
**DETERMINATION OF THE GLYCOSYLATION
REQUIREMENTS FOR ACTIVE HUMAN TESTIS
ANGIOTENSIN-CONVERTING ENZYME**

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ABSTRACT

Secreted and membrane-bound proteins are often glycosylated and these sugars confer stability to the protein, protect it from proteolytic attack and have been shown to be involved in determining correct protein folding. However, the overall protein heterogeneity afforded by glycans lends a great deal of difficulty in the isolation and analysis of glycoproteins. In particular, 3-dimensional structural analysis through X-ray crystallography is hampered. This relies on the formation of crystals, more effectively obtained from components with strong conformational similarity. Removal of sugars increases the protein homogeneity, however complete removal often results in incorrectly folded protein, which is rapidly degraded intracellularly.

In our studies with the membrane-bound ectoprotein angiotensin-converting enzyme (ACE), we have sought to determine the minimal glycosylation requirements to maintain structural integrity in an attempt to produce a crystallisable candidate. We have produced a series of glycosylation mutants of human testis ACE containing one, two or three N-terminal sites. These were constructed by site-directed mutagenesis of the six glycosylated N-linked recognition sequons in tACE. These mutants were stably transfected into CHO-K1 cells and the enzyme activity assessed to determine whether structural integrity was maintained.

Results show that removal of sites at the C-terminus does not impair tACE expression and activity and the presence of glycosylation at a single site at the N-terminus is sufficient to produce enzymatically active tACE. There is a preference for the first or third sequon as glycosylation at either of these sites was sufficient to produce enzymatically active tACE and a mutant lacking both these sites was degraded intracellularly. Decreased ACE activity was observed for mutants that were glycosylated at one or two sites, whereas those containing three or four sites exhibited high levels of activity. Inhibition of glycosylation by tunicamycin produced unglycosylated tACE, which was rapidly degraded intracellularly. Thus, the presence of N-linked glycans at the N-terminus is required for the expression of enzymatically active human tACE.

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
sACE	somatic angiotensin-converting enzyme
tACE	testis angiotensin-converting enzyme
RAAS	Renin-angiotensin-aldosterone system
Hip-His-Leu	hippuryl-L-histidyl-L-leucine
CHO	Chinese hamster ovary
NMR	Nuclear magnetic resonance
ASM	antibiotic selection mix
RER	rough endoplasmic reticulum
GalNac	N-acetylgalactosamine
GlcNac	N-acetylglucosamine
OST	oligosaccharyl transferase
HPLC	high performance liquid chromatography
MALDI-TOF	matrix-assisted laser desorption ionisation time of flight

CHAPTER 1: INTRODUCTION

1.1 PHYSIOLOGICAL IMPORTANCE OF ACE

Hypertension is a term used to refer to persistent high blood pressure (Swales, 1995). It has been implicated in an increased risk of stroke and cardiovascular disease, through chronic renal dysfunction, and myocardial infarction (Castelli, 1984). Diagnosis and treatment of hypertension is poor, thus doing little to combat these conditions. This is reflected in the incidence of cardiovascular disease and stroke, which are two of the greatest causes of death the world over, responsible for 30% of deaths in 1999 (World Health Report, 2000). Heart disease and hypertension were listed in the top 15 causes of death in the United States in 1999, ranking at one and thirteen respectively (Kochanek et al, 2001). The incidence of stroke and cardiovascular disease are the results of numerous other factors, but effective treatment of hypertension may greatly reduce the risks in sufferers. Traditionally, hypertension has been treated by targeting the Renin-Angiotensin-Aldosterone System (RAAS) and with beta-blockers or Ca^{+2} channel blockers (Swedberg, 2000). The RAAS plays a major role in blood pressure regulation and aberrations in this system are a leading cause of hypertension (Swales, 1995). It is controlled through the activity of two enzymes: Renin and Angiotensin-I Converting Enzyme (ACE). Renin is produced in the kidneys as an inactive precursor and is cleaved to produce an active form in response to stress. Active renin circulates in the blood plasma, where it is exposed to angiotensinogen, which is continuously produced by the liver. Renin cleaves angiotensinogen to produce angiotensin I (AI), a decapeptide (Fig. 1.1). ACE, a membrane-bound protease, converts circulating AI into the octapeptide angiotensin II (AII), through cleavage of a dipeptide from the C-terminus. AII is a potent vasoconstrictor that promotes the secretion of aldosterone, a hormone, which increases Na^+ and water retention in the kidneys, all acting to increase blood pressure. ACE also inactivates bradykinin, a vasodilator that decreases blood pressure.

Because of its pivotal role in the RAAS, the most effective RAAS targeted anti-hypertensive drugs are ACE inhibitors (Swales, 1995). Numerous ACE inhibitors exist in the market, including enalaprilat, ramiprilat and captopril, the latter being one of the first examples of structure-based drug design. A potent inhibitor of blood pressure increase was isolated from pit-viper venom and subsequently modified to produce captopril (Ondetti et al, 1977).

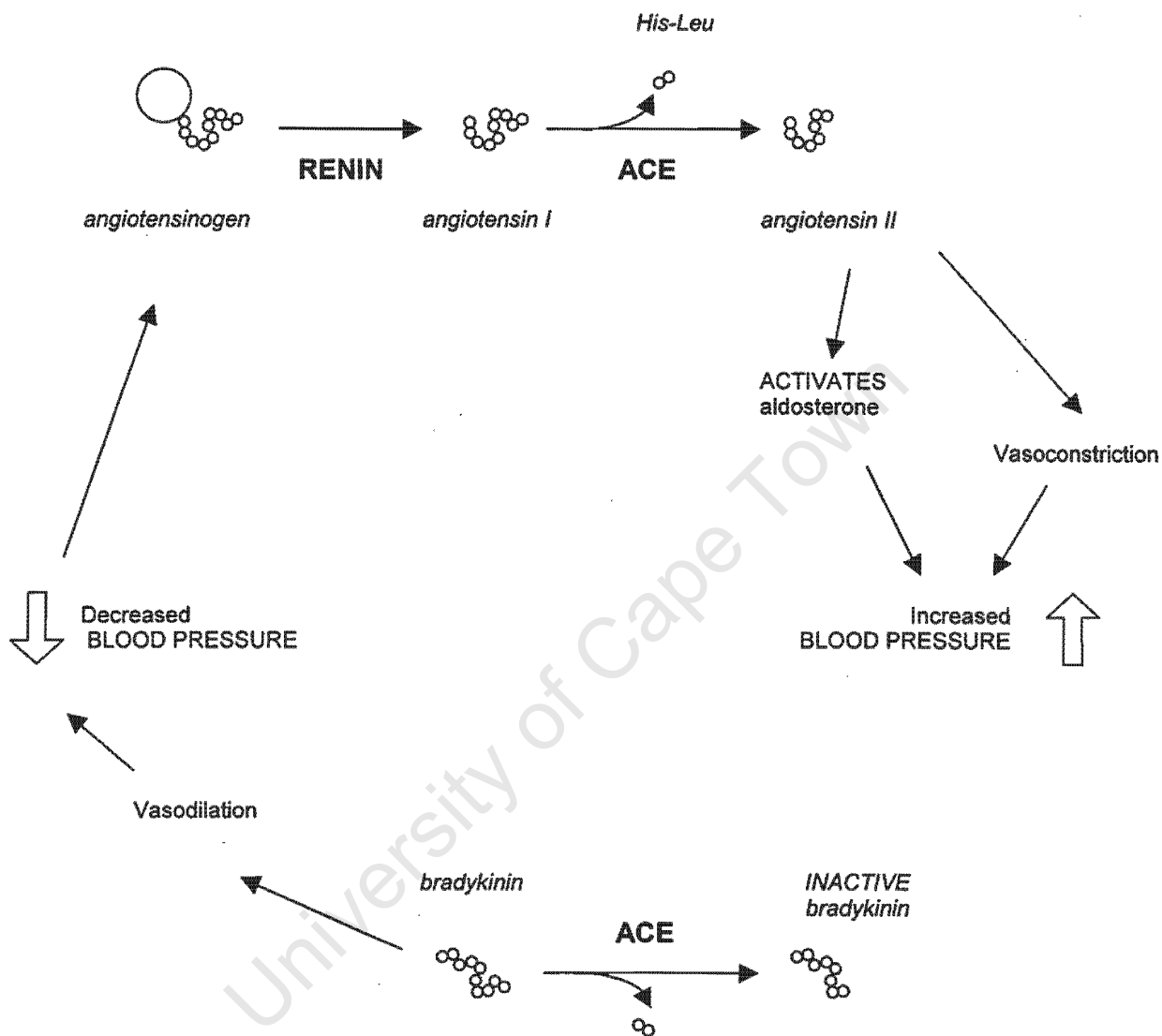


Figure 1.1. The Renin Angiotensin Aldosterone System. In response to a decrease in blood pressure, Renin is released by the kidney and converts angiotensinogen to angiotensin I, which is cleaved by Angiotensin-I converting enzyme (ACE) to produce angiotensin II. This potent vasoconstrictor promotes the secretion of aldosterone, which together act to increase blood pressure. At the same time the potent vasopressor, bradykinin, is inactivated by cleavage by ACE.

Much of the information used to modify the compound to increase its specificity were based on assumptions made about the active site of ACE extrapolated from the 3-dimensional structural data of a related enzyme Carboxypeptidase A (CPA) (Ondetti et al, 1977). However, there are differences between the catalytic mechanisms of CPA and ACE, particularly regarding anion activation. ACE is known to be activated in the presence of anions such as chloride (Skeggs et al, 1954), whereas CPA binds anions in a competitive manner and is inhibited upon chloride binding (Williams & Auld, 1986), both of which appear to be mediated by different residues in the active site (Shapiro, 1983; Williams & Auld, 1986). The differences within the active sites of these two enzymes suggests that CPA may not be a good model from which to extrapolate information about the catalytic mechanism of ACE. This demonstrates the importance of a thorough knowledge of an enzyme both in understanding its function and in the design of a specific, potent drug. To date, no "wonder" drug has been designed that effectively combats hypertension. Ideally, this drug would have to be effective for long periods and confer an advantage to the cardiovascular system, thereby decreasing the risks of cardiovascular disease (Meredith, 2000).

Determining the structural, chemical and kinetic components of the active site of ACE would greatly enhance our understanding of the interaction of current ACE inhibitors and aid the design of increasingly effective antagonists. Currently no 3-dimensional model of ACE exists to clarify the structure-function relationship of this enzyme, the elucidation of which has been marred by the large size of ACE, its hydrophobicity and the presence of glycosylation, which is discussed later in this review. What follows is an introduction to what is known regarding the structure-function relationship of ACE with respect to its structure, catalytic properties and post-translational modification including solubilisation and glycosylation, with a focus on the latter.

1.2 BIOCHEMISTRY OF ACE

1.2.1 Physical Properties

The deduced amino acid sequence of ACE comprises 1306 residues which give rise to a 170 kDa protein. The sequence contains a 29-residue signal sequence; a 17-residue hydrophobic transmembrane region at the C-terminus; a 30-residue cytoplasmic tail; and a 1230-residue ectodomain (Fig. 1.2a) (Soubrier et al, 1988; Wei et al, 1991a). This large ectodomain has high internal homology between two domains (termed the N- and

a) Somatic ACE



b) Testis ACE

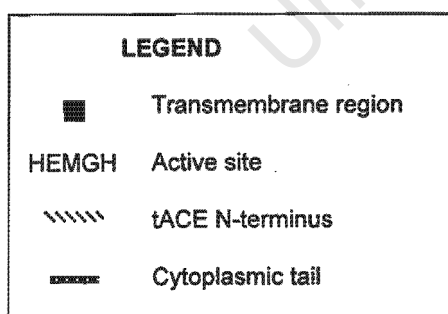
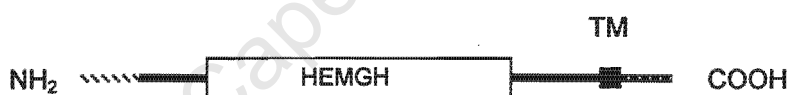


Figure 1.2. The two isoforms of ACE. The testis isoform is homologous to somatic ACE, except for the N-terminus, as indicated by the dashed line.

C-domains) which appear to be the result of a gene duplication event. Each domain contains a conserved binding motif (HEMGH) known to be part of the active sites of zinc-metalloproteases such as thermolysin, neutral endopeptidase and collagenase (Soubrier et al, 1988).

Interestingly, purification of ACE from rabbit testes revealed a second, smaller isoform (El Dorry et al, 1982a), which is expressed uniquely in sperm cells (Strittmatter & Snyder, 1984; Yotsumoto et al, 1984; Brentjens et al, 1986). This isoform, referred to as testis ACE (tACE), is structurally and catalytically identical to the C-terminal domain of the somatic isoform, except for a 36-residue region adjacent to the signal peptide (El Dorry et al, 1982b) (Fig. 1.2b). It has similar immunological properties (El Dorry et al, 1982b; Kumar et al, 1989) and is transcribed from the same gene (Bernstein et al, 1988; Soubrier et al, 1988) from an intronic promoter (Lattion et al, 1989; Howard et al, 1990)

1.2.2 Catalytic properties

The inferred enzyme action of ACE as a dipeptidyl carboxypeptidase (Soffer & Sonnenblick, 1978), from structure activity studies using snake venom inhibitors, led to the development of assays using synthetic peptides to monitor ACE activity, which were invaluable in kinetic studies and the establishment of an effective method to purify ACE. These include a spectrophotometric assay which monitors the cleavage of the compound furylacryloyl-L-phenylalanyl-glycylglycine (Holmquist & Vallee, 1976) and an assay which determines ACE activity through the cleavage of hippuryl-L-histidyl-L-leucine (Hip-His-Leu), a C-terminal analogue of angiotensin I (Friedland & Silverstein, 1976)

Before the sequence of ACE was determined, it was assumed from kinetic data that sACE contained a single active site as shown by competitive inhibitor binding studies (Bull et al, 1985; Strittmatter & Snyder, 1986; Cumin et al, 1989) and the observation that lung ACE was thought to bind a single zinc atom per molecule enzyme (Das & Soffer, 1975; Bunning & Riordan, 1985). Furthermore, the presence of a catalytically active isoform that lacks the N-domain indicates that one active site is sufficient for dipeptidyl carboxypeptidase activity (Ehlers et al, 1989; Kumar et al, 1989; Lattion et al, 1989). This implies an alternate role for the N-terminal active site in sACE. A more stringent re-evaluation of the kinetics of both isoforms purified from various tissues and of purified recombinant ACE showed that two zinc atoms bound per molecule enzyme and that each active site has specific substrate preferences (Ehlers & Riordan, 1991). It appears that the C-terminal active site

predominantly maintains the dipeptidyl carboxypeptidase function, whereas the N-terminal active site catalyses other substrates, including the luteinising hormone-releasing hormone (LHRH), through tripeptidyl carboxypeptidase hydrolysis (Ehlers & Riordan, 1991; Wei et al, 1991b).

These observations led to further analysis of the active sites through the use of site-directed mutagenesis. The first set of mutants designed to confirm that both sites were active consisted of full-length sACE containing a single active site – where the zinc-binding motif of the second site had been disrupted – and truncated mutants containing a single active domain (Wei et al, 1991b). Disruption of the zinc-binding motif resulted in a failure to convert angiotensin I to angiotensin II – as shown with double mutants where both sites had been disrupted. This proved that the zinc-binding motif is an integral part of the active site. Both sites were able to hydrolyse angiotensin I and bound Hip-His-Leu with the same specificity as wild-type, indicating that these sites act independently. Interestingly, the effect appeared to be additive, where the amount of substrate converted by both the N-domain and C-domain mutants was equivalent to that converted by wild-type. The significant difference noted between the two domains was in the chloride ion dependent hydrolysis of Hip-His-Leu, where the C-domain site appeared to be more chloride dependent (Williams et al, 1992). Another study endeavoured to determine the role of the flanking sequence motifs by creating N-del-ACE, a chimera containing a single active site (Marcic et al, 2000). The N-domain active site and the region between the two active sites were removed, linking 141 residues of the N-terminal region directly to the active site of the C-domain. By determining the effects on the hydrolysis of Hip-His-Leu, Z-Phe-His-Leu and natural substrates angiotensin I and bradykinin, it was shown that the presence of N-terminal sequences affects the activity of the C-terminal domain with respect to these substrates, implicating the role of flanking sequences in ligand binding and catalysis.

It remains unclear why the two active sites within sACE have different substrate preferences and requirements and what physiological importance these have. A greater understanding would be provided from the structural determination of both active sites and any adjacent motifs that could be involved in ligand binding.

1.2.3 Purification and Cloning

The structural determination of ACE lies in recombinant DNA technology and the development of protein chemistry, specifically in the purification of ACE.

Affinity chromatography using inhibitors bound to a sepharose matrix to isolate ACE from tissue homogenate proved to be an efficient method to obtain highly purified ACE (Harris et al, 1981; El Dorry et al, 1982b). The addition of a spacer between the column matrix and ligand allowed for access to the active site of ACE, thus increasing the yield obtained. This was supported by the work of Pantoliano et al (1984), where a 28 Å spacer was used to bind a sepharose matrix to lisinopril (*N*-[1(S)-carboxy-5-amino-pentyl]-L-phenylalanylglycine). This workable method allowed for the purification of tACE from numerous sources and tissues, including rabbit lung (DeLuca-Flaherty et al, 1987) and testes (El Dorry et al, 1982b; Roy et al, 1988), mouse kidney (Bernstein et al, 1988) and human kidney (Soubrier et al, 1988).

This paved the way for the cloning of the full-length cDNA of sACE. Purified protein from human kidney was digested and the amino acid sequence of the N-terminal peptide and peptide fragments determined by N-terminal sequencing. Degenerate oligonucleotides were designed from selected peptide sequences and used to screen a cDNA library of human umbilical vein endothelial cells (Soubrier et al, 1988). DNA sequencing of the positive clones gave the full sequence of the sACE gene transcript, from which the amino acid sequence was derived.

As with somatic ACE, cloning of tACE revealed much about its structure. The full-length human cDNA was constructed by screening a human testicular library both with antibodies raised against bovine lung ACE and with ACE cDNA probes (Ehlers et al, 1989). tACE is a distinct protein, translated from a unique mRNA transcript and is not a cleavage product of sACE. This was evidenced by a 3 kb mRNA transcript in human testis RNA detected with a sACE cDNA probe, that was smaller than the 4.5 kb transcript detected in lung tissue (Soubrier et al, 1988). Furthermore, *in vitro* translation of testis mRNA in reticulocytes synthesised a ~83 kDa protein, which corresponds to estimated unglycosylated tACE (El Dorry et al, 1982a).

The isolation of full-length cDNA facilitated the expression of recombinant sACE and tACE in cultured cells. Human sACE was successfully cloned into a mammalian expression vector and stably transfected into Chinese hamster ovary (CHO) cells (Wei et al, 1991a). Similarly the cloning and expression of full-length tACE in CHO cells yielded 70 % and 85 % of ACE from detergent solubilised cell pellets and culture medium, respectively (Ehlers et al, 1991a). This recombinant protein was structurally, chemically and immunologically identical to ACE expressed in testes (Ehlers et al, 1991a).

Expressing recombinant ACE has two advantages: 1) protein that is expressed in quantities exceeding those found *in vivo* can be easily purified from culture medium or detergent-solubilised cells; and 2) ACE mutants can be constructed from recombinant cDNA. These can be expressed in an effort to understand various aspects of ACE activity, physiology and structure (Ehlers et al, 1991a; Wei et al, 1991a).

1.3 POST-TRANSLATIONAL PROCESSING OF ACE

1.3.1 Ectodomain Shedding

When full-length tACE was expressed in CHO cells, a significant percentage of protein was observed in culture medium, indicating that tACE was being solubilised (Ehlers et al, 1991a). Furthermore, truncated forms of sACE and tACE, lacking the C-terminal anchor, were observed predominantly in the medium, implicating the release of membrane bound ACE (Ehlers et al, 1991a; Wei et al, 1991a). Purified ACE from plasma, which is catalytically and immunologically identical to ACE isolated from cells (Das et al, 1977; Lanzillo et al, 1985), was not detected with antibodies raised against 20 amino acids at the C-terminus (Wei et al, 1991a), suggesting that solubilisation of ACE is a naturally occurring phenomenon.

The understanding of ACE solubilisation has made significant progress with the use of molecular engineering and improved protein chemistry. Studies have largely exploited the mutagenesis and expression of tACE for three reasons: 1) tACE is structurally and catalytically identical to the C-terminal domain of sACE (El Dorry et al, 1982b; Ehlers et al, 1989); 2) its smaller size facilitates cloning and expression (Ehlers et al, 1991a) and 3) recombinant tACE expressed in cultured cells is solubilised (Ehlers et al, 1991b) and is released more efficiently than sACE (Beldent et al, 1995; Woodman et al, 2000).

