2O and add 147 mg $CaCl_2.2H_2O$ and 200 mg $MgCl_2.6H_2O$. Make up to 1 L with H_2O.

PenStrep stock (100x)
[10000 U/ml penicillin, 10000 µg/ml streptomycin]
Mix 1 ml NovoPen with 3 ml NovoStrep and 96 ml PBS. Filter-sterilize, freeze in 5 ml aliquots.

8% PFA (100ml)
(paraformaldehyde . Use in fume hood only.)
Add 8 g paraformaldehyde to 90 ml H_2O while stirring. Warm to 60°C, then check pH – should be acidic. Neutralise with 1 M NaOH, stir until dissolved (some always remains insoluble). Cool to RT, adjust volume to 100 ml, filter through 0.22 µm filter. Store in 5 ml aliquots at -20°C.

3% PFA in PBS (10ml)
Thaw one 5 ml aliquot of 8% PFA to 60°C. Mix 3.75 ml 8% PFA, 1 ml 10× PBS and 5.25 ml H_2O in fume hood.

Phenol Tris- Buffered
Mix 500 ml liquefied phenol or melted crystals of redistilled phenol (melt at 65°C) with 0.5 g 8-hydroxyquinolone, 2 L water and 500 ml 50 mM Tris, pH 8 and stir at RT for 10 min. Allow phases to separate, remove aqueous phase and replace with 50 mM Tris, pH 8 and repeat until phenol reaches pH 8. Add 250 ml Tris, pH 8 and decant into tubes wrapped in aluminium foil and store at 4°C.

PMSF (100 mM)
348 mg phenylmethylsulfonyl flouride (PMSF) [Sigma: P-7626] was dissolved in to 20 ml methanol.

2 µM Propidium Iodide
A 1:2500 dilution of a 5 mM stock of propidium iodide was made. Invert coverslip on 50 µl drop, in the dark.

5 mM Propidium Iodide stock
5 mg of propidium iodide (668.4 g/mol) was dissolved in 1.5 ml isopropanol. Store at -20°C in foil.

2× SDS Loading buffer (20 ml)
[0.0625M Tris, pH 6.8, 2% SDS, 10% Glycerol, 0.001% Bromophenol Blue]
Mix 625 µl of 2 M Tris (pH 6.8), 400 mg of SDS, 2 ml of Glycerol, 0.2 µg of Bromophenol Blue and 14 ml of H_2O. Adjust to 20ml with H_2O.

2× SDS Reducing buffer (2.5 ml)
[0.0625 M Tris (pH 6.8), 2% SDS, 10% Glycerol, 0.001% Bromophenol Blue, 5% β-mercaptoethanol]
Add 125µl of β-mercaptoethanol to 2.375 ml of 2× SDS loading buffer. Mix and use immediately.

5× SDS Loading Buffer (20 ml)
[0.1563M Tris (pH 6.8), 5% SDS, 25% Glycerol, 0.0025% Bromophenol Blue]

Mix 1.563 ml of 2 M Tris (pH 6.8), 1 g of SDS, 5 ml of Glycerol, 0.5 µg of Bromophenol Blue and 10 ml of H_2O. Adjust to 20 ml with H_2O.

5× SDS Reducing Buffer (4 ml)
[0.1563 M Tris (pH 6.8), 5% SDS, 25% Glycerol, 0.0025% Bromophenol Blue, 12.5% β-mercaptoethanol]
Add 500 µl β-mercaptoethanol to 3.5 ml 5× SDS loading buffer. Mix and use immediately.

SDS Running Gel Buffer (250 ml)
[1.125 M Tris (pH 8.8), 0.3% SDS]
Dissolve 34.07 g of Trisma Base and 0.75 g of SDS in 200 ml H_2O. Adjust the pH to 8.8 with HCl and the volume to 250 ml with H_2O.