From the observation that ACE is solubilised by a membrane-bound secretase (Hooper et al, 1987; Ehlers et al, 1991b) advances in protein analysis have allowed for the identification of the cleavage site within tACE. With human tACE, the C-terminal peptide of soluble tACE expressed in CHO cells was identified through N-terminal sequencing of cyanogen bromide digested peptides and analysed by mass spectrometry by comparison of its mass with a hypothetical N-terminal peptide ladder generated from the published amino acid sequence (Ehlers et al, 1996). The cleavage site between Arg627 and Ser628 corresponded to that identified with rabbit tACE – at Arg663 (Ramchandran et al, 1994).

Human sACE was expressed in CHO cells, purified and analysed to determine the cleavage site. As with tACE, cleavage occurred at Arg1203 (Arg627 in tACE), confirmed by MALDI-TOF mass spectrometry of peptide fragments and with antibodies that identified the C-terminal region of the soluble protein (Woodman et al, 2000). This cleavage site was corroborated in an investigation of a sACE mutant, which was solubilised at Arg1203 (Eyries et al, 2001).

Another approach towards characterising the proteolytic release of ACE is directed at identifying a sequence or structural recognition motif. A study of the effects of different motifs in the juxtamembrane (or 'stalk') region on solubilisation was performed by expressing chimeric forms of human tACE (Ehlers et al, 1996; Schwager et al, 1998; Schwager et al, 1999). Initially a set of deletion mutants was constructed where the juxtamembrane region was consecutively shortened to determine whether cleavage occurred at a specific distance from the membrane (Ehlers et al, 1996). There seemed to be no specific preference regarding the length or composition of the juxtamembrane region, provided the stalk was ~10 residues, as all mutants were solubilised at the same cleavage site as wild-type tACE (Ehlers et al, 1996). Interestingly, two reports show marked differences in proteolysis after point mutations in the stalk region. A Pro to Leu mutation identified in purified plasma ACE has been attributed to increased shedding efficiency resulting in high levels of plasma ACE observed (Eyries et al, 2001). Proteolysis occurred at the same site reported for recombinant sACE, at Arg1203. Furthermore, shedding could be stimulated in the presence of phorbol esters (Arribas et al, 1996) and was inhibited by the hydroxamate based inhibitor TAPI, both characteristics of the ACE secretase (Ramchandran & Sen, 1995; Schwager et al, 1998). In light of these findings, this aberrant solubilisation is thought to be the result of increased stalk accessibility, rather than the action of a distinct secretase. The disruption of a putative N-linked glycosylation site seven residues upstream of the cleavage site increased proteolysis of human tACE expressed in a neuronal cell line (IMR32 cells) (Alfalah et al, 2001). This appeared to be the action of a distinct secretase as a novel cleavage site was observed and solubilisation was not inhibited by batimastat, which inhibits the ACE secretase in IMR32 cells (Parvathy et al, 1997; Alfalah et al, 2001). The effect of this mutation on proteolysis could not be attributed to an altered glycosylation pattern, as this site is not glycosylated in wild-type tACE, as is discussed below (Yu et al, 1997). However, the presence of glycosylation does seem to affect shedding. A series of mutants were constructed to determine the role of both O-linked and N-linked glycosylation in the

solubilisation of tACE (Schwager et al, 1999). Shedding was not inhibited by the introduction of an N-linked glycosylation site adjacent to Arg627, although the cleavage site was repositioned 14 residues closer to the membrane. Furthermore, the presence of O-linked glycosylation did not hamper expression and seemed to increase solubilisation (Schwager et al, 1999). It appears that solubilisation of ACE can accommodate various disruptions within the juxtamembrane region.

As yet, the specific recognition motif and the ACE secretase have not been identified. The 3-dimensional structure of ACE will provide insight into these two aspects of ACE solubilisation by identifying potential recognition domains that can be targeted for investigation.

1.3.2 Glycosylation

1.3.2.1 *The structure and synthesis of glycosylation*

1.3.2.1.1 *Structure of oligosaccharide chains*

Most extracellular proteins are post-translationally modified by the addition of oligosaccharides to specific amino acids in the protein backbone. These oligosaccharides consist of linked sugars that can be highly varied. Some of the determinants are the number of sugars that are linked, the sequence in which they are linked and the conformation with which they interact. Sugars can assume numerous conformations that is the result of the site on each sugar at which the glycosidic linkage occurs, the conformation of that linkage (either α or β) and the conformation of the sugar, which can be in either the pyranose or furanose form (Kobata, 1992). Thus, compared to amino acids whose variability depends on the peptide sequence, a great degree of structural multiplicity can be derived from a few different sugars. Furthermore, multiple sites for linkage allows for more than one sugar to bind to a single residue resulting in branched chains, extending the structure.

The two classes of oligosaccharides predominantly associated with extracellular proteins are N-linked and O-linked glycosylation, which are distinguished by their basic core structures and the specific peptide sequences to which they are attached. The branched N-linked pentasaccharide core, of two N-acetylglucosamine and three mannose (GlcNac₂Man₃), is bound to an asparagine residue within the sequon Asn-X-Ser/Thr via N-acetylglucosamine (Ronin et al, 1981). O-linked sugars are bound to threonine or serine residues via an N-acetylgalactosamine disaccharide (GalNac₂), in regions of the protein

where these residues occur in clusters. The oligosaccharide chain (antennae) extends from these core sugars.

Proteins are glycosylated in the rough endoplasmic reticulum (RER) and Golgi apparatus (reviewed by Lis & Sharon, 1993; Gahmberg & Tolvanen, 1996; Wyss & Wagner, 1996; Sears & Wong, 1998). The pathway that synthesises N-linked oligosaccharides has been well characterised (Kornfeld & Kornfeld, 1985) and can be divided into two stages: 1) oligosaccharide chain synthesis in the RER and 2) chain elongation in the Golgi apparatus.

The synthesis of O-linked sugars is not as well characterised. It is thought that initiation of O-linked glycosylation occurs by the attachment of GalNAc to the protein backbone during transfer of the glycoprotein from the RER to the Golgi apparatus (Sears & Wong, 1998).

1.3.2.1.2 Synthesis of N-linked glycoproteins

N-linked oligosaccharide chain synthesis begins with the formation of a precursor oligosaccharide block on dolichol pyrophosphate embedded in the RER membrane. The precursor contains the pentasaccharide core, an additional six mannose residues and a chain of three terminal glucose molecules ($\text{GlcNAc}_2\text{Man}_9\text{Glu}_3$). These sugars are transferred co-translationally from dolichol pyrophosphate by oligosaccharyl transferase (OST) to the nascent protein that is being translocated to the RER (Fig. 1.3). The Asn-X-Ser/Thr sequon adopts a particular motif, an Asx-turn, that is a better substrate for OST (Rees et al, 1983). The Asx-turn is formed by macrolactamisation of the second side chain in the sequon with a main chain amine and stabilised by hydrogen bonding between the amide proton of serine or threonine and the carbonyl oxygen in the side chain of asparagine (Fig. 1.4) (Imperiali et al, 1999). This structure is transiently formed as the nascent protein is translated into the lumen of the RER and is dependent on the properties of the side chain in the second position (Bause, 1983; Imperiali & Rickert, 1995). As a result, not all potential N-linked sites are glycosylated (roughly 90 % are), which increases the heterogeneity of specific proteins (Helenius & Aebi, 2001). The precursor oligosaccharide undergoes processing under the action of specific enzymes whilst the nascent polypeptide chain is folded. The terminal glucose is removed by α -glucosidase I, followed by the stepwise removal of the remaining glucose residues by α -glucosidase II. Finally, α -mannosidase I removes a single terminal mannose residue.



monensin

kituensis



castanospermin



N-butyldeoxyfructin

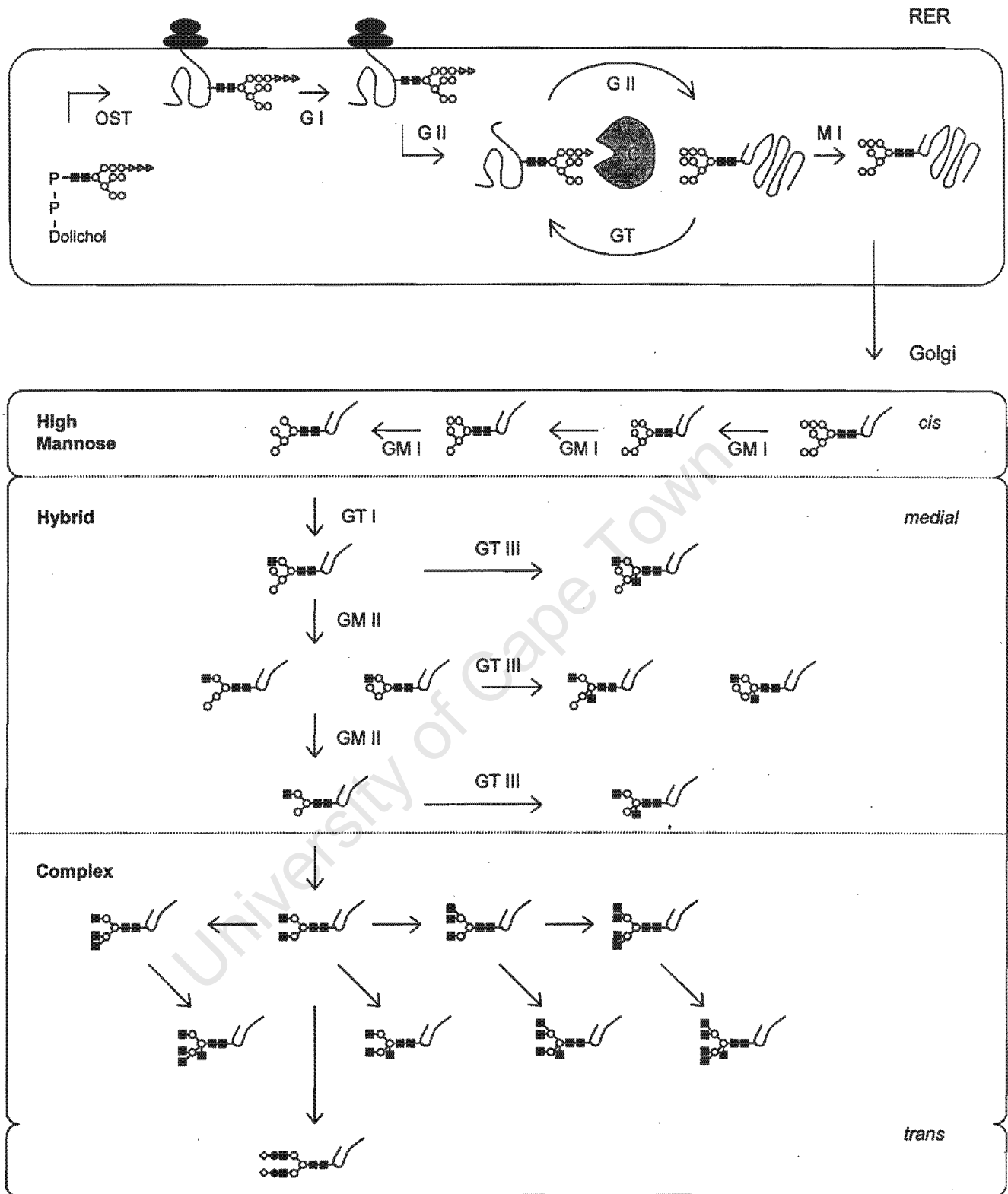


triacetin



amphotericin B

University of Cape Town



LEGEND







-  ribosome
-  GlcNAc
-  glucose
-  sialic acid
-  galactose
-  mannose

Figure 1.3. Synthesis of N-linked glycosylation, as explained in the text. RER: rough endoplasmic reticulum, Dolichol-P-P: dolichol pyrophosphate, OST: oligosaccharyl transferase, G I: α -glucosidase I, G II: α -glucosidase II, GT: glucosidase, C: Calnexin/ calreticulin, M I: α -mannosidase I, GM I & II: golgi α -mannosidases I & II, GT I & III: N-acetylglucosaminyltransferases I & III. **Insert:** Specific glycosylation inhibitors and the sites at which they act. Figure adapted from Kendrew & Lawrence (1994), pg 442.

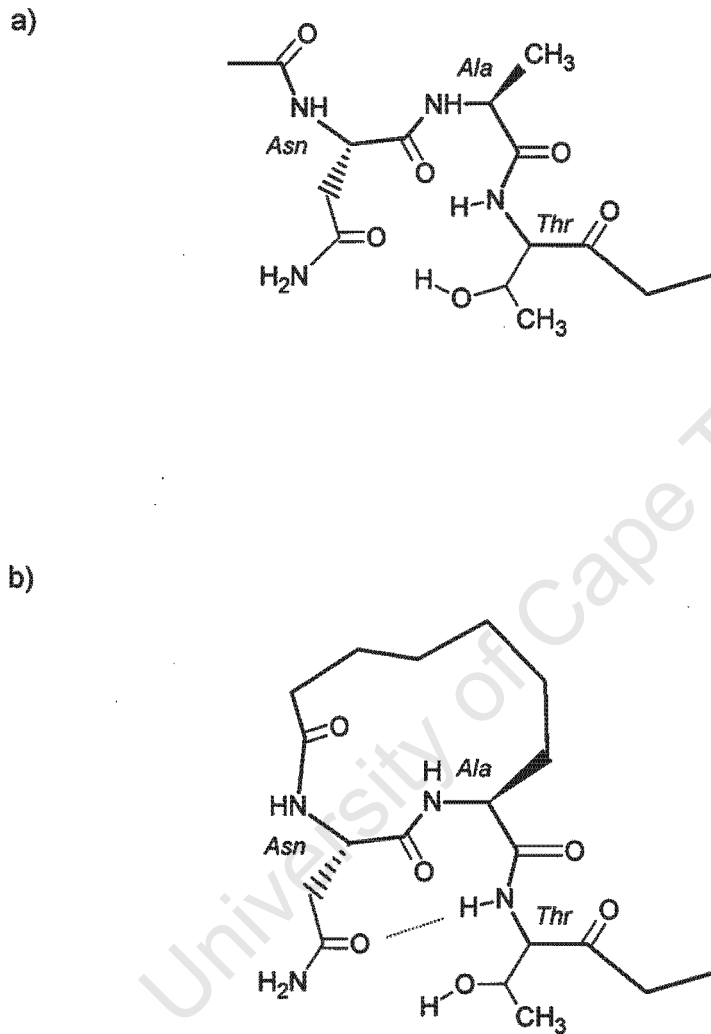


Figure 1.4. The conformation of the N-linked glycosylation sequon, using Asn-Ala-Thr as an example. a) Linear Asn-Ala-Thr, b) The Asx-turn recognised by oligosaccharyl transferase, indicating the stabilising hydrogen bond between Asn and Thr. Figure adapted from Imperiali et al, 1999.

N-linked glycosylation has been found to play a significant role in protein folding in the RER. When oligosaccharides attached to the nascent polypeptide undergo deglycosylation by α -glucosidase I and II, the single terminal glucose can interact with the lectins calreticulin and calnexin (reviewed by Parodi, 2000 and Helenius & Aebi, 2001). This interaction seems to be a control point for the synthesis of correctly folded proteins. The binding of glycoproteins to these lectins delays their processing to some degree, thus may allow time for correct folding or retain incorrectly folded proteins in the RER for degradation. Once the proteins are correctly folded, the terminal glucose is removed by α -glucosidase II and the protein undergoes further processing. If folded incorrectly, the protein is sent back along the pathway by addition of a terminal glucose to the oligosaccharide chain by a glucosyltransferase, thereby reinstating its interaction with calreticulin and calnexin (Fig. 1.3) (Ruddon & Bedows, 1997; Zhang et al, 1997). This process of reglycosylation and deglycosylation is repeated until the protein is correctly folded or degraded.

Some evidence from NMR studies indicates that N-linked oligosaccharides may play an active role in defining the conformation of the peptide backbone, where the presence of sugars converts the Asx-turn motif to a more compact β -turn (Imperiali & O'Connor, 1999). Furthermore, the extended structure of the sugar chains would necessitate that the region of the polypeptide to which it is attached is located on the surface of the protein (Helenius & Aebi, 2001).

At this point, glycoproteins have been synthesised, glycosylated at N-linked sequons, disulphide bridges formed and the folded proteins, containing $\text{GlcNac}_2\text{Man}_6$ N-glycans, are now transferred via vesicles to the Golgi apparatus for further processing of the oligosaccharide chain.

1.3.2.1.3 *Elongation of the oligosaccharide chain*

Chain elongation for both classes of oligosaccharides is the result of the action of specific enzymes, or glycosyltransferases, expressed in the Golgi apparatus (Hirschberg & Snider, 1987). Each glycosyltransferase recognises a specific glycosidic linkage and attaches or removes a specific sugar. Thus, the sugar sequence is not encoded, but is a direct result of the host of enzymes present in the Golgi apparatus. This is a highly dynamic environment as the action of these enzymes is interdependent. Two enzymes may compete for the same substrate, the action of one enzyme may result in the addition of a sugar that inhibits a second enzyme or, in the same way, the action of one enzyme may facilitate the

action of second one. Because the processing of the antennae is determined by the glycosyltransferases present the resultant oligosaccharide chain varies between different proteins, between different glycosylation sites in the same protein and between the same protein expressed in different cell types. This is particularly evident when comparing the glycosylation patterns of endogenous proteins to their recombinant counterparts, such as with human interferon- β 1 which, when expressed in three cells lines, showed three distinct sets of glycosylation that differed from the natural protein (Kagawa et al, 1988). Furthermore, carcinoma cells express glycoproteins with altered glycosylation patterns (Kobata, 1989), which is a consideration when investigating the glycosylation of recombinant proteins as most cultured cells are derived from tumours (Kobata, 1992).

Although the oligosaccharides produced are highly variable, N-linked sugar chains can be divided into three subgroups according to their structure: high mannose, complex and hybrid (Kobata, 1992), which are derived from the action of glycosyltransferases in the Golgi apparatus. There is a degree of variation within these subgroups. As the term suggests, high mannose sugars strictly contain mannose residues in the antennae, which vary according to their number and location on the branched pentasaccharide core. These oligosaccharides are the products of a series of reactions in the *cis* Golgi whereby terminal mannose residues are sequentially removed by Golgi α -mannosidase I from the GlcNac₂Man₈ chain, synthesised in the RER, to generate the heptasaccharide GlcNac₂Man₅, from which complex and hybrid sugars are derived in the *medial* Golgi (Fig. 1.3). Complex sugars are branched chains containing GlcNac, GalNac, fucose, glucose, galactose, mannose and sialic acid residues linked to the pentasaccharide core, which contains the only mannose residues. The sugars are synthesised by the action of a group of N-acetylglucosaminyltransferases (GTs). For example, a GlcNac is added to the terminal mannose residue in the heptasaccharide by GT I, allowing for the action of Golgi α -mannosidase II, which removes the two terminal mannose residues to generate the pentasaccharide core containing a terminal GlcNac. Further processing by specific GTs generates a host of intermediate sugars. The addition of a side-chain GlcNac to the first mannose by GT III terminates the addition of further sugars. This can occur on all intermediates in the *medial* Golgi and is the genesis of hybrid oligosaccharides, whose structures have features common to both high mannose and complex sugars. Glycosylation is terminated in the *trans* Golgi by the addition of side-chain GlcNac or terminal sialic acid residues.

When glycoproteins are expressed on the cell surface, their degree of variability is vastly increased by the presence of oligosaccharides. This is due to the microheterogeneity of sugars at each site, where the sequence, number and conformation of oligosaccharides vary. Further heterogeneity is the result of distinct sequences at each site and the structural dynamics of the oligosaccharide chain. The overall conformation of the glycoprotein is increasingly heterogeneous due to the spatial orientation of sugar chains as a result of the degree of flexibility within the oligosaccharide chain and the asparagine side chain (Rudd et al, 1995).

1.3.2.2 *The Role of Oligosaccharides in Protein Expression and Function*

The presence of oligosaccharides in general increases the heterogeneity of proteins; specifically, this plays a significant role in the chemistry and biology of glycoproteins, which has been extensively reviewed (Kobata, 1992; Sears & Wong, 1998; Helenius & Aebi, 2001). Glycosylation plays a diverse role in protein chemistry and function ranging from folding to specific immunological functions (Lis & Sharon, 1993; Gahmberg & Tolvanen, 1996; Wyss & Wagner, 1996; Sears & Wong, 1998). Additionally, the diverse glycosylation patterns observed between recombinant proteins expressed in different cell lines (Kagawa et al, 1988; Charlwood et al, 2001) imply that the role of glycosylation must be determined specifically for each protein in a particular expression system (Lis & Sharon, 1993).

The effect of N-linked glycosylation on the expression and activity of numerous proteins has been well characterised through the use of glycosylation inhibitors, purified glycosidases and glycosyltransferases, cell lines that contain defects in the glycosylation pathway, and recombinant DNA technology (Lis & Sharon, 1993). Furthermore, these approaches have been invaluable in ascertaining the minimal glycosylation requirements for the correct expression of fully functional glycoproteins.

O-linked sugars have not been as extensively studied as their structures are less defined than N-linked oligosaccharides and few specific inhibitors and glycosidases exist to expand such investigations (Lis & Sharon, 1993).

1.3.2.2.1 *Glycosylation Inhibitors*

A variety of inhibitors have been isolated, which are used to arrest the glycosylation pathway at particular points (Fig. 1.3, insert) (reviewed by Elbein, 1991; Winchester & Fleet, 1992). They include tunicamycin (Heifetz et al, 1979), N-butyldeoxynojirimycin (Karlsson et

al, 1993), castanospermine (Tsuji et al, 1996), monensin (Harold et al, 1974), kifunensine (Elbein et al, 1990), swainsonine (Dorling et al, 1980) and mannostatin A (Tropea et al, 1990). Tunicamycin inhibits the action of N-acetylglucosaminyl phosphate transferase, an enzyme involved in synthesising the precursor oligosaccharide block on dolichol pyrophosphate (Heifetz et al, 1979; Keller et al, 1979). The absence of a precursor prevents N-linked glycosylation of nascent polypeptides in the RER (Struck et al, 1978; Helenius, 1994). N-butyldeoxynojirimycin and castanospermine can be used to minimise the heterogeneity of sugars present on a recombinant protein as they inhibit α -glucosidase I and II respectively in the RER, resulting in the accumulation of glycoproteins containing the high mannose precursor in cells (Loch et al, 1992). Monensin and kifunensine can also be used in this way as the former prevents vesicular traffic between the RER and Golgi apparatus and the latter inhibits Golgi α -mannosidase I, thereby preventing chain elongation, causing an accumulation of oligomannose GlcNac₂Man₈ sugars. Finally, mannostatin A has been shown to prevent the formation of complex sugars as it inhibits Golgi α -mannosidase II.

Tunicamycin is the most widely used inhibitor, particularly in determining the effect of complete deglycosylation on the activity and expression of proteins. Treatment of CHO cells expressing the human thyrotropin receptor (TSHR) with tunicamycin produced a deglycosylated form of the receptor, which was incapable of ligand binding and signal transduction. Immunoprecipitation showed that this deglycosylated protein was improperly folded and rapidly degraded intracellularly, preventing its surface expression and activity (Nagayama et al, 1998). Tunicamycin treatment of CD4-positive human acute lymphoblastic leukemia cells decreased cell surface expression of CD4 and caused accumulation of the protein inside cells, which was attributed to the lack of glycosylation (Konig et al, 1988). The same observations were made with HeLa cells expressing tACE, where tunicamycin treatment resulted in a deglycosylated form that was rapidly degraded intracellularly (Kasturi et al, 1994). Thus, treatment of cells with tunicamycin prevents N-linked glycosylation, which, in many cases, results in intracellular sequestering of proteins and their subsequent degradation. This implicates N-linked glycosylation in the processing and expression of proteins.

1.3.2.2.2 *Mutant cell lines*

Another development in determining the role of glycosylation has been in the expression of glycoproteins in various cell lines. This approach exploits the different

glycosylation pathways present in different cells to produce glycoproteins containing particular types of oligosaccharide chains. Unglycosylated proteins can be expressed in *E. coli*, which lacks the machinery to glycosylate proteins, as was performed with rabbit tACE (Sadhukhan & Sen, 1996). The heterogeneity of oligosaccharides is reduced by the expression of recombinant proteins in yeasts, which produce N-linked sugars as the high mannose type exclusively (Tanner & Lehle, 1987). Certain CHO cell mutants have deficiencies in the glycosylation pathways. CHO-Lec 1 cells lack N-acetylglucosaminyl-transferase I activity, resulting in the accumulation of high mannose (GlcNac₂Man₅) sugars at sites where complex sugars are usually present (Stanley & Siminovitch, 1977). CHO-Lec 2 cells are deficient in Golgi sialic acid transporters, preventing acquisition of CMP-sialic acid from the cytoplasm and as a result formulate oligosaccharides which are sialic acid deficient (Stanley & Siminovitch, 1977). In addition to this mutation, CHO-Lec 8 cells lack Golgi galactose transporters, resulting in galactose and sialic acid deficient sugars (Stanley & Siminovitch, 1977). Human thyrotropin receptor (THSR) was expressed in these three cell lines to characterise its glycosylation (Nagayama et al, 1998). The effect of altered glycosylation on surface expression, binding affinity and signal transduction was determined. Decreased cell surface expression was observed with TSHR expressed in CHO-Lec 1 and -Lec 2 cells, whereas protein expressed in all three cell lines were able to bind ligand and induce cAMP production as well as wild-type. This validated the importance of N-linked glycosylation in surface expression of THSR, where the presence of oligomannose sugars decreases surface expression (Nagayama et al, 1998).

CHO *ld/D* cells have a reversible defect, which can be exploited to define the glycosylation requirements for functional glycoproteins. These cells lack UDP-Gal/UDP-GalNac 4 epimerase, which converts glucose and GlcNac into galactose and GalNac, respectively (Kingsley et al, 1986). As a result, these cells are unable to produce complex N-linked sugars, which require galactose and GalNac, and the synthesis of O-linked oligosaccharides is blocked by the absence of an initiating GalNac disaccharide. However, this can be overcome by the addition of galactose or GalNac to culture medium. This is one of the first applications for manipulating the synthesis of O-linked sugars (Krieger et al, 1989). The effect of each class can be determined by inhibiting both N-linked and O-linked sugars in the absence of additives or by reinstating glycosylation, allowing N-linked oligosaccharides to be synthesised by the addition of galactose and the synthesis of O-glycans by the addition of GalNac (Krieger et al, 1989).

The use of inhibitors and mutant cell lines has been instrumental in defining the glycosylation requirements for recombinant proteins. However, there are disadvantages to these two approaches. First, inhibitors may be toxic to cells, as was demonstrated with tunicamycin and swainsonine in sympathetic neurons and pig tissues, respectively (Tulsiani et al, 1984; Chang & Korolev, 1996), making the effect of glycosylation on protein expression disadvantageous to investigate in this system. Second, both approaches target the entire glycosylation pathway, which may encumber the elucidation of glycosylation of a particular protein as the result of effects on cell viability (Lis & Sharon, 1993).

1.3.2.2.3 *Mutagenesis of N-linked Glycosylation Sites*

A powerful approach used to target specific proteins is site-directed mutagenesis, where glycosylation sites on a protein can be altered and the effects monitored by expression in cultured cells. This approach enables the role of each site to be determined with respect to protein processing, expression and function. Site-directed mutagenesis was performed on influenza virus haemagglutinin (HA) to clarify the interaction between calnexin and calreticulin and specific N-linked sites in the protein, and the role these sites play in protein folding (Hebert et al, 1997). Combinations of mutants were constructed by mutagenesis of the seven potential N-linked sequons and were translated in canine pancreas microsomes in the presence of [³H] methionine. Three distinct conformers were observed upon immunoprecipitation of HA and autoradiography, which corresponded to the protein in different stages of folding. With each mutant, the presence of these conformers was determined with respect to their association with the lectins. Removal of glycosylation at a single site in the N-terminal domain disrupted folding, whereas the lack of glycosylation in the C-terminal region had the opposite effect (Hebert et al, 1997). Thus, targeting specific sites highlighted regions of HA that determine correct protein folding.

The role of N-linked glycosylation at particular sites in protein expression and function has been exhaustively investigated in numerous proteins, including rhodopsin (bovine opsin; (Kaushal et al, 1994), the rat lutenizing hormone receptor (Zhang et al, 1995), human Alzheimer's β -secretase protein, Asp-2 (Charlwood et al, 2001) and the human thyroid Na⁺/I⁻ symporter (Levy et al, 1998). Site-directed mutagenesis of rat dipeptidylpeptidase IV (DPPIV) was used to highlight the role of N-linked oligosaccharide in the different domains of this glycoprotein (Fan et al, 1997). This type II membrane bound enzyme exists as a homodimer, each monomer consisting of three domains from the transmembrane region at

the N-terminus: an N-glycan rich domain adjacent to the cell membrane, a cysteine-rich domain and the active site domain at the C-terminus. An N-linked glycosylation sequon was removed from each domain to ascertain the role of sugars in cell surface expression, enzymatic activity and dimerisation by immunoprecipitation and immunoblotting of mutants expressed in CHO cells. The removal of sugars in the two outer domains had little effect as these mutants were expressed as enzymatically active homodimers. A remarkable effect was seen when one of the two N-glycans was abolished in the cysteine-rich domain. This mutant was enzymatically inactive and rapidly degraded intracellularly, implicating this site in correct protein folding (Fan et al, 1997). Specific sites essential for correct targeting of human vasoactive intestinal peptide I receptor were investigated by site-directed mutagenesis of the four potential N-linked sites (Couvineau et al, 1996). Mutants were transiently expressed in COS-7 cells and the expression of active protein determined by immunoblotting, ligand binding and confocal microscopy. The presence of glycosylation on one of the two sites in the N-terminal region that extends from the membrane was shown to be essential for proper cell surface expression and ligand binding (Couvineau et al, 1996).

Other investigations using site-directed mutagenesis of target sites for N-linked and O-linked glycosylation have highlighted the minimal glycosylation requirements for the expression of functional glycoproteins. Combinatorial site-directed mutagenesis of the four putative N-linked glycosylation sites on rat parathyroid hormone and parathyroid-related peptide (PTH/PTHrP) receptor was performed to determine the sites that were essential for normal processing and function, monitored by ligand binding assays and cAMP production (Zhou et al, 2000). The removal of individual sites had no significant effect on ligand binding or signal transduction and a mutant containing a single N-linked site was still functional, indicating that the PTH/PTHrP receptor requires glycosylation at a single site for cell surface expression and function, with a preference for sites at the N-terminus.

Specific glycosidases have been purified and have been instrumental in defining the types of glycosylation present on purified proteins or recombinant proteins expressed in cultured cells (Maley et al, 1989). They have defined the extent of glycosylation and complement studies using site-directed mutagenesis and expression of proteins in mutant cell lines or in the presence of inhibitors.

The use of purified glycosidases, mutant cell lines, specific inhibitors and site-directed mutagenesis has increased our knowledge and understanding of glycoproteins. What is

clear from these investigations is that with the majority of proteins, some form of glycosylation, particularly N-glycans, is required for the production of functional proteins. There appears to be a delicate equilibrium between removing glycans to reduce the heterogeneity of glycoproteins and retaining sufficient oligosaccharides to ensure protein viability. Furthermore, each protein expressed in a particular cell line has unique glycosylation requirements for its folding, targeting and expression.

1.3.2.3 Glycosylation of ACE

An understanding of the role that glycosylation plays in expression and solubilisation of ACE has seen great advances through the use of the techniques described. The majority of investigations have targeted tACE because it is catalytically and structurally identical to the C-terminal domain of sACE and it contains fewer potential sites for glycosylation.

The unique 36 amino acid N-terminal region of tACE is rich in serine and threonine residues, which are potential sites for O-linked glycosylation (Ehlers et al, 1989; Kumar et al, 1989). A deletion mutant, lacking these 36 residues, was expressed in CHO cells to determine their role in the synthesis and function of tACE (Ehlers et al, 1992). Enzymatic deglycosylation of the purified protein showed that wild-type tACE was heavily glycosylated with both N-linked and O-linked sugars, whereas the mutant contained N-linked sugars and lacked O-linked sugars, but remained catalytically active (Ehlers et al, 1992). Thus, O-linked glycosylation at the N-terminus of tACE, accounting for 15 kDa, does not seem to play a role in enzyme stability or function when expressed in CHO cells. However, this form of glycosylation may play a specific role in the testes (Ehlers et al, 1992), such as in fertility (Hagaman et al, 1998).

Altered glycosylation patterns between the two isoforms led to an investigation into the role of N-linked sugars in the expression and activity of ACE. Initially, the effect of glycosylation was determined by exposure of cells expressing rabbit tACE to tunicamycin and monitored by the sensitivity of expressed protein to various deglycosidases. Unglycosylated tACE was expressed, but rapidly degraded intracellularly as shown by transient expression of rabbit tACE in HeLa cells in the presence of tunicamycin (Kasturi et al, 1994). Furthermore, expression of rabbit tACE in *E. coli* resulted in the expression of inactive protein, probably the result of incorrect folding (Sadhukhan & Sen, 1996). Thus, rabbit tACE requires N-linked glycosylation to be expressed as an enzymatically active protein. To determine the extent of glycosylation required, rabbit tACE was expressed in CHO *Id/D* cells and monitored by

enzymatic deglycosylation of immunoprecipitated protein. The absence of O-linked sugars and presence of less complex N-linked sugars did not affect tACE activity and surface expression or solubilisation (Kasturi et al, 1994).

To further elucidate the glycosylation requirements for the expression and processing of active tACE, specific glycosylation sites were disrupted (Sadhukhan & Sen, 1996). There are 5 potential N-linked glycosylation sites in the rabbit tACE sequence (Fig. 1.5), with an additional 6 in the somatic form (Kumar et al, 1989). These 5 sites were combinatorially mutated and their role evaluated by expression in HeLa cells by pulse-chase analysis using [³⁵S] methionine-labelled tACE (Sadhukhan & Sen, 1996). A null mutant, where all 5 sites had been disrupted, behaved similarly to wild-type tACE expressed in the presence of tunicamycin. It was degraded intracellularly and failed to be detected in culture medium, confirming previous findings that tACE requires N-linked glycosylation to be expressed in an active form (Kasturi et al, 1994; Sadhukhan & Sen, 1996). Expression of the remaining mutants showed a preference for N-linked glycosylation at the N-terminus and that the presence of sugars at a single N-terminal site was necessary and sufficient to produce enzymatically active tACE that was solubilised. The presence of glycosylation is not site-specific, as mutants that have either the first site or second site intact are expressed and active. However, glycosylation at the third site alone was not sufficient to produce active protein in HeLa cells, albeit this mutant was expressed in yeast (Sadhukhan & Sen, 1996), indicating that the requirements for glycosylation are cell-specific.

It appears that this may extend to species-specificity as analysis of purified sACE from four species (guinea-pig, rabbit, rat and human) revealed differential glycosylation patterns. Of the 17 potential N-linked glycosylation sites in human sACE, 7 appear to be glycosylated in ACE expressed in seminal plasma (Ripka et al, 1993). Limited proteolysis and sequencing of tryptic fragments of purified human kidney ACE identified seven peptides containing potential N-linked sites, six of which were glycosylated (Fig. 1.5) (Yu et al, 1997). Four of these sites are clustered at the N-terminus and the unglycosylated site lies seven residues proximal to the reported cleavage site.

The number of sites that are glycosylated vary between 7 and 8 over these four species. Furthermore, the pattern of glycosylation with respect to O-linked glycosylation, high mannose and complex N-linked sugars differs (Ripka et al, 1993). Considering these findings, altered glycosylation patterns would be expected across different species, across

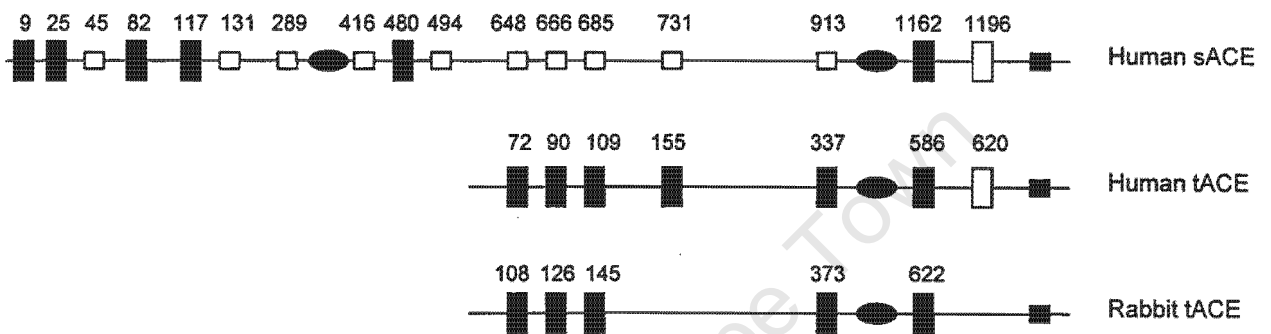


Figure 1.5. The N-linked glycosylation profiles of human and rabbit ACE.

ACE expressed in different tissues and across recombinant protein expressed in different cell lines (as was observed for rabbit tACE in HeLa cells and yeast).

In human tACE O-linked sugars are not necessary for tACE expression, implicating the N-linked sugars in this role (Ehlers et al, 1992). N-linked glycosylation of human tACE expressed in CHO cells at each site has been identified by MALDI-TOF mass spectrometry (Yu et al, 1997). There are 7 potential N-linked sites in human tACE, five of which are complementary to the sites in rabbit tACE (Fig. 1.5) (Yu et al, 1997). The unique sites lie within the ectodomain (the fourth site) and in the juxtamembrane stalk region, adjacent to the cleavage site (the seventh site). As with the rabbit form, there appears to be a preference for glycosylation at the N-terminus. The peptides containing the first three sites were not detected by limited proteolysis, but were resolved on HPLC after enzymatic deglycosylation of unidentified peaks (Yu et al, 1997), indicating that they are glycosylated. Both unglycosylated and glycosylated forms of the fourth, fifth and sixth sites were identified in the same way. The presence of partial glycosylation at these sites may be the result of protein folding or the formation of a disulphide bridge that may occlude glycosylation (Yu et al, 1997). The peptide containing the seventh site was isolated in its unmodified form, indicating that this site is not glycosylated (Yu et al, 1997). The role of each of these sites has not been determined and an investigation into the glycosylation requirements of human tACE would prove insightful in light of what was observed in rabbit tACE (Sadhukhan & Sen, 1996) and with the pattern of glycosylation in human tACE (Yu et al, 1997).

1.4 STRUCTURAL DETERMINATION OF ACE

A structural understanding of ACE would provide much insight into its biochemical properties by identifying the exact structure of the active site and structural motifs that are involved in ligand binding and interaction with other proteins, such as the ACE secretase.

There are four main approaches to elucidating the 3-dimensional structure of macromolecules: 1) nuclear magnetic resonance (NMR), 2) electron microscopy, 3) X-ray crystallography and 4) molecular modelling. Compared to X-ray crystallography, electron microscopy is still in its infancy although this approach has been used successfully to resolve large protein complexes such as the chaperonins GroEL and GroES (Chen et al, 1998), which appear to be more readily defined using this method. NMR has been invaluable in identifying the structures of oligosaccharides as it can afford some degree of flexibility and

sequence heterogeneity (Lis & Sharon, 1993). However, it is limited to small molecules and information from proteins larger than 150 residues can not be elucidated (Smutzer, 2001).

The 3-dimensional structures of many proteins have been determined using X-ray crystallography. Diffraction patterns are used to extrapolate information about the structure of the molecules that make up the crystal (van Holden, 1985). This technique has been used extensively to find the spatial arrangements of peptides within proteins. The incorporation of computer-based data analysis has augmented X-ray crystallography greatly, rapidly advancing the rate at which the final 3-dimensional structure is determined.

However, the limiting step in this approach is the formation of crystals that are suitable for X-ray diffraction studies. In particular difficulties are encountered when trying to grow crystals from hydrophobic membrane glycoproteins (Giege, 1994), such as ACE. This has been overcome by resolving structures of the ectodomain or truncated proteins lacking the hydrophobic transmembrane domain. tACE can be expressed as a soluble, truncated protein that would be a better candidate for crystallisation (Ehlers et al, 1991a). In spite of available resources for producing large quantities of truncated tACE in expression systems, crystals suitable for analysis have not been obtained.