SDS Stacking Gel Buffer (250 ml)
[0.375 M Tris (pH 6.8), 0.3% SDS]
Dissolve 11.36 g of Trisma Base and 0.75 g of SDS in 200 ml H_2O. Adjust the pH to 6.8 with HCl and the volume to 250 ml with H_2O.

10× SDS Tank Buffer (500 ml)
[1% SDS, 0.25 M Tris (pH 8.3), 1.92 M Glycine]
Dissolve 15.14 g of Tris, 72.06 g of Glycine and 5 g of SDS in 400 ml of H_2O. Adjust the pH to 8.3 using 10 M NaOH and adjust the volume to 500 ml with H_2O.

[^{35}S] labelling medium
(2% dialysed FCS in minimal Eagles medium (MEM) supplemented with 4 mM L-glutamine and 100 µCi [^{35}S]-methionine/[^{35}S]-cysteine per ml)
5× aliquots of 200 µCi [^{35}S]-methionine/[^{35}S]-cysteine were added to 10 ml starvation medium. 500 µl of this was added to confluent cells in 6-well dishes for metabolic labelling (i.e. 50 µCi per well).

2M Sodium Acetate (20 ml, pH 4.0)
Dissolve 3.28 g anhydrous Sodium Acetate (NaAc) in 10 ml dsH_2O. Adjust to pH 4.0 with pure Glacial Acetic acid, then 20 ml with H_2O. Autoclave. Store at RT.

3M Sodium Acetate (20 ml, pH 5.2)
Dissolve 4.92 g anhydrous Sodium Acetate (NaAc) in about 10 ml dsH_2O. Adjust the pH to 5.2 using Pure Glacial Acetic acid (~ 7 ml). Make up to 20 ml with sdH_2O. Autoclave. Store at RT.

Solvent A (HPLC)
[0.1% trifluoroacetic acid (TFA) in H_2O; pH ~1.0]

Solvent B (HPLC)
[0.1% TFA in acetonitrile]

20× SSC (1 L)
[3 M NaCl, 0.3 M sodium citrate, pH 7.0]
Dissolve 175 g of NaCl and 88 g of sodium citrate (Na_3citrate.$2H_2O$) [Sigma, S4641] in 800 ml H_2O. Adjust the pH to 7.0 with HCl, then the volume to 1000 ml with H_2O. Autoclave and store at RT.

Starvation medium
(2% dialysed FCS in minimal Eagles medium (MEM) supplemented with 4 mM L-glutamine)
400 µl dialysed FCS [GibcoBRL, 10110-153], 400 µl 200 mM L-glutamine [GibcoBRL, 21051-024] and 19.2 ml minimal Eagles medium [ICN, 1641447]. 10 ml is used for starving 3× 6-well dishes for 30 min (500 µl per well), while the remainder is used for metabolic labelling (5× [^{35}S] methionine/cysteine aliquots [200 µCi each] are added) – 500 µl of mixture per well.

TBE (1 L)
[89 mM Tris (pH 8.0), 89 mM boric acid, 2.5 mM EDTA]

Add 100 ml 10× TBE to 900 ml H_2O.

TBE [EtBr]
[89 mM Tris (pH 8.0), 89 mM boric acid, 2 mM EDTA and 0.5 µg/l EtBr]
Add 1 ml 0.5 mg/l Ethidium Bromide Stock to 1 L of TBE.

10× TBE (1 L)
[890 mM Tris (pH 8.0), 890 mM boric acid, 25 mM EDTA]
Dissolve 108 g Tris, 55g boric acid and 9.3g EDTA in 800 ml H_2O. Adjust the pH to 8.0 with concentrated HCl. Adjust the volume to 1000 ml with H_2O and autoclave.