To obtain high resolution crystal structures, high quality crystals are required, which are grown from an extremely pure, homogeneous protein with relative ease (Giege, 1994). The use of expression systems in protein purification has enhanced purity, as vast quantities of recombinant protein can be isolated from culture medium. However, purified tACE is not structurally homogeneous, due to the presence of glycosylation (Yu et al, 1997). The degree of conformational flexibility of the sugar backbone and the heterogeneity of the sugar sequences at each site is responsible for hampering crystallisation (Wyss & Wagner, 1996). The formation of high quality crystals requires a set of compact, uniform structures and the sequence heterogeneity of the sugars combined with the variation of their spatial positioning increases the overall heterogeneity of the monomers. The extended conformation of sugars prevents the formation of tight packing of the protein and their flexibility decreases the degree of diffraction of light passing through the crystals. One solution may be to produce a deglycosylated form of tACE resulting in a homogeneous protein that may facilitate crystallisation; however, enzymatic removal of the oligosaccharide from tACE has not been successful in completely deglycosylating the enzyme without denaturing it (Lis & Sharon, 1993). An alternative would be to prevent the glycosylation of tACE by chemical or molecular means. The expression of a truncated form of tACE lacking the O-glycosylated N-terminal

region and the transmembrane domain (tACE- Δ 36sol) was attempted in the presence of N-butyldeoxynojirimycin, an inhibitor that limits glycosylation to oligomannose chains. The protein was purified and enzymatically deglycosylated with endoglycosidase H (Yu et al, 1997). Although sufficient quantities of underglycosylated tACE- Δ 36sol were produced for crystallogenesis, low yields of the deglycosylated protein have led us to explore the use of other tACE glycoforms. tACE lacking oligosaccharides has not been produced (Kasturi et al, 1994; Sadhukhan & Sen, 1996), indicating that glycosylation plays a role in ensuring the expression of tACE.

The production of a crystallisable candidate of human tACE would require the removal of glycans to decrease the overall heterogeneity and the retention of some N-linked glycans to facilitate protein expression. In light of what has been discussed regarding the role of glycosylation, this requires some insight into the minimum glycosylation requirements for the production of correctly folded enzymatically active human tACE.

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CHAPTER 2: DNA MANIPULATION

2.1 INTRODUCTION

To investigate the role of glycosylation in the expression of enzymatically active human tACE, minimally glycosylated isoforms were constructed by the removal of a combination of N-linked glycosylation sites. Glycosylation was abolished by site-directed mutagenesis of the site of attachment in the recognition sequon (Asn-X-Ser/Thr) through a conserved transition of the asparagine to a glutamine residue.

The three N-terminal sites were targeted based on previous observations in rabbit tACE. Testis ACE isoforms from human and rabbit have 85% sequence homology, with all five glycosylation sequons present in rabbit tACE being identical to complementary sequons in the human isoform (Appendix A8, Fig.A1). Glycosylation mutants of rabbit tACE indicate that either the first or the second site is sufficient to produce enzymatically active tACE when expressed in HeLa cells (Sadhukhan & Sen, 1996). Furthermore, similar studies on the role of glycosylation in the expression of the insulin receptor and rat PTH/PTHrP receptor implicate the importance of glycosylation at the N-terminus (Elleman et al, 2000; Zhou et al, 2000).

tACE- Δ 36 cDNA was divided into four fragments and introduced into a cloning vector to facilitate site-directed mutagenesis (Fig. 2.1). The first fragment contained the three N-terminal sequons from which a series of mutants was generated having either one or two of these sites. The fourth, fifth and sixth sites were removed from the second, third and fourth fragments, respectively. The seventh site was not mutated as it is not glycosylated when tACE is expressed in CHO cells (Yu et al, 1997). These four fragments were reassembled to produce eight tACE- Δ 36 mutants that lacked particular glycosylation recognition sequons. The mutants were introduced into the mammalian expression vector, pLEN, to facilitate the production of underglycosylated tACE protein. Additional cloning was required due to the lack of unique restriction enzyme sites in pLEN. The fourth fragment, containing the mutated sixth sequon, was cloned directly into pLEN-tACE- Δ 36, whereas the first three fragments were assembled in pBS-tACE- Δ 36, producing the desired mutants of the N-terminal sites, and cloned as the N-terminus of tACE into pLEN-tACE- Δ 36-g12345.

wild-type tACE

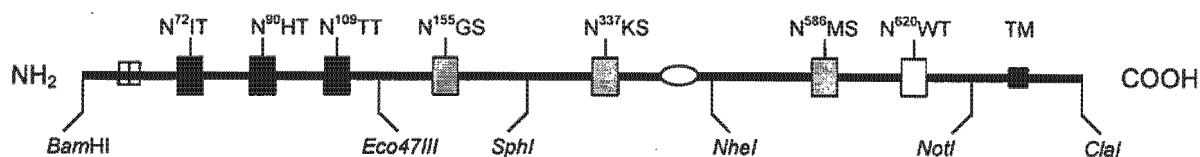
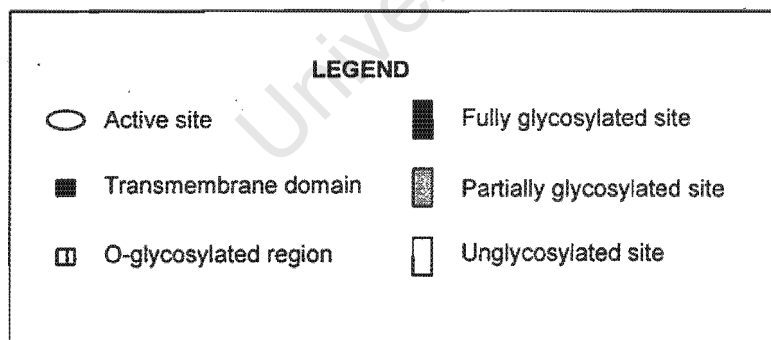
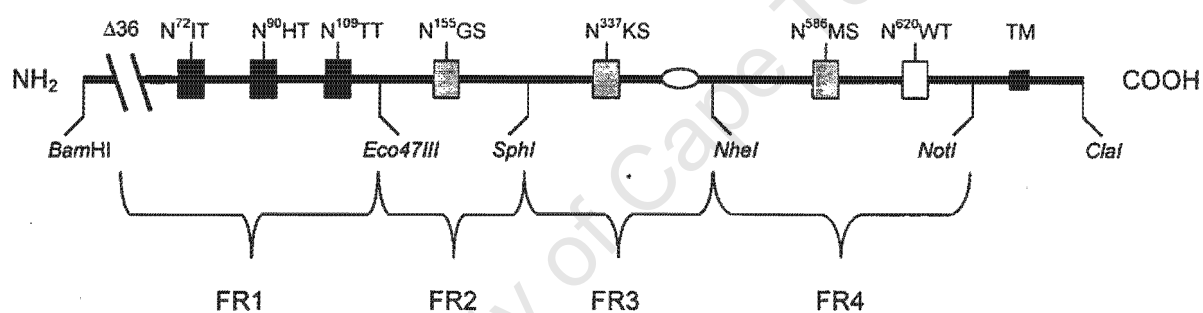
tACE- Δ 36

Figure 2.1. Schematic representation of wild-type testis ACE and tACE- Δ 36, indicating the sites of O-linked and N-linked glycosylation, the latter shown as fully glycosylated, partially glycosylated and unglycosylated sites. The restriction sites used to clone the four fragments (indicated as FR1, FR2, FR3 and FR4) into pGEM are shown. The protein sequences of wt-tACE and tACE- Δ 36 are detailed in Figure A1 of Appendix A8.

2.2 METHODS

2.2.1 Subcloning of human tACE fragments into a cloning vector for site-directed mutagenesis

Human tACE- Δ 36 cDNA was subcloned into pGEM-11Zf(+) according to standard subcloning techniques, detailed in Appendix A2-A4. Briefly, compatible restriction enzyme digested insert and vector DNA fragments were ligated and transformed into bacterial cells for amplification. Transformed cells were isolated by ampicillin selection on bacterial plates and small scale plasmid DNA isolation was performed on colonies (detailed in Appendix A1.1). Plasmids bearing the desired insert were identified by restriction enzyme digests and the relevant insert and vector bands visualised in agarose gels. Large scale DNA isolation was performed on positive clones with QIAGEN midiprep kits (DIAGEN, GmbH, Germany) according to the manufacturer's instructions (Appendix A1.2).

2.2.1.1 Introduction of an *Eco*47III site into pGEM to facilitate cloning

An *Eco*47III site needed to be introduced into pGEM to facilitate cloning of the first and second tACE fragments (Fig. 2.2). A 146 bp *Bam*HI/*Nhe*I digested pBR329 fragment that contained an *Eco*47III site was ligated to the compatible ends of *Bam*HI/*Xba*I digested pGEM, to generate pGEMeco, which was identified by digestion of isolated DNA with *Eco*47III and *Bsa*AI/*Sph*I.

2.2.1.2 Introduction of FR1 into pGEMeco

The N-terminal region of tACE- Δ 36, which contains the first three glycosylation sites (Asn72, Asn90 and Asn109), was introduced into pGEMeco (Fig. 2.3). It is derived from a 450 bp *Bam*HI/*Eco*47III digested pLEN-tACE- Δ 36 fragment that was ligated to similarly digested pGEMeco. Plasmid DNA containing FR1 was detected by visualisation of the excised fragments in an agarose gel after a *Bam*HI/*Hind*III digestion.

2.2.1.3 Introduction of FR2 into pGEMeco

A tACE fragment containing the fourth glycosylation site (Asn155) was cloned into pGEMeco (Fig. 2.4). This 389 bp fragment was generated from a *Eco*47III/*Sph*I digested

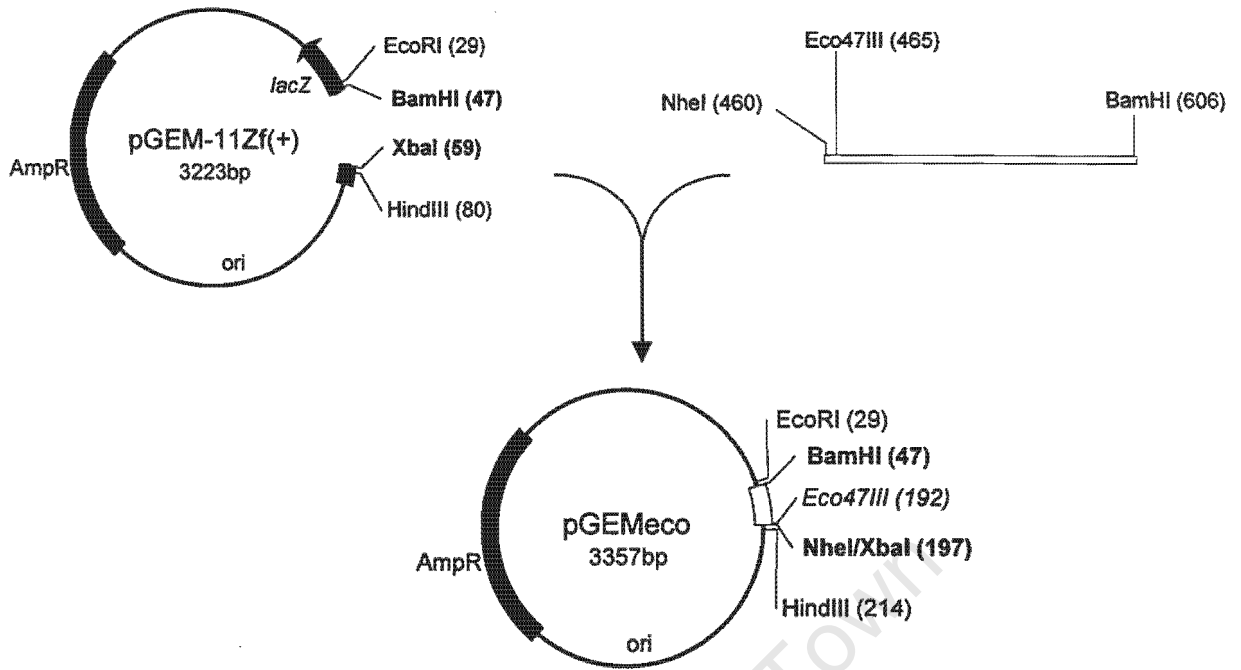


Figure 2.2. Schematic diagram of the construction of pGEMeco. A *Bam*HI/*Nhe*I pBR329 fragment containing an *Eco*47III site was introduced into *Bam*HI/*Xba*I digested pGEM-11Zf(+). Restriction enzymes cloning sites used to introduce the pBR329 fragment into pGEM are indicated in bold. The *Eco*47III site introduced is indicated in italics.

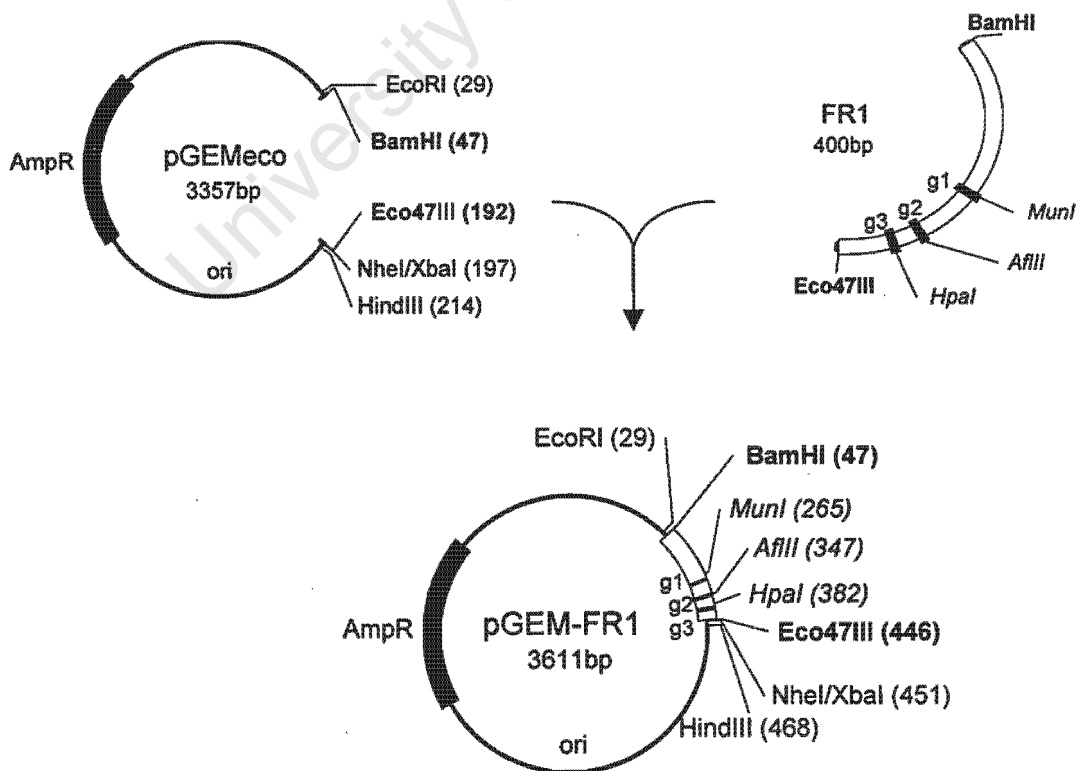


Figure 2.3. Schematic diagram of the construction of pGEM-FR1. A *Bam*HI/*Eco*47III fragment of tACE, containing the first three glycosylation sites was introduced into pGEMeco. Restriction enzymes cloning sites are indicated in bold. g1: N⁷²IT, g2: N⁹⁰HT, g3: N¹⁰⁹TT. The *Mun*I, *Afl*II and *Hpa*I sites introduced during site-directed mutagenesis are indicated in italics.

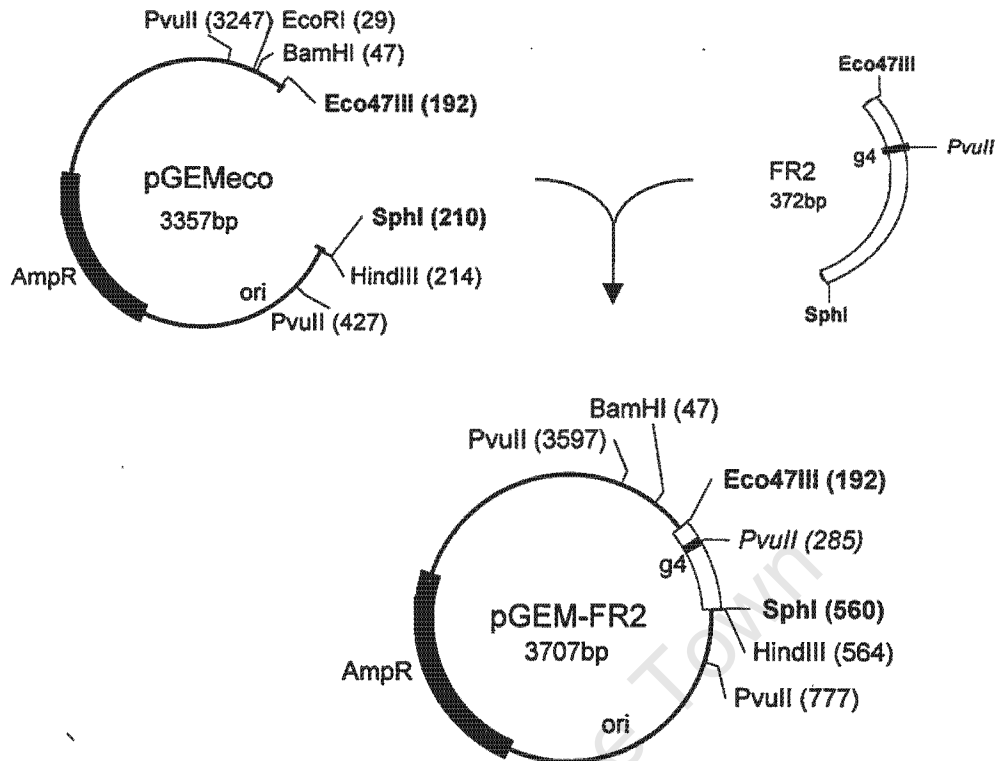


Figure 2.4. Schematic diagram of the construction of pGEM-FR2. An *Eco47III/SphI* fragment of tACE, containing the fourth glycosylation site, was introduced into pGEMeco. Restriction enzymes cloning sites are indicated in bold. g4: N¹⁵⁵GS. The *PvuII* site removed during site-directed mutagenesis is indicated in italics.

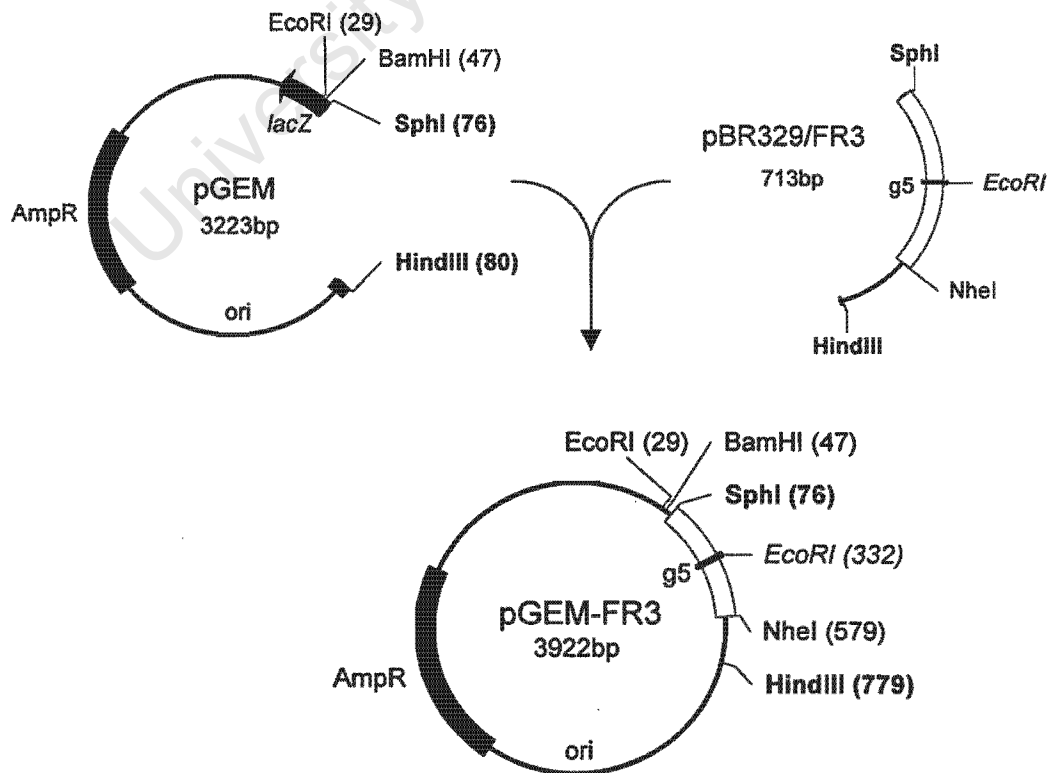


Figure 2.5. Schematic diagram of the construction of pGEM-FR3. A *SphI/HindIII* fragment of pBR329/tACE, containing the fifth glycosylation site, was introduced into pGEM. Restriction enzymes cloning sites are indicated in bold. g5: N³³⁷KS. The *EcoRI* site introduced during site-directed mutagenesis is indicated in italics.

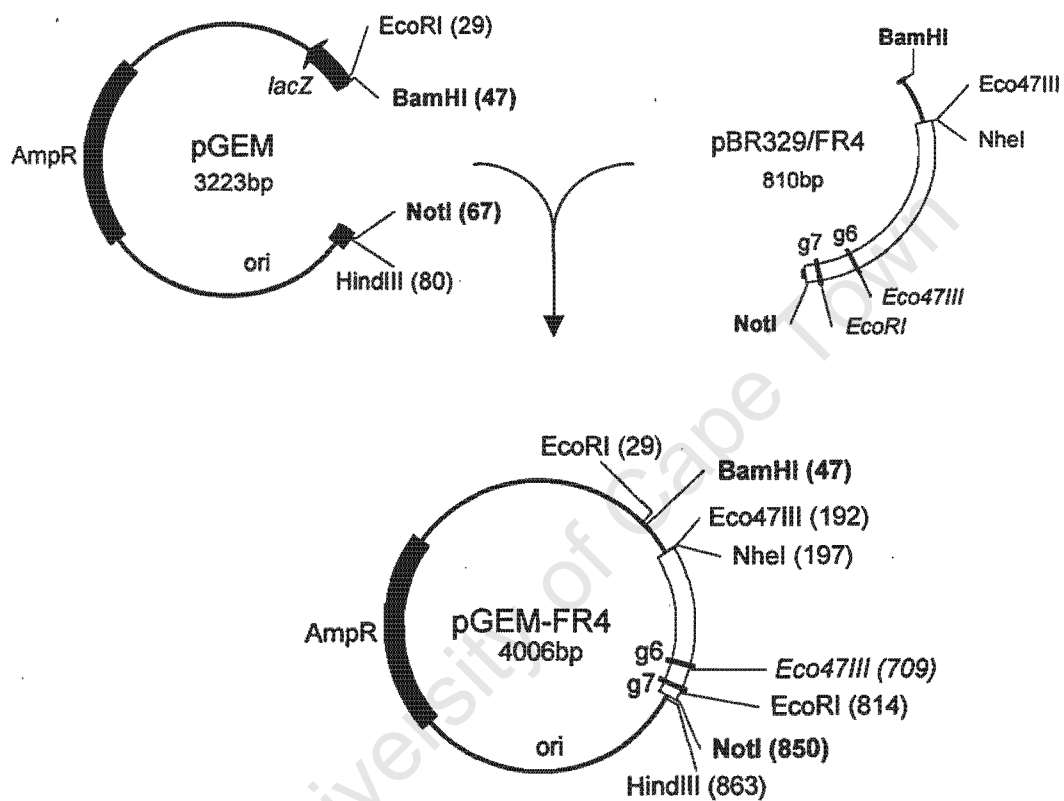


Figure 2.6. Schematic diagram of the construction of pGEM-FR4. A *Bam*HI/*Not*I fragment of pBR329/tACE, containing the sixth and seventh glycosylation sites, was introduced into pGEM. Restriction enzymes cloning sites are indicated in bold. g6: N⁵⁸⁶MS, g7: N⁶²⁰WT. The *Eco*47III site introduced during site-directed mutagenesis is indicated in italics.

pBS-tACE, and ligated to similarly digested pGEMeco. The presence of FR2 in vector DNA was detected with *Bam*HI/*Hind*III digests of isolated DNA.

2.2.1.4 Introduction of FR3 and FR4 into pGEM

The last two tACE fragments that were introduced into pGEM are FR3, which contains the fifth glycosylation site (Asn337); and FR4, which is the C-terminal region of tACE containing the sixth and seventh glycosylation sequons (Asn586 and Asn620). To facilitate the cloning of FR3 and FR4 into pGEM, it was necessary to introduce a *Nhe*I site into the vector. The most effective approach was to clone the *Nhe*I site into pGEM with FR3 and FR4. Thus, the two fragments were initially cloned into pBR329, which contains a *Nhe*I site.

2.2.1.4.1 Construction of pGEM-FR3

A 489 bp *Nhe*I/*Sph*I digested pBS-tACE fragment was introduced into pBR329 (Fig. 2.5). FR3 was excised from pBR329 with a *Hind*III/*Sph*I digestion and cloned into pGEM. *Bam*HI/*Hind*III digests were performed to confirm the presence of FR3 in pGEM.

2.2.1.4.2 Construction of pGEM-FR4

A 1.1 kb *Nhe*I/*Cla*I digested pBS-tACE fragment was ligated to pBR329 (Fig. 2.6). FR4 was introduced into pGEM as a *Bam*HI/*Not*I digested fragment. *Bam*HI/*Hind*III digests confirmed the presence of FR4 in pGEM.

2.2.2 Site-Directed Mutagenesis of the Glycosylation Recognition Sequons in Human tACE

2.2.2.1 Mutagenic Oligonucleotides

Glycosylation recognition sequons were removed by altering the asparagine residue to a glutamine, thereby abolishing glycosylation. This was encoded on mutagenic oligonucleotides: g1-F, g2-F, g3-F, g4-F, g5-F, g6-F, g1-R, g2-R and g3-R, with the number referring to each complementary recognition sequon. The oligonucleotides contain a silent mutation that either introduces or removes a restriction enzyme recognition site, to facilitate screening for mutants. The sequences of these oligonucleotides are shown in Appendix A5.

The optimal annealing temperature of each oligonucleotide was determined in replicated PCR reactions run on a temperature gradient block. The PCR profile is described in Appendix A7.1.

2.2.2.2 Site-Directed Mutagenesis of Asn155 and Asn586 using Altered β -Lactamase Specificity

Mutagenesis of Asn155 and Asn586 (the fourth and sixth sites) was performed according to the Gene-Editor *In Vitro* Site-Directed Mutagenesis system (Promega, USA); a non-PCR based technique that uses antibiotic selection to isolate mutants (Fig. 2.7), detailed in Appendix A6. A selection oligonucleotide is used to introduce an alteration in the β -lactamase gene of pGEM, which confers a broader resistance to β -lactam antibiotics. After transformation of bacterial cells with mutated plasmid DNA, exposure to a cocktail of antibiotics (the Antibiotic Selection Mix - ASM) isolated positives. The selection oligonucleotide and g4-F or g6-F were simultaneously annealed to alkaline denatured pGEM-FR2 or pGEM-FR4 at 56°C. Second strand synthesis was performed with T4 DNA polymerase (Promega, USA) and double-stranded DNA was transformed into *E. coli* BMH 71-18 *mutS* cells for amplification. Mini-preparations of DNA from overnight cultures were performed and DNA was transformed into JM109 cells, grown in the presence of ASM for the selection of mutants.

2.2.2.3 Site-Directed Mutagenesis of Asn90 and Asn337 using "megaprimer" PCR

Removal of the second glycosylation site (Asn90) was performed using a "megaprimer" PCR reaction with primers, g2-R and M13-F, amplifying the 5' region of FR1 (Fig. 2.8) (Sarkar & Sommer, 1990). The 428 bp product was used as a forward primer in a second PCR reaction with M13-R to amplify FR1. The 615 bp product was *Bam*HI/*Not*I digested and cloned into *Bam*HI/*Not*I digested pBS. Ligated DNA was transformed into *E. coli* XL1-blue competent cells for amplification and selection of mutants. PCR was performed on a Hybaid Sprint thermocycler, details of each PCR reaction are in Appendix A7.2.

The fifth glycosylation site (Asn337) was removed by amplification of the 3' region of FR3 (in pGEM) with M13-R and g5-F (Fig. 2.9). The 528 bp PCR product digested with *Eco*RV, generating a 350 bp fragment that was used in a second PCR reaction as a reverse primer with M13-F to amplify the upstream region of FR3. The 691 bp product was digested with *Bam*HI and *Nhe*I, generating a 532 bp band which was introduced into *Bam*HI/*Nhe*I

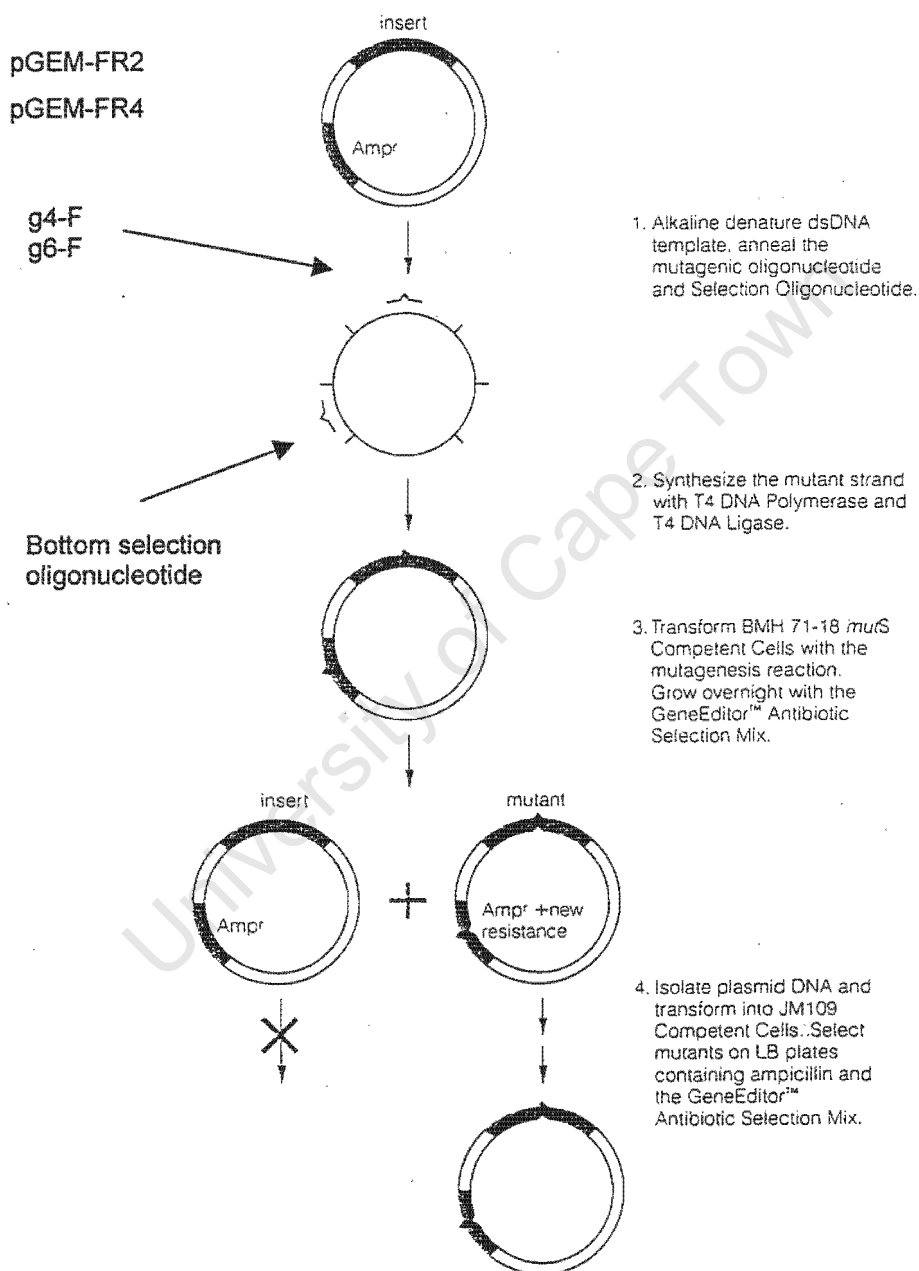


Figure 2.7. Schematic diagram of the GeneEditor *in vitro* mutagenesis system, which was used to remove the fourth and sixth sites. The oligonucleotides used to introduce mutations in pGEM-FR2 and pGEM-FR4 are indicated.

Figure taken from Gene Editor *in vitro* mutagenesis protocol handbook (Promega)

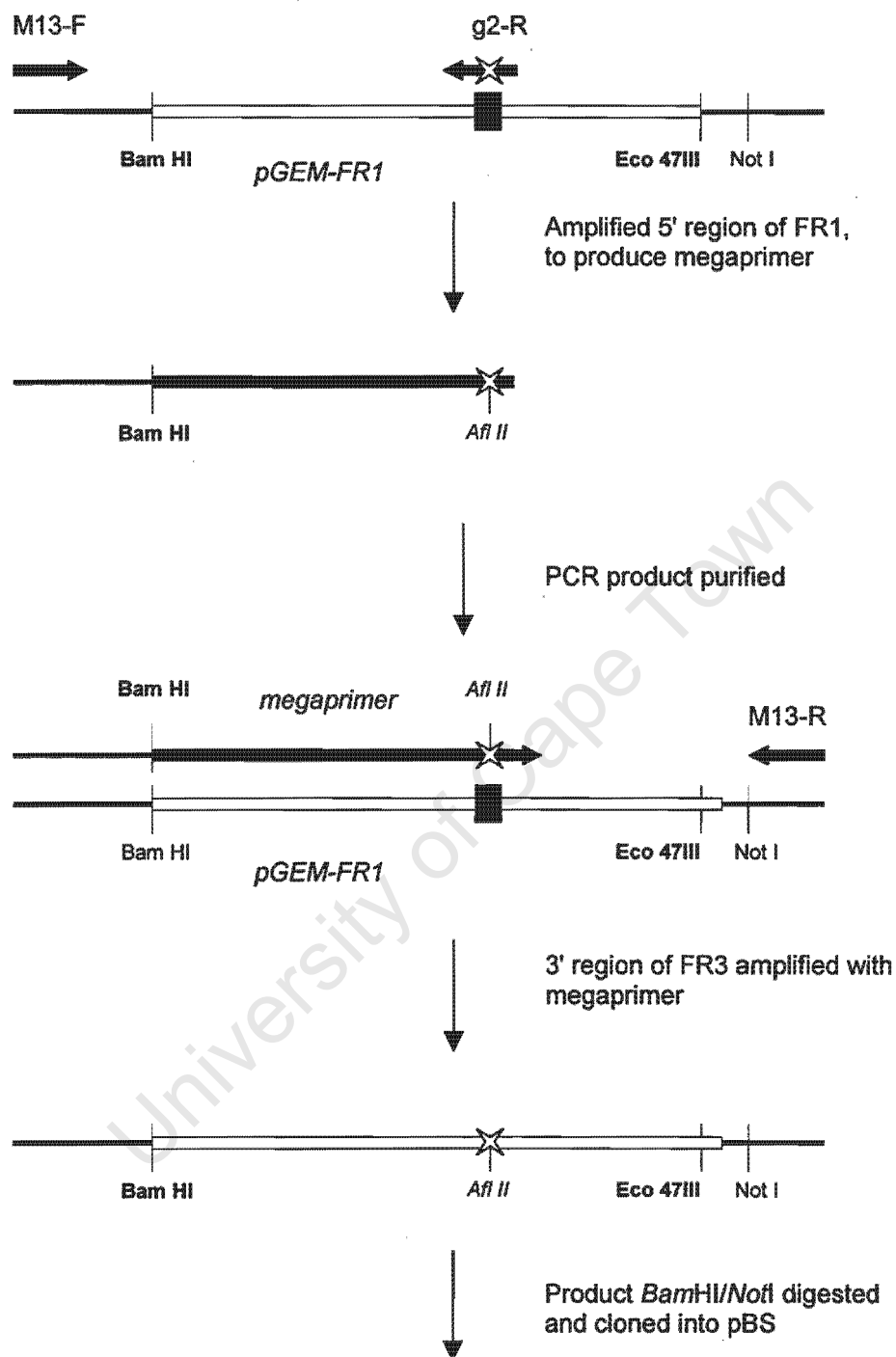


Figure 2.8. PCR mutagenesis of the second glycosylation site (Asn72). FR1 was amplified in two PCR reactions to produce the full fragment containing N72Q. The mutations introduced by g2-R are indicated as an open cross. Restriction Enzymes used for cloning are indicated. FR1 was introduced into pGEM with *Bam*HI/*Not*I sites (in bold). The *Afl*II site introduced by g2-R is shown in italics.

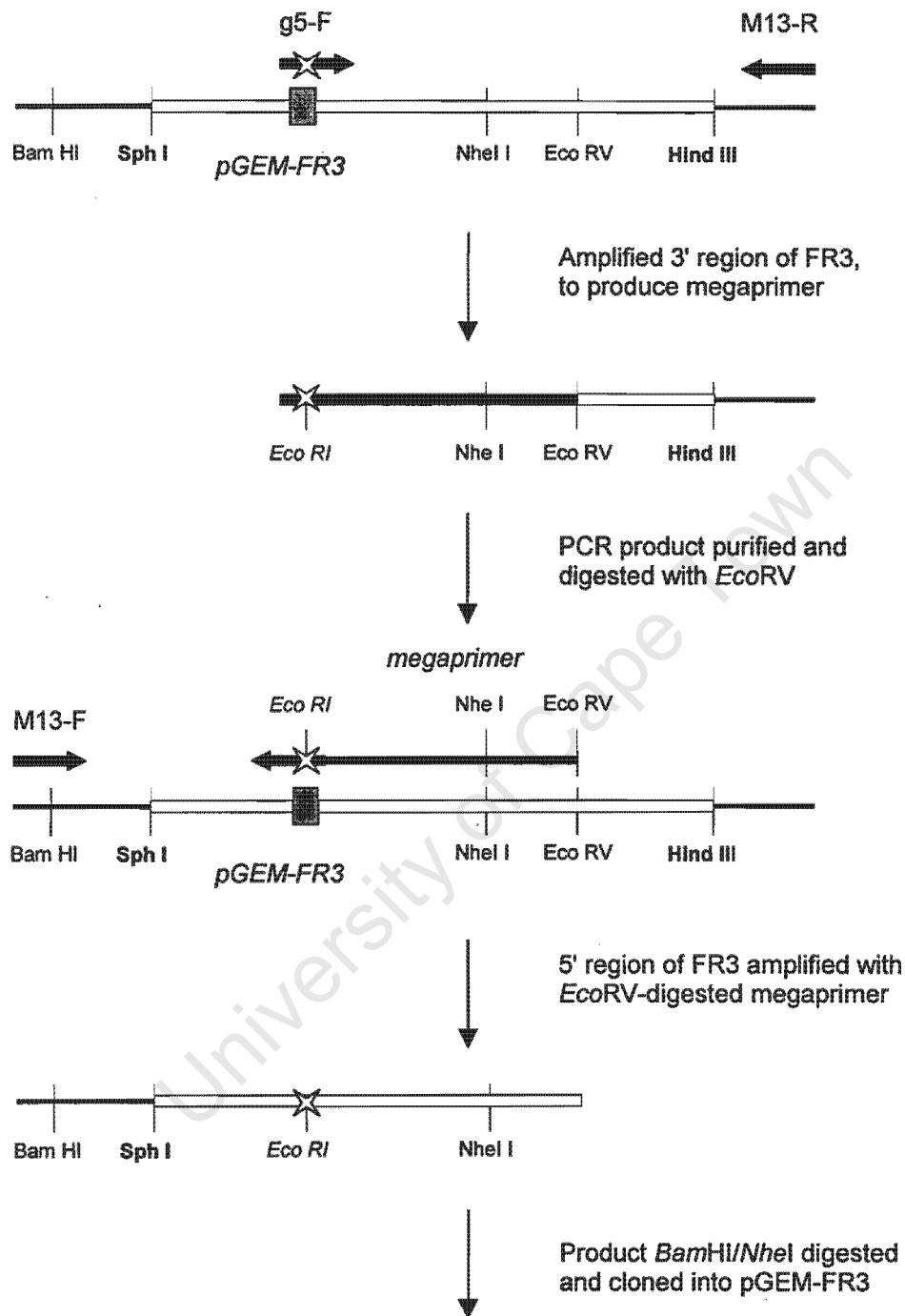


Figure 2.9. PCR mutagenesis of the fifth glycosylation site (Asn337). FR3 was amplified in two PCR reactions to produce the full fragment containing N337Q. The mutation introduced by g5-F is indicated as an open cross. Restriction Enzymes used for cloning are indicated. FR3 was introduced into pGEM with *SphI/HindIII* sites (in bold). The *EcoRI* site introduced by g5-F is shown in italics.

2.2.2.4 Site-Directed Mutagenesis by Overlap-Extension PCR

Five mutants were made using this approach (Ge & Rudolph, 1997). One mutant lacks the first site (N72Q) and another lacks the first and second site (N72Q/N90Q). The third mutant, which lacks the third site (N109Q), was used as a template for two subsequent mutations, at the first and second sites, resulting in two double mutants in FR1 (N72Q/N109Q and N90Q/N109Q).

PCR was performed on a Hybaid Sprint thermocycler, details of each PCR reaction are in Appendix A7.3.