Tris EDTA (TE) (10x)
10 mM EDTA, 100 mM Tris (pH 8.0)

Terrific Broth (Ψ-broth)
[2% (w/v) Difco Bacto Tryptone, 0.5% (w/v) Difco Bacto Yeast Extract, 0.4% (w/v) $MgSO_4$, 10 mM KCl]

TFB1
100 mM RbCl, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% glycerol

TFB2
10 mM MOPS pH 7.0, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol

Tris [Tris(hydroxymethyl)aminomethane]
Merck, MW = 121.1g/mol.

0. 2M Tris (100 ml, pH8.5)
Dissolve 2.42 g Tris in ~70 ml H_2O. Adjust to pH 8.5 with concentrated HCl, and 100ml with H_2O. Autoclave.

2M Tris (100 ml, pH 6.8)
Dissolve 24.2 g Tris in ~70 ml H_2O. Adjust to pH 6.8 with concentrated HCl, and 100ml with H_2O. Autoclave.

Triton-lysis buffer
[1% Triton X-100, 50 mM Hepes, pH 7.5, 0.5 M NaCl, 1 mM PMSF].
Mix 29.5 ml H_2O, 2.5 ml 1 M Hepes, pH 7.5, 12.5 ml 2 M NaCl, 5.0 ml 10 % Triton X-100, 500 µl 100 mM PMSF. Store at RT.

0.1% Triton X-100 (100 ml)
Dilute 100 µl Triton X-100 in 100 ml PBS^{++}.

Triton X-114 (pre-condensed, 1 L)
[11% final in 10mM Tris, pH 7.5, 150 mM NaCl]
Dissolve 20g Triton X114 in 980ml on ice. Place the clear solution at RT (to allow micelle formation) and allow it to stand until the phases have separated. Remove the aqueous phase and replace with fresh Triton extraction buffer and allow it to stand overnight. Repeat this 3 times, and remove the aqueous phase thereafter. This results in an 11% solution of Triton X114 which can then be diluted to 2 % for phase separations.

Triton X-114 extraction buffer (200ml, pH 7.5)
[10mM Tris, pH 7.5, 150 mM NaCl]
Dissolve 242mg Trisma base (121.1 g/mol) and 1,75 g NaCl (58.4 g/mol) in 160ml H_2O. Adjust the pH to 7.5 using HCl, and make up to 200ml.

2% Triton X-114 (100 ml, pH 7.5)
[2% Triton X-114, 10mM Tris, pH 7.5, 150 mM NaCl]

Dilute 18.2 ml pre-condensed Triton X-114 (11%) in 100 ml Triton X-114 extraction buffer [10 mM Tris, pH 7.5, 150 mM NaCl].

Trypsin/EDTA
[0.5 mg/ml Trypsin in 5 mM EDTA/PBS]

Wash buffer
[20 mM Hepes, pH 7.5; 0.5 M NaCl]
29.22 g NaCl was dissolved in 980 ml H_2O, to which 20 ml 1 M Hepes (pH 7.5) was added, and then sterilised by autoclaving.

Western Blocking Buffer (500 ml)
[5% Skim milk, 0.1% Tween-20, 0.2 M NaCl, 0.05 M Tris (pH 7.4)]
Mix 25 g of Skim Milk powder, 500 µl of Tween-20, 50 ml of 2 M NaCl, 25 ml of 1 M Tris (pH 7.4) and 400ml H_2O. Adjust to 500ml with H_2O.

Western Blotting Buffer (1 L)
Dissolve 3.025 g Tris and 14.4 g of Glycine in 800 ml H_2O, then add 200 ml methanol. This should have a pH of 8.3.

0.1 M $ZnCl_2$
Dissolve 682 mg $ZnCl_2$ (Sigma, Z0152; 136.3 g/mol) in 50 ml H_2O. Add a 10 µl drop of conc. HCl to aid dissolution. Filter sterilise.

z-PHL stock Solution
Dissolve 220 mg of z-PHL in 2 ml 0.28 M NaOH and add water to a final volume of 20 ml and store aliquots (2 ml) at -20°C to obtain a 20 mM stock solution.

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