Three PCR reactions were performed to remove the first site at Asn72 (Fig. 2.10). In the first reaction, the region of FR1 upstream of Asn72 was amplified with M13-F and g1-R, generating a 370 bp fragment. The region downstream of Asn72 was amplified with g1-F and SP6 to give a 240 bp product. These fragments overlapped by 40 bp, because g1-F and g1-R are complementary, and together encompass FR1. Thus, they were used as a template in a third reaction to amplify FR1-N72Q, with M13-F and SP6. The 570 bp product was digested with *Bam*HI and *Not*I and cloned into complementary sites of pBS for sequencing.

The same three-step PCR approach was used to generate FR1-N72Q/N90Q, using pBS-FR1-N90Q as a template, g1-F and g1-R as the mutagenic primers and M13-F and T3 as the flanking primers (Fig. 2.11). The 580 bp product was *Bam*HI /*Not*I digested and cloned into complementary sites in pBS for sequencing.

The third site (Asn109) was removed in the same way (Fig. 2.10). The first PCR reaction amplified the region of FR1 upstream of Asn109 with M13-F and g3-R, generating a 168 bp product. The second reaction generated a 485 bp product, comprising the region downstream of Asn109, amplified with M13-R and g3-F. Because these products were amplified with complementary primers (g3-F and g3-R), they overlapped by 36 bp and together encompassed FR1. The products were annealed and used as a template in a third PCR reaction with M13-F and M13-R to amplify FR1 containing the mutation N109Q. The 615 bp product was digested with *Bam*HI/*Not*I and cloned into *Bam*HI/*Not*I digested pBS for sequencing.

The double mutant lacking the first and third site (N72Q/N109Q) was also the result of three PCR reactions (Fig. 2.11). The first two products were generated from pBS-FR1-N109Q, which amplified the region 5' to Asn72 with T7 and g1-R and the region 3' to Asn72 with T3 and g1-F. The 350 bp upstream region and the 250 bp downstream region

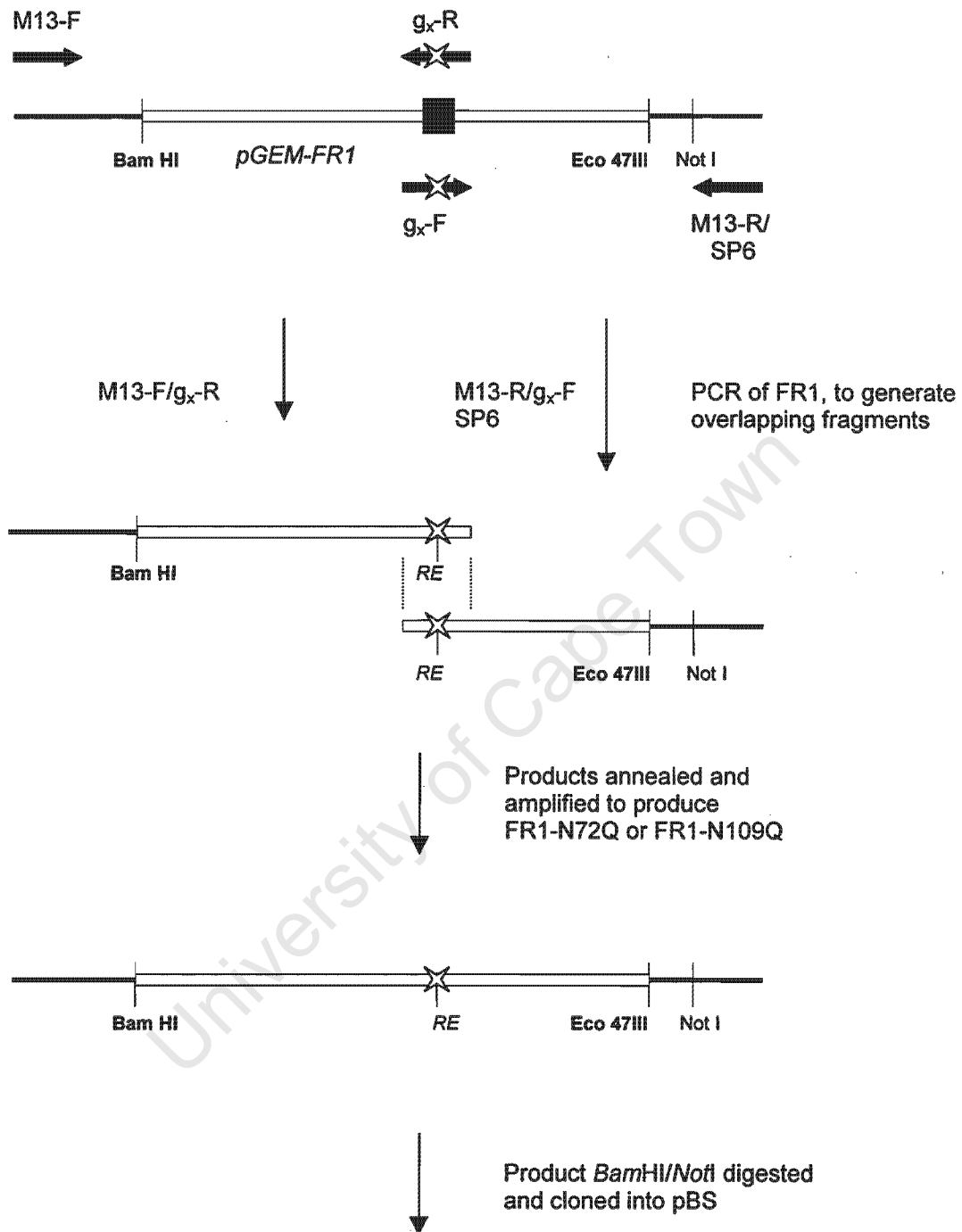


Figure 2.10. Overlap-extension PCR of FR1 to introduce a single mutation. N72Q and N109Q were introduced by amplification of two overlapping products of FR1, which were annealed and used as a template in a third PCR reaction, to produce complete FR1. The mutations introduced by the mutagenic primers (g_x-F and g_x-R , where x represents a glycosylation site) are indicated as an open cross. Restriction enzymes used for cloning are indicated in bold. The restriction enzyme site introduced during site-directed mutagenesis is shown in *italics* (RE).

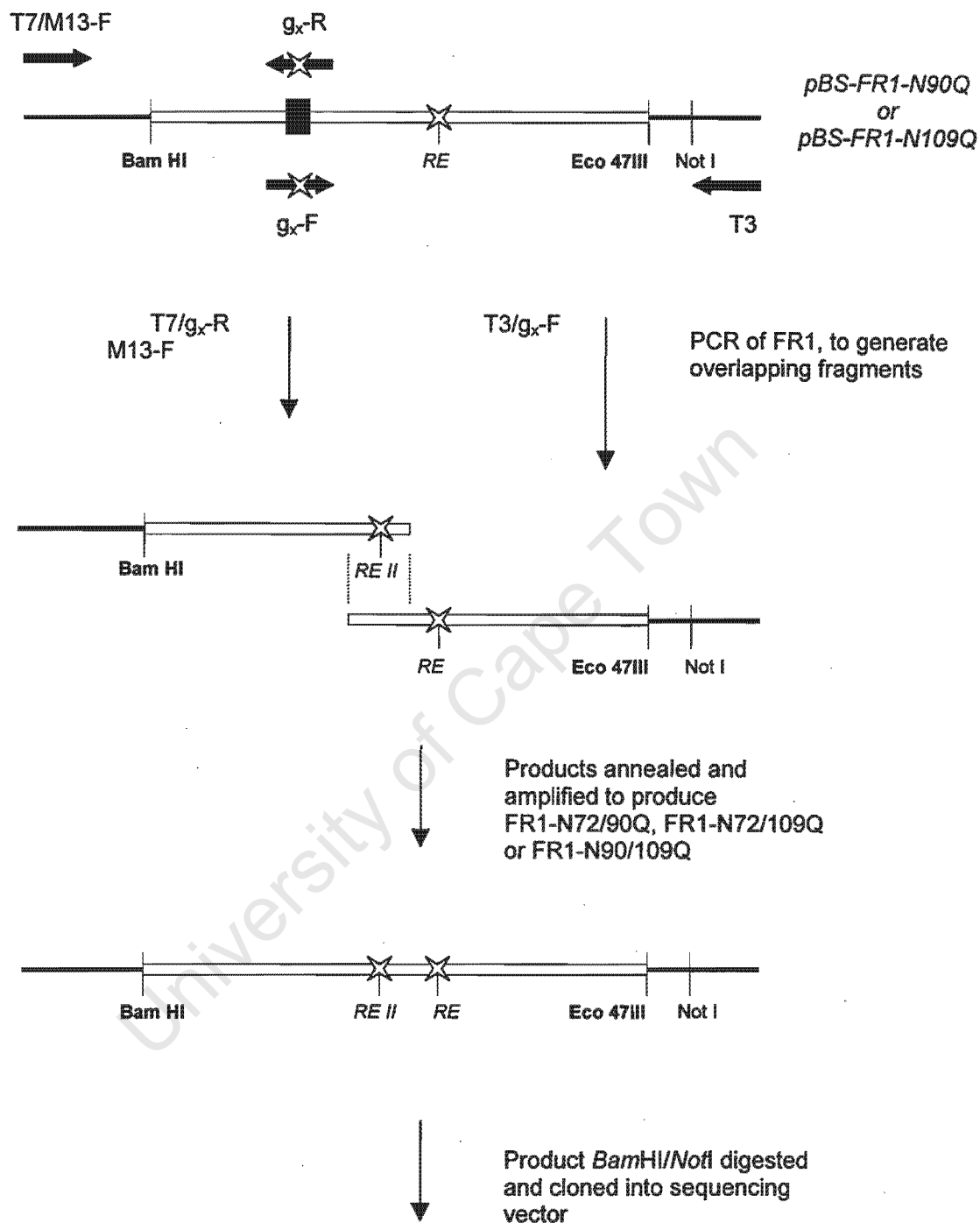


Figure 2.11. Overlap-extension PCR of FR1 to produce double mutants. Mutations were introduced into FR1-N90Q and FR1-N109Q by amplification of two overlapping products, which were annealed and used as a template in a third PCR reaction, to produce complete FR1. The mutations introduced by the mutagenic primers are indicated as an open cross. Restriction enzymes used for cloning are indicated in bold. The restriction enzymes sites introduced are shown in *italics* (RE II).

overlapped by 40 bp, because g1-F and g1-R are complementary, and together encompass FR1. Thus, the two products were annealed and used as a template with T7 and T3 to amplify FR1-N72Q/N109Q. The 560 bp product was *Bam*HI/*Not*I digested and cloned into *Bam*HI/*Not*I digested pGEMeco for sequencing.

A second double mutant (N90Q/N109Q) was made in a similar manner, from pBS-FR1-N109Q. A 183 bp product of the region 3' to Asn90 was amplified with g2-F and T3. This was complementary to 40 bp of a 413 bp product of the region 5' to Asn90, amplified with g2-R and T7. These two products were ligated and used as a template to amplify FR1 containing N90Q/N109Q with T7 and T3, generating a 560 bp product that was cloned into *Bam*HI/*Not*I digested pGEMeco for sequencing.

2.2.2.5 Screening of Plasmid DNA for Mutants

Silent mutations encoding restriction enzyme sites on the mutagenic oligonucleotides were used to screen plasmid DNA for glycosylation mutants after antibiotic selection in bacterial cells. Mini-preparations of plasmid DNA were performed on colonies grown in the presence of ampicillin. Restriction enzyme digests were performed and compared to that of wild-type DNA, to identify mutants. FR1 clones were digested with *Mun*I, *Afl*III and *Hpa*I to screen for mutations at Asn72, Asn90 and Asn109, respectively. *Pvu*II digests detected mutations at Asn155 on FR2. Mutations at Asn337 on FR3 were identified by digestion with *Eco*RI. Finally, FR4 clones were screened for mutations at Asn586 with *Eco*47III digests. Positive clones were cultured for preparation of plasmid DNA.

2.2.2.6 DNA Sequencing to Confirm the Presence of Mutations in tACE Fragments

tACE- Δ 36 DNA fragments containing glycosylation mutants were sequenced in both orientations, primed with universal sequencing primers complementary to the vector DNA into which the fragments were cloned. DNA in pGEM was sequenced using T7 and SP6 and DNA in pBS was sequenced with T7 and T3.

DNA was sequenced both manually and with an automated sequencer, and methods are outlined in Appendix A8.

2.2.3 Subcloning of Glycosylation Mutants into pLEN-tACE

tACE fragments in pGEM containing the glycosylation mutants were assembled in pLEN-tACE to produce minimally glycosylated isoforms (Fig. 2.12). The mutants are designated as tACE-g(x), where x refers to the intact glycosylation recognition sites. For simplicity, the seventh site is not included in the nomenclature, although this site was not mutated.

2.2.3.1 Construction of tACE-g1234

A tACE isoform lacking the fifth (N337Q) and sixth (N586Q) glycosylation sites was assembled in pLEN-tACE- Δ 36. *NheI/NotI* digested FR4-N586Q was cloned into pLEN-tACE- Δ 36 digested with the same enzymes, to give tACE-g12345 (Fig. 2.12E). Due to the presence of more than one *SphI* site in pLEN-tACE, FR3-N337Q was introduced into *SphI/NheI* digested pBS-tACE- Δ 36 (Fig. 2.12B) and cloned as a larger *BamHI/NheI* digested fragment into pLEN-tACE-g12345, generating tACE-g1234 (Fig. 2.12F). Constructs were selected for in bacterial cells in the presence of ampicillin and DNA isolated. *BamHI/BglIII*, *PvuII*, *EcoRI* and *Eco47III* digests were performed on DNA to detect the presence of Δ 36, the transmembrane domain, N155Q, N337Q and N586Q, respectively.

2.2.3.2 Construction of tACE-g123

tACE-g123 was constructed by introducing FR2-N155Q and FR3-N337Q into pLEN-tACE-g1234. The presence of additional *Eco47III* and *SphI* sites in pLEN-tACE prohibited direct cloning of these fragments. Thus, they were first assembled in pBS-tACE- Δ 36, to generate pBS-tACE-N155Q/N337Q. *Eco47III/SphI* digested FR2-N155Q was cloned into pBS-tACE-N337Q (Fig. 2.12C). DNA was cloned into tACE-g1234 as a 1.5 kb *BamHI/NheI* digested fragment, comprising tACE DNA extending beyond the fifth site, containing the first 3 glycosylation sites (Fig 2.12G).

Restriction enzyme digests were performed to confirm the presence of tACE- Δ 36, N155Q, N337Q and N586Q.

2.2.3.3 Construction of tACE-g1, -g2, -g3, -g12, -g13 and -g23

As with the previous isoforms, FR1 mutants had to be introduced into pLEN-tACE via *BamHI* and *NheI* sites, due to additional *Eco47III* sites in pLEN-tACE. Thus, the six FR1

mutants were introduced into pBS-tACE-N337Q/N586Q (Fig. 2.12D). *Bam*HI/*Eco*47III digested FR1-N90Q/N109Q, FR1-N72Q/N109Q, FR1-N72Q/N90Q, FR1-N109Q, FR1-N90Q and FR1-N72Q were introduced into pBS-tACE-N337Q/N586Q. These constructs were *Bam*HI/*Nhe*I digested and introduced into tACE-g1234 to generate tACE-g1, tACE-g2, tACE-g3, tACE-g12, tACE-g13 and tACE-g23, respectively (Fig. 2.12H).

Restriction enzyme digests described were performed to characterise the constructs, including as *Mun*I, *Afl*III and *Hpa*I digests to detect N72Q, N90Q and N109Q.

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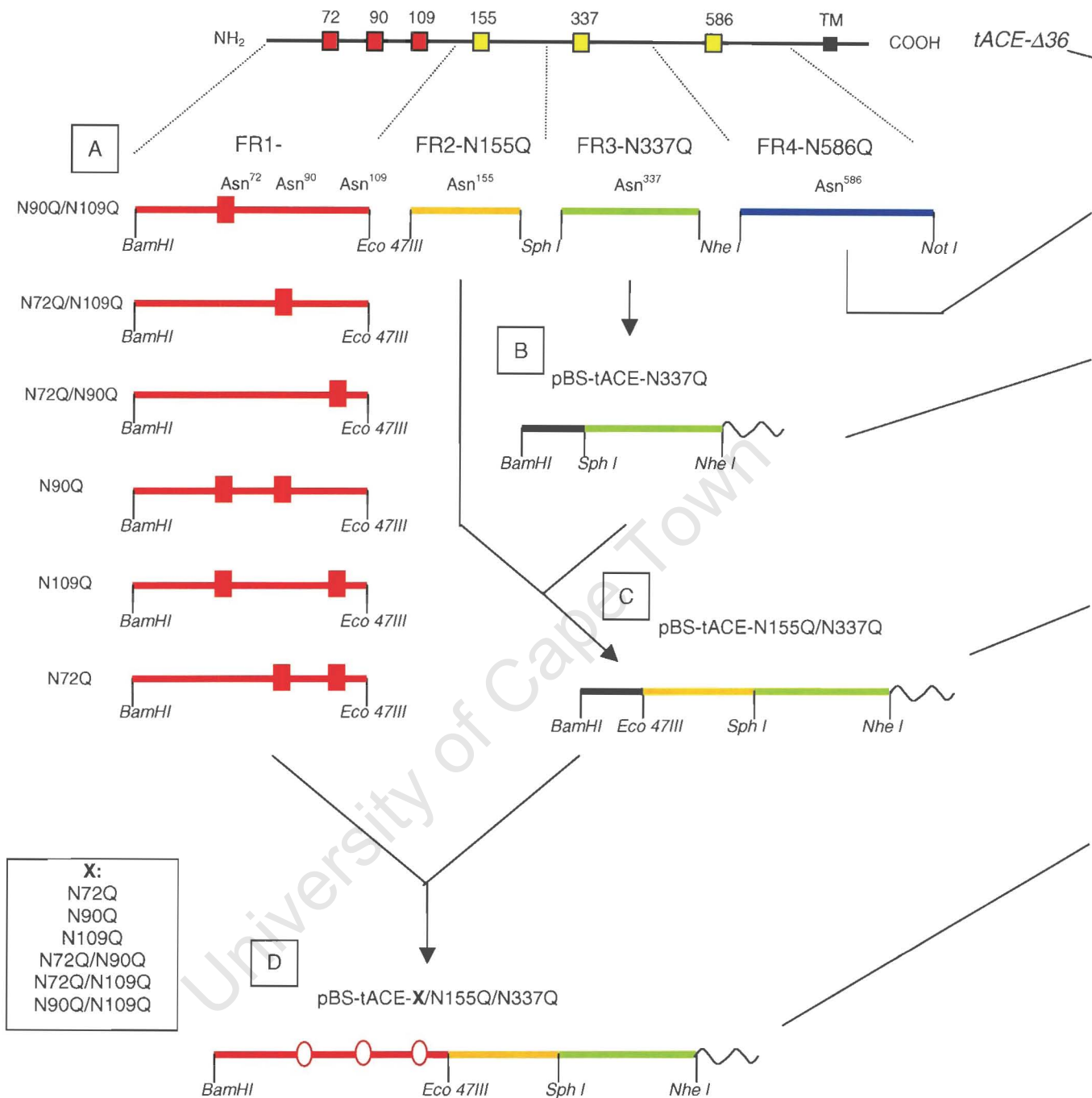


Figure 2.12. The cloning strategy used to introduce tACE fragments into pLEN. A: Nine mutants were constructed from four fragments of tACE-Δ36 cDNA, cloning sites used to generate the four fragments are shown. tACE glycoforms were compiled from these nine mutants, using pBS-tACE-Δ36 as an intermediate due to the lack of unique *Eco47III* and *SphI* sites in pLEN. B: The fifth site was removed from tACE by introducing FR3-N337Q into pBS-tACE-Δ36, from which the fourth site was removed by introducing FR2-N155Q into this construct (C). D: The six FR1 mutants were cloned into pBS-tACE-N155Q/N337Q. These eight constructs were introduced into pLEN-tACE-Δ36 as the N-terminal *BamHI*/*NheI* region of tACE-Δ36. E: The fourth site was cloned directly into pLEN-tACE-Δ36, removing the sixth site. F: pBS-tACE-N337Q was introduced into pLEN-tACE-g12345, generating pLEN-tACE-g1234, which was used as a template to construct the remaining tACE glycosylation mutants: tACE-g123 (G) and tACE-g1, -g2, -g3, -g12, -g13 and -g23 (H).

E

tACE-g12345



F

tACE-g1234



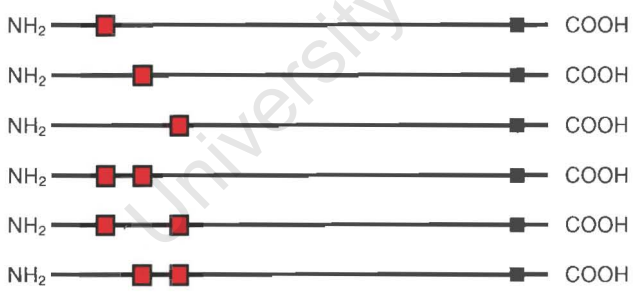
G

tACE-g123



H

tACE-g1, -g2, -g3, -g12, -g13, -g23



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2.3 RESULTS

2.3.1 Introduction of Human tACE Fragments into pGEM

tACE- Δ 36 DNA was divided into four fragments and each fragment was introduced into pGEM to facilitate site-directed mutagenesis. The use of fragments of tACE as opposed to the full-length gene decreases the chance of any possible spurious mutations occurring within the DNA during replication. Additionally, the smaller size of each fragment allowed for complete sequencing, which would confirm the presence of each mutation and detect any spurious mutations. The fragments were defined by the unique restriction enzyme sites available in vectors used for mutagenesis, sequencing and expression (Fig. 2.1).

The first fragment (FR1) comprises the amino terminus of tACE, containing the first three glycosylation sites (Asn72, Asn90 and Asn109), and lacks the O-glycosylated 36-residue N-terminal region. This fragment is derived from *Bam*HI/*Eco*47III digested pLEN-tACE- Δ 36. The second fragment (FR2) contains the fourth glycosylation site (Asn155) and is an *Eco*47III/*Sph*I digested fragment of pBS-tACE- Δ 36. The third fragment (FR3) contains the fifth glycosylation site (Asn337) and is a *Sph*I/*Nhe*I digested pBS-tACE- Δ 36 fragment. The fourth fragment (FR4) lies upstream of the C-terminal transmembrane region of tACE and contains the sixth and seventh glycosylation sites (Asn586 and Asn620). FR4 is a *Nhe*I/*Not*I digested pBS-tACE- Δ 36 fragment. The cloning strategy used to introduce these four fragments is summarised in Figures 2.2 to 2.6.

2.3.2 Site-Directed Mutagenesis of the Glycosylation Recognition Sequons

Sites were removed from tACE- Δ 36 fragments in pGEM and the mutated fragments recombined in pLEN-tACE- Δ 36 to produce the desired constructs. The lack of unique restriction enzyme sites prevented the three N-terminal sites from being separated into three fragments. Consequently, these sites could not be mutated individually and recombined to produce the desired series of constructs. To facilitate this non-PCR based mutagenesis was used as simultaneous site-directed mutagenesis could be performed (Fig. 2.7). Only mutagenesis of pGEM-FR2 with g4-F and pGEM-FR4 with g6-F produced positive clones. Sequencing data confirmed the presence of N155Q and N586Q. Consistently, with the remaining sites, no colonies were obtained after the second round of transformation into

JM109 cells. This was most likely a result of poor growth of *BMutS* cells, producing low concentrations of poor quality DNA for transformation into JM109 cells. Optimisation of this system became too time-consuming and this approach was abandoned, albeit the fourth and sixth sites (Asn337 and Asn586) were removed with this system.

Two alternative methods were investigated for removal of the first, second, third and fifth sites. Both approaches involved PCR based site-directed mutagenesis. PCR based approaches entail amplification with primers (one of which contains the mutation) that contain inherent restriction enzyme sites, which are used for reintroducing the product into the template DNA. However, the lack of unique restriction enzyme sites flanking the glycosylation sequons in tACE DNA meant that mutagenesis could not be performed in a single PCR reaction. Thus, both approaches entailed a two-stage PCR protocol to incorporate restriction enzyme sites.

Firstly, an approach termed the “megaprimer” method (Sarkar & Sommer, 1990) was used to remove the second and fifth sites (at Asn90 and Asn337), generating FR1-N90Q and FR3-N337Q, respectively (Figs. 2.8 and 2.9).

Secondly, three-step overlap extension PCR (Ho et al, 1989) was used to introduce further mutations at the first three sites, producing five mutants: FR1-N72Q, FR1-N109Q, FR1-N72Q/N90Q, FR1-N72Q/N109Q and FR1-N90Q/N109Q (Figs. 2.10 and 2.11).

After sequence confirmation of each mutant, the four fragments were reintroduced into tACE- Δ 36 in the expression vector pLEN.

2.3.3 Subcloning of Glycosylation Mutants into pLEN-tACE

Assembly in pLEN-tACE- Δ 36 of FR1, FR2, FR3 and FR4 containing mutants produced tACE- Δ 36 constructs lacking particular N-linked glycosylation sequons. Due to the lack of unique cloning sites, mutants were compiled in pBS-tACE- Δ 36 and introduced into pLEN-tACE-g12345 as FR1, FR2 and FR3 combined (Fig. 2.12). Eight isoforms were constructed in pLEN-tACE- Δ 36 (Fig. 2.13). tACE-g1234 contains FR3-N337Q and FR4-N586Q. Introduction of FR2-N155Q into this construct produced tACE-g123. Each of the six FR1 mutants were introduced into tACE-g123, to give tACE-g1, tACE-g2, tACE-g3, tACE-g12, tACE-g13 and tACE-g23.

A series of restriction enzyme digests was performed on each isoform (Fig. 2.14). This served to confirm that the mutants had been introduced into pLEN-tACE- Δ 36, that they

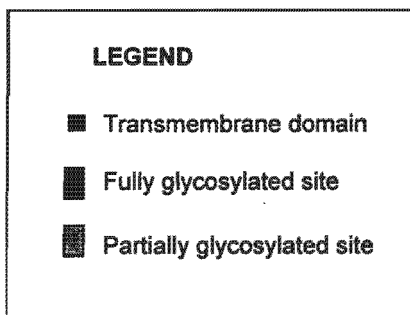
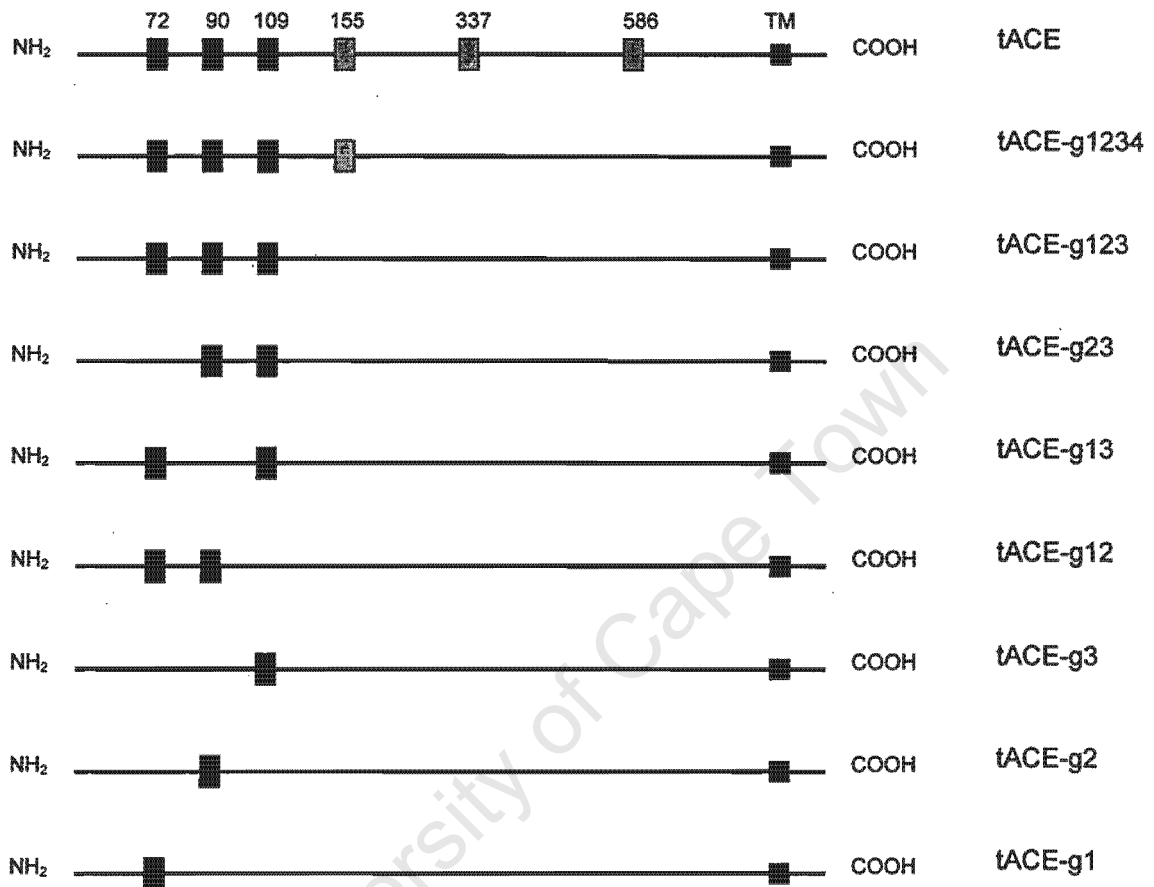


Figure 2.13 The minimally glycosylated tACE isoforms prepared for expression in mammalian cells.

Table 2.1. Glycosylation profiles for pLEN-tACE isoforms

Digests:	<i>Bam</i> HI/ <i>Bg</i> II		<i>Mun</i> I	<i>Cl</i> AI/ <i>A</i> III	<i>Hpa</i> I	<i>Pvu</i> II	<i>Eco</i> RI	<i>Eco</i> 47III	Sites removed
Mutation:	Δ36	TM	N72Q	N90Q	N109Q	N155Q	N337Q	N586Q	
tACE-g1	Y	Y	-	+	+	+	+	+	23456
tACE-g2	Y	Y	+	-	+	+	+	+	13456
tACE-g3	Y	Y	+	+	-	+	+	+	12456
tACE-g12	Y	Y	-	-	+	+	+	+	3456
tACE-g13	Y	Y	-	+	-	+	+	+	2456
tACE-g23	Y	Y	+	-	-	+	+	+	1456
tACE-g123	Y	Y	-	-	-	+	+	+	456
tACE-g1234	Y	Y	-	-	-	-	+	+	56

+ : digestion pattern differs from wild-type, indicating a positive result for the characteristic

- : digestion pattern same as wild-type, thus the construct lacks the mutation/feature.

TM : constructs containing the transmembrane region and cytoplasmic tail.

NXQ : glycosylation site mutation

contained the transmembrane domain and that they had the correct profile of glycosylation sites. The profile of each full-length isoform is summarised in Table 2.1. The 36 residue N-terminus deletion was detected by a 110 bp shift from 630 bp in wild-type pLEN-tACE to 520 bp in pLEN-tACE-Δ36 when DNA was digested with *Bam*HI and *Bg*II (Fig. 2.14A). A 520 bp band in the mutant lanes confirmed the presence of this deletion in the eight isoforms. Further, *Bam*HI/*Bg*II digests distinguished full-length isoforms from the truncated pLEN-tACE-Δ36sol through a 400 bp shift from 1.9 kb in the former to 1.5 kb in the latter, clearly shown with pLEN-tACE and pLEN-tACE-Δ36sol (Fig. 2.14A). This served to confirm that the mutants had been introduced into pLEN-tACE-Δ36.

The glycosylation site profile for the eight isoforms was confirmed by digestion with restriction enzymes whose sites had been introduced or removed by the mutagenic oligonucleotides during site-directed mutagenesis. A *Mun*I site was introduced adjacent to the first site, thereby digesting a 4.6 kb fragment in wild-type to 3.1 kb and 1.5 kb fragments in isoforms containing N72Q, as with tACE-g2, -g3 and -g23 (Fig. 2.14B). An *A*III site was introduced downstream of the second site. Thus *Cl*AI/*A*III digests distinguished linearised wild-type from DNA containing N90Q, which had a 2.2 kb fragment, such as tACE-g1, -g3 and -g13 (Fig. 2.14C). A 4.3 kb *Hpa*I digested fragment in wild-type was cut into 2.5 kb and 1.8 kb fragments in isoforms containing N109Q due to the insertion of a *Hpa*I site upstream of the third site, seen with tACE-g1, -g2 and -g12 (Fig. 2.14D). A *Pvu*II site was abolished

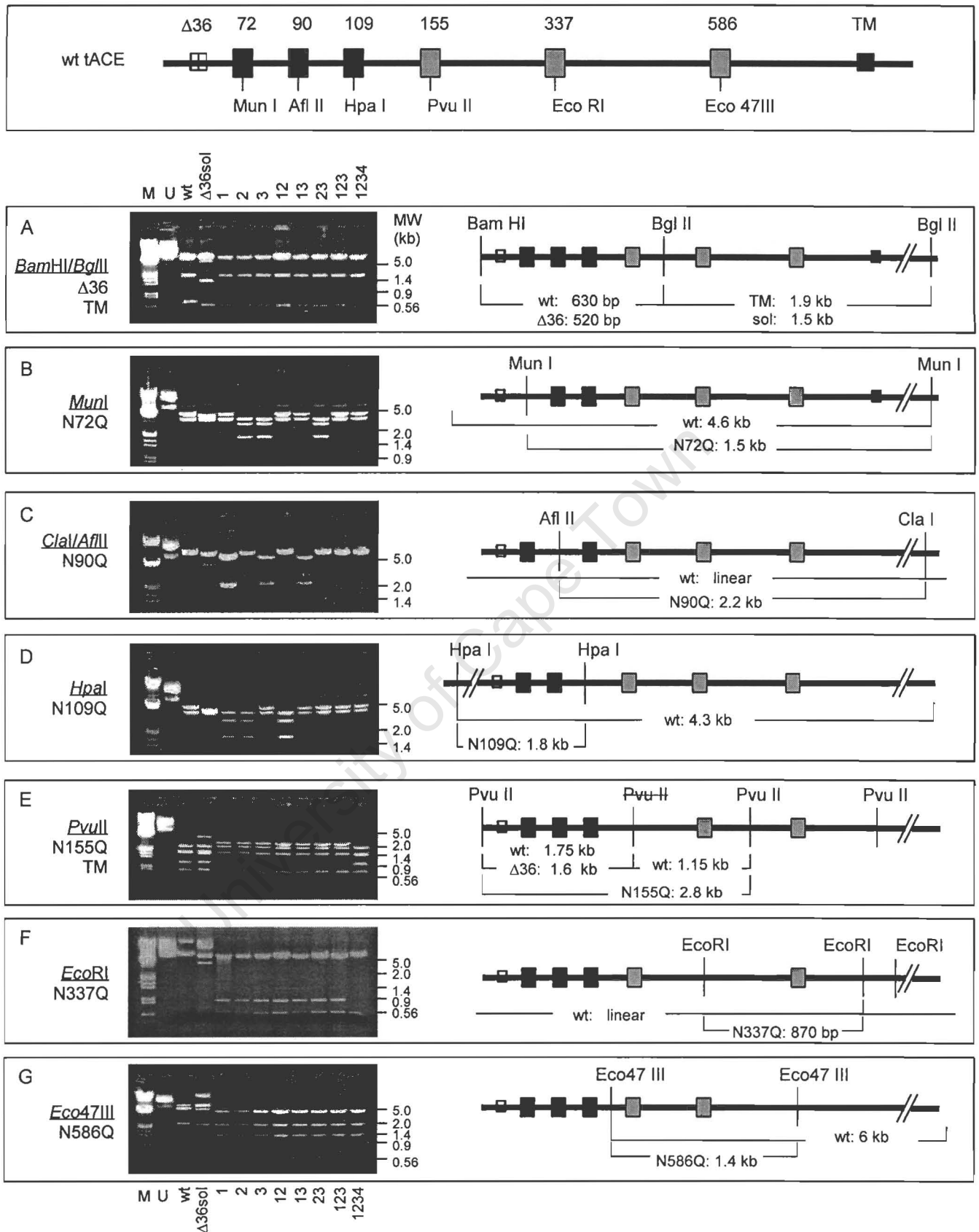


Figure 2.14. Restriction enzyme profile of full-length pLEN-tACE constructs. Each construct was digested with a panel of restriction enzymes to detect the glycosylation mutants, $\Delta 36$ and the transmembrane region. Top panel: schematic of wild-type tACE indicating the restriction enzymes sites used to screen for mutants; the glycosylated Asn are represented as the residue number. Panels A-G: Restriction enzyme digests performed on each isoform and the mutation being screened for. M: *HindIII/EcoRI* digested Lambda DNA marker. U: undigested pLEN-tACE, wt: pLEN-tACE, $\Delta 36$ sol: pLEN-tACE $\Delta 36$ sol. 1: tACE-g1, 2: tACE-g2, 3: tACE-g3, 12: tACE-g12, 13: tACE-g13, 23: tACE-g23, 123: tACE-g123, 1234: tACE-g1234. A schematic in the right of each panel indicates the expected fragments sizes for wild-type and mutant restriction digests.

adjacent to the fourth site, thus DNA containing N155Q could be distinguished from wild-type by the disappearance of 1.65 kb and 1.15 kb fragments to produce a 2.8 kb fragment, as with tACE-g1, -g2, -g3, -g12, -g13, -g23 and -g123 (Fig. 2.14E). An *EcoRI* site inserted upstream of the fifth site identified N337Q by the presence of an 870 bp band in all eight mutants, compared to linearised wild-type DNA (Fig. 2.14F). Note, pLEN-tACE- Δ 36sol is not digested as the nascent *EcoRI* site lies within the 400 bp transmembrane region truncation. Finally, the introduction of an *Eco47III* site adjacent to the sixth site gave 4.6 kb and 1.4 kb fragments in DNA with N586Q from a 6 kb fragment in wild-type, seen in all eight isoforms (Fig. 2.14G).

In summary, glycosylation profiles for each of the six isoforms were confirmed by a series of restriction enzyme digests (Table 2.1).

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2.4 DISCUSSION

2.4.1 Construction of Minimally Glycosylated Isoforms of tACE

The aim of this study was to create underglycosylated human testis ACE from which N-linked glycosylation had been progressively abolished so that the minimal glycosylation requirements for enzymatically active human tACE could be determined. Consequently, tACE- Δ 36 cDNA was divided into manageable fragments, each containing one or more glycosylation recognition sequon. Site-directed mutagenesis was performed on each of these fragments to remove the site of attachment of glycosylation through a conserved Asn to Gln transition. The approaches used were dictated by the speed and simplicity with which mutagenesis could be performed, as well as the degree of sequence fidelity that could be maintained. The mutated fragments were re-assembled as tACE- Δ 36 containing the transmembrane domain.

The panel of mutants were designed to determine the role of the N-terminal glycosylated sites in the expression of active tACE, particularly those at residues 72 and 90. This was based on two observations: firstly that glycosylation at either one of these sites is sufficient to produce active rabbit tACE in HeLa cells (Sadhukhan & Sen, 1996), and secondly that in human tACE only the three sites clustered near the N-terminus are fully glycosylated, indicating that glycosylation in this region may be important (Yu et al, 1997). This is supported by studies of the role of glycosylation in the expression of the insulin receptor and the rat PTH/PTHrP receptor, where removal of glycosylation sites near the N-terminus inhibited the action of these proteins (Elleman et al, 2000; Zhou et al, 2000). tACE-g123 was designed to determine whether the three fully glycosylated sites alone are necessary for expression of active tACE. Six further constructs were designed to assess the role of these three sites in the expression of enzymatically active tACE. The first three contain only two sites, two have the first site and either the second or the third (tACE-g12 and -g13) and the third lacks the first site (tACE-g23). These were produced to determine whether glycosylation at two sites is sufficient to produce active tACE and to distinguish whether there is a preference for glycosylation at any site. The second set of mutants (tACE-g1, -g2 and -g3) was designed to determine whether active human tACE could be expressed containing glycosylation at a single site and, if this occurs, whether this is site-specific.

In order to assess the role of these sites in the production of enzymatically active tACE, the constructs were introduced into the eukaryotic expression vector, pLEN, and transfected into mammalian cells.

2.4.2 Technical Difficulties Encountered

A non-PCR based approach was employed for the site-directed mutagenesis - in the form of the Gene-Editor *In Vitro* Site-Directed Mutagenesis System (Promega) - because more than one oligonucleotide could be used in a single reaction to generate multiple site-directed mutations. An advantage this technique has over PCR based approaches is that no additional cloning is required for DNA sequencing. Mutations are introduced during replication of the single stranded template, which comprises the target DNA in a cloning vector that contains an ampicillin resistance gene. This is also an effective tool in that a high yield of mutants is ensured by the use of antibiotic selection.

However, preferential annealing of the mutagenic oligonucleotide is essential to ensure that all resistant clones contain the desired mutation, which requires some optimisation. But compared to most PCR based methods - particularly those in which multiple site-directed mutations are introduced (Ling & Robinson, 1995; Ge & Rudolph, 1997) - this optimisation is minimal and is further reduced by the use of fewer oligonucleotides (only the antibiotic selection oligonucleotide and a mutagenic oligonucleotide for each site are required).

The yield of mutants is further enhanced by an additional transformation step where newly synthesised double stranded DNA containing mismatches is amplified in a repair negative cell line. Further, high sequence fidelity is maintained by the use of T4 DNA polymerase.

Considering these advantages, this system complied with the requirements for rapid, efficient mutagenesis with high sequence fidelity. However, application of this technique to mutagenesis of the glycosylation sites of human tACE proved tedious, generating only two mutants. It is not clear why the remaining mutants failed. Consistently, few or no colonies were observed on selection plates, most likely due to poor growth of the repair negative cell line (*E. coli* BMH 71-18 *mutS*). Growth was not inhibited by too high a concentration of Antibiotic Selection Mix (ASM) as experiments in the presence of 50 % ASM failed to produce colonies. Contributing factors, including too low a concentration of alkaline denatured DNA and the failure of oligonucleotides to anneal and prime second strand synthesis, were

investigated. However, the author could not determine the cause of failure of the Gene-Editor *In Vitro* System in our hands. This approach was abandoned in view of the problems encountered and considering that the method for site-directed mutagenesis required high throughput efficiency, thus alternative methods were investigated.

Two PCR based methods were attempted. Firstly, the "megaprimer" method has an advantage over other PCR based methods used to introduce mutations internally in that it only requires three oligonucleotides (two flanking the region to be amplified and one

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CHAPTER 3: PROTEIN EXPRESSION AND PURIFICATION

3.1 INTRODUCTION

The role of N-linked glycosylation in the expression and processing of tACE was investigated to establish the minimal glycosylation requirements for the expression of correctly folded enzymatically active protein. The eight glycosylation mutants constructed (Fig. 2.13) were transfected into CHO cells and the effect deglycosylation had on the enzyme activity, expression and processing of tACE was assessed by enzyme assays, immunodetection and mass spectrometry.

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3.2 METHODS

3.2.1 Expression of tACE glycosylation mutants

DNA was transfected into CHO-K1 cells (ATCC: CCL-61), which have no endogenous ACE expression. Cells were cultured in 50 % DMEM, 50 % HAMS supplemented with 10 % foetal calf serum (heat-inactivated at 56°C for 30 minutes), 20 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5 % CO₂.

Approximately 4×10^6 cells were plated in 100 mm culture dishes and transfected with DNA according to the calcium phosphate protocol (ProFection® Mammalian Transfection System, Promega). tACE-wt, -g1, -g2, -g3, -g12, -g13, -g23, -g123 and -g1234 DNA were co-transfected with pSV2neo, a selection vector which confers neomycin resistance to eukaryotic cells. Briefly, a CaCl₂-DNA mix (comprising 20 µg tACE:pSV2neo, at a molar ratio of 20:1) was added to 2 × HEPES-buffered saline [25 mM HEPES, 25 mM NaCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, pH 7.9], to form a DNA-CaPO₄ precipitate over a 30 minute incubation at room temperature. The precipitate was added dropwise to cells and incubated at 37°C with 5 % CO₂ for 4 hours. Cells were washed with 1 × phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3] and glycerol shocked at room temperature for 2 minutes in 15 % glycerol, 1 × HBS. The cells were incubated overnight in fresh culture medium, after thorough washing with 1 × PBS. Culture medium containing 0.8 mg/ml G418 (Sigma) was added 24 hours after transfection. Cells were incubated until individual colonies were observed in transfected dishes. Individual colonies were selected from transfected dishes with cloning rings, trypsinised [0.13 M NaCl, 10mM Na₂HPO₄·2H₂O, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.5 % (w/v) trypsin, 0.1 % (w/v) EDTA, pH 7.4] and re-seeded into fresh flasks for amplification. These clones were screened for tACE expression by assessing ACE activity.

3.2.2 Determination of Enzyme Activity

CHO cells expressing tACE-wt, -g1, -g2, -g3, -g12, -g13, -g23, -g123 and -g1234 were grown to confluency in 6 well plates. Expression of tACE was induced in the presence of 40 µM ZnCl₂, which activates the HMTIIA promoter upstream of the tACE gene. Transfected cells were induced overnight in 50 % DMEM, 50 % HAMS supplemented with 2 % foetal calf

serum (heat-inactivated at 70°C for 15 minutes), 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 40 µM ZnCl₂. Medium was harvested, cells were detergent solubilised in triton lysis buffer [1 % (v/v) Triton X-100, 50 mM HEPES, 0.48 mM NaCl, 1 mM PMSF], the samples assayed for enzyme activity and tACE detected by Western blotting. Results were compiled from two experiments of triplicate samples.

A fluorometric assay was used to assay samples for ACE activity (Helenius, 1994). The assay is based on the formation of the fluorescent adduct of O-phthaldialdehyde and the histidyl moiety of L-histidine-L-leucine, which is the product of ACE cleavage of the hippuryl-L-histidine-L-leucine (Hip-His-Leu) substrate. The samples were incubated at 37°C for 15 minutes in the presence of 4 mM Hip-His-Leu. After addition of 280 mM NaOH to stop the reaction, 7.7 mM O-phthaldialdehyde was added and the samples were incubated at room temperature for 10 minutes, to allow for formation of the fluorescent adduct. The reaction was terminated by the addition of 3 M HCl. The samples were analysed with a fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 485 nm.

Flourescence units were used to calculate ACE units (U), where 1 U is equivalent to 1 mmol of product (His-Leu) produced per minute.

3.2.3 Isolation and Purification of mutant protein

3.2.3.1 Bulk cultures

Transfected CHO cells were seeded into four T175 cm² culture flasks and grown to 80% confluency, whereupon they were induced in 2 % culture medium containing 40 µM ZnCl₂. Medium was harvested every 48hrs for 4 days, pooled and frozen. The cells were incubated overnight in culture medium to recover, fresh 2 % medium was added and the harvesting was repeated.

3.2.3.2 Affinity chromatography of ACE

Soluble tACE-g1, -g13 and -g1234 were purified from harvested 2 % medium by affinity chromatography (Ehlers et al, 1989). Medium was thawed at room temperature and applied to sepharose-28-lisinopril affinity columns. The columns were washed thoroughly with wash buffer [0.5 M NaCl, 20 mM HEPES, pH 7.5] to remove unbound protein, whereupon tACE was eluted with 50 mM Borate (pH 9.5). 2 ml fractions were collected and assayed for ACE activity. Fractions containing significant ACE activity were pooled and

dialysed against distilled water for 2 days, with frequent changes of water. Total protein of the pooled fractions was determined using a Bradford test (BIO-RAD Protein Assay). After dialysis, the protein was freeze-dried overnight and weighed.

3.2.4 Analysis of mutants

3.2.4.1 Western blots

3.2.4.1.1 Assessment of antiserum using dot blots

Polyclonal rabbit anti-human tACE antiserum raised against wild-type tACE was tested to determine the optimum antiserum concentration necessary to detect tACE for Western blots. Approximately 1 µg of purified tACE-Δ36 was applied to nitrocellulose membranes (Hybond-C, Amersham) and allowed to dry. The membrane was blocked for 1 hour at room temperature in 1×tris-buffered saline [200 mM NaCl, 50 mM Tris-HCl, 0.1 % Tween-20, pH 7.4] with 5 % skim milk. Blocking reagent was washed off with 1×TBS and blots were incubated for 1 hour at room temperature in 1×TBS with 5 % skim milk containing 1:500, 1:1000 and 1:1500 dilutions of anti-serum. Unbound anti-serum was removed by thorough washing with 1×TBS. Blots were incubated for 1 hour at room temperature in a 1:2000 dilution of a horseradish peroxidase-rabbit Ig conjugate (raised in goat) in 1×TBS with 5 % skim milk. After thorough washing in 1×TBS, antibody was detected with ECL Western blotting reagents (Amersham) and visualised on autoradiographic film, as per instructions.

3.2.4.1.2 Western blots of mutant proteins

tACE-wt, -Δ36, -g1, -g2, -g3, -g12, -g13, -g23, -g123 and -g1234 were immunodetected by Western blotting of cell lysate and harvested medium collected from cultures of transfected CHO cells. Samples were mixed in 6× SDS sample buffer [0.35 M Tris-HCl/SDS, pH 6.8; 3 % (v/v) glycerol, 1 % (w/v) SDS, 1 % (w/v) bromophenol blue, 0.6 % (v/v) β-mercaptoethanol] and denatured for 5 minutes in boiling water. Proteins were separated on a 10 % SDS-PAGE gel. Protein was transferred to nitrocellulose membrane (Hybond-C, Amersham) for 1 hour at 100 V constant, using the PROTEAN II gel transfer system (BIO-RAD). Non-specific binding sites were blocked with 5 % skim milk 1×TBS for 1 hour at room temperature. After thorough washing in 1×TBS, rabbit anti-serum was bound by incubation of the membrane for 1 hour at room temperature in 5 % skim milk 1×TBS containing 1:2000 anti-serum. Unbound anti-serum was washed off and the secondary

antibody was applied at a 1:2000 dilution in 5 % skim milk 1×TBS for 1 hour at room temperature. After thorough washing, antibody was detected using ECL Western blotting reagents (Amersham) and visualised on autoradiographic film, as per instructions.

3.2.4.2 HPLC and Maldi-TOF Mass Spectrometry

Purified tACE-g1, -g13 and -g1234 was reduced, the cysteine groups protected with vinyl pyridine, and the protein cleaved enzymatically with endoproteinase Lys-C (Ehlers et al, 1996). Peptides were fractionated by reversed-phased high performance liquid chromatography (HPLC) and the eluted fractions were analysed by matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry using a Perceptive Voyager Elite Biospectrometry Workstation, using a nitrogen laser at 337 nm and a 4-hydroxy- α -cyanocinnamic acid matrix (10 mg/ml). Peptides were identified according to the expected masses generated with PAWS (ProteoMetrics) and GPMaw (ver 4.23, Lighthouse data) software.

3.2.5 Inhibition of tACE glycosylation

Confluent 6 well plates containing CHO tACE-wt cells were induced overnight in 2 % medium in the presence of 5 μ g/ml tunicamycin. Fresh medium was added and cells were grown in the presence of 5 μ g/ml tunicamycin for 24 hours. Medium and detergent-solubilised cell samples were collected at 6, 8, 12, 16, 20 and 24 hours. Duplicate samples were assayed for enzyme activity and tACE immunodetected by Western blotting.

3.3 RESULTS AND DISCUSSION

3.3.1 Determination of the minimal glycosylation requirements for enzymatically active tACE

3.3.1.1 Expression of glycosylation mutants in CHO cells

The effect of deglycosylation on expression of the correctly folded active recombinant protein was determined by ACE activity assays of the detergent-solubilised cells.

CHO cells transfected with both tACE-g123 and -g1234 exhibited ACE activity (Table 3.1), indicating that the absence of sugars at the fourth, fifth and sixth sites (Asn155, Asn337 and Asn586) does not prevent the expression of active tACE, i.e. the presence of sugars at the three N-terminal sites is sufficient to produce tACE.

This finding directed our investigations to the role of the first three sites, principally to determine the minimal number of sites that need to be glycosylated for active tACE to be expressed. The presence of glycosylation at two sites was sufficient to produce active tACE, with all three mutants tACE-g12, -g13 and -g23 having cellular enzyme activity. Of the three mutants containing a single site, tACE-g1 and -g3 had cellular enzyme activity. No enzyme activity was observed with CHO cell transfected with tACE-g2 (Table 3.1).

This data does not illustrate quantitatively the effect glycosylation has on the enzyme activity of tACE. Whilst ACE activity in samples indicates that enzymatically active tACE is being expressed, the level of activity between samples is not directly comparable. Cellular ACE activity fluctuated extensively amongst the mutants, most notably the 11-fold increase in activity between wild-type and tACE-g1234 expressed in CHO cells. The level of enzyme activity measured is determined by the amount of protein present and the catalytic activity of the expressed protein, both of which could be affected by removal of glycosylation.

The comparatively high levels of activity in tACE-g1234 cells could be the result of a number of factors, for example: 1) the transfection efficiency of this cell-line may be higher than that of wild-type tACE, resulting in a larger percentage of neomycin-resistant cells expressing tACE, thus increasing protein concentration in cell lysate and consequently total ACE activity. 2) The absence of sugars at the fifth and sixth sites may improve protein expression by allowing tACE to be more effectively processed, thereby increasing protein yield and ACE activity in turn. 3) In the enzyme assay, the rate at which tACE cleaves

Table 3.1. Activity of tACE mutants in CHO cells

	Activity* (mU/well)	
	Cellular	Solubilised
untransfected CHO	0	0
tACE-wt	35.3 (\pm 10.8)	6.6 (\pm 4.4)
tACE-g1	13.5 (\pm 2.1)	6.1 (\pm 3.5)
tACE-g2	0	0
tACE-g3	43.7 (\pm 5.2)	6.3 (\pm 0.5)
tACE-g12	22.7 (\pm 2.1)	6.7 (\pm 0.6)
tACE-g13	151.8 (\pm 16.0)	23.0 (\pm 5.0)
tACE-g23	49.2 (\pm 14.1)	5.0 (\pm 2.3)
tACE-g123	322.8 (\pm 16.5)	90.8 (\pm 26.7)
tACE-g1234	395.2 (\pm 36.1)	80.0 (\pm 9.7)

*n = 6

Hip-His-Leu may be improved by the absence of glycosylation, thereby increasing the specific activity. In this way, the same amount of protein may be present in both cell-lines, but tACE-g1234 has a higher specific activity, yielding higher values for cellular enzyme activity. However, this is purely speculative, as there is no precedent in the literature to indicate how glycosylation affects tACE enzyme kinetics.

In the same way, tACE-g3, -g13, -g23 and -g123 showed increased ACE activity compared to wild-type. The comparative decrease in activity with tACE-g1 and -g12 is most likely the result of the opposite effects: lower transfection efficiency, poor protein expression or a decrease in specific activity caused by the absence of sugars.

Thus, the effect deglycosylation has on the enzyme kinetics of tACE cannot be determined unequivocally from the data obtained. These enzyme assays served only to confirm the presence of enzymatically active tACE in mutant cell-lines, indicating which sites are sufficient to produce active protein. The absence of activity is in itself not conclusive. As with tACE-g2, the reason for the absence of activity is not clear, it could be due to the lack of protein expression as a result of a failed transfection or the lack of activity caused by an expressed inactive protein. Western blotting analysis, discussed later, was used to address this further.

3.3.1.2 Processing of glycosylation mutants

The enzyme activity in medium harvested from mutant cell-lines was determined to clarify the effect glycosylation has on the cell-surface expression and processing of tACE. *In vitro* tACE is expressed on the cell surface where it undergoes cleavage-secretion by an as yet unidentified secretase (Ehlers et al, 1991b). This event is exhibited by an appearance of

soluble ACE in culture medium harvested from cells expressing ACE, detected by enzyme assays of harvested medium. All mutants that exhibited cellular ACE activity had levels of active soluble tACE in harvested medium namely tACE-g123 and -g1234, mutants containing two sites: tACE-g12, -g13 and -g23 and those containing a single site: tACE-g1 and -g3 (Table 3.1). Thus, mutants that are expressed intracellularly as enzymatically active protein appear to be processed correctly. No soluble tACE was detected in CHO cells transfected with tACE-g2.

3.3.1.3 Immunodetection of cellular and soluble tACE

The expression and glycosylation of mutant tACE was further investigated by Western blot analysis performed on cell lysate and harvested medium, using anti-tACE serum prepared from immunised rabbits.

The optimal antibody concentration was determined by dot blots with purified soluble tACE- Δ 36. Protein was detected at an anti-serum concentration of 1 in 1500, indicating that the anti-serum is highly immunogenic. The specificity of the anti-serum was determined by performing Western blots on cell lysate from CHO cells transfected with wild-type (Fig. 3.1). With this anti-serum (Fig. 3.1 anti-ACE serum I), purified tACE- Δ 36 was detected as a broad band at 90 kDa, with wild-type tACE appearing at 105 kDa, consistent with previous findings (Schwager et al, 1999). The proteins appear as broad bands due to the heterogeneity in size introduced by the presence of glycosylation. The 15 kDa difference between wild-type and tACE- Δ 36 is due to O-linked glycosylation at the N-terminus, which has been removed in tACE- Δ 36. At concentrations of 1 in 500, this anti-serum appeared to have higher specificity for tACE compared to a second anti-serum (Fig. 3.1 anti-ACE serum II) prepared simultaneously, which detected various smaller bands, indicating a greater degree of non-specific binding. Thus, anti-tACE serum I was selected for immunodetection of tACE glycoforms at dilutions of 1:1000 and 1:2000.

Western blot analysis of CHO cell lysate from all eight tACE glycoforms detected protein which had increased mobility compared to tACE- Δ 36 (Fig. 3.2). The calculated molecular mass of monomeric soluble, unglycosylated tACE- Δ 36 was 70 kDa, thus the tACE glycoforms migrated as proteins between 70 kDa and 90 kDa, depending on the amount of glycosylation present. Sequential deglycosylation by the removal of two, three, four or five sites resulted in an increased mobility of the glycosylation mutants on SDS-PAGE (Fig. 3.2).

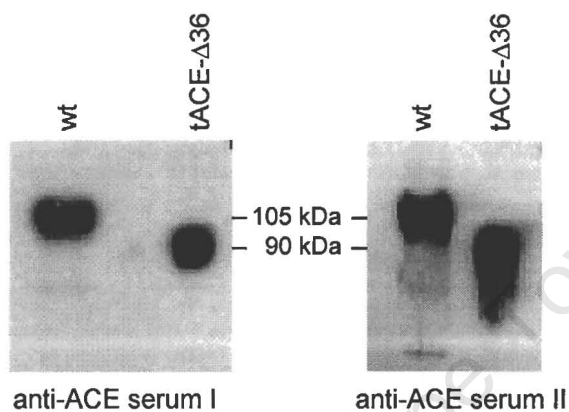


Figure 3.1. Specificity of anti-ACE sera. Bleeds from two rabbits immunised against tACE were used to detect purified tACE- Δ 36 and tACE from transfected CHO cell lysate (wt). The left panel was tACE detected with the first anti-serum and the right panel was detected with the second anti-serum at an antibody concentration of 1:500.

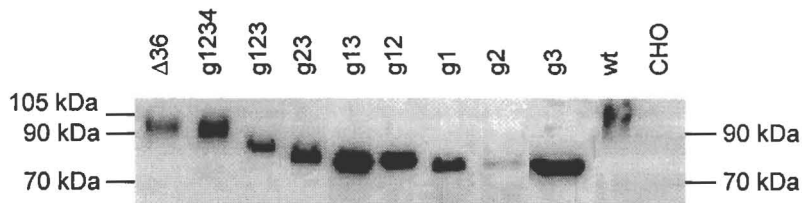


Figure 3.2. Expression of tACE glycosylation mutants. Protein was immunodetected from detergent-solubilised cells with anti-serum (at 1:2000). The estimated protein size is indicated. Lanes contain tACE- Δ 36 (Δ 36), untreated CHO (CHO) and wild-type tACE (wt) cell lysate, and cell lysate of cells transfected with the mutants, with numbers representing the sites that are glycosylated.

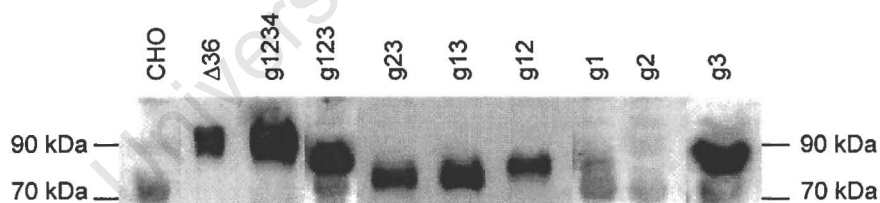


Figure 3.3. Processing of tACE glycosylation mutants. Protein was immunodetected from harvested medium with anti-serum (at 1:2000). The estimated protein size is indicated. Lanes contain purified soluble tACE- Δ 36 (Δ 36), wild-type tACE harvested medium (wt), untreated CHO medium (CHO) and harvested medium from CHO cells transfected with the mutants, with numbers representing the sites that are glycosylated.

Comparatively low amounts of tACE-g2 were detected in CHO cell lysate at 75 kDa, at the same mobility as tACE-g1. This confirmed that tACE-g2 transfections into CHO cells were successful and that this mutant is expressed intracellularly as an underglycosylated protein.

Protein bands of similar sizes to those observed in the cell lysate were detected in harvested medium from CHO cells expressing tACE-g123 and -g1234 (Fig. 3.3), indicating that these mutants are solubilised. Protein was also detected in medium from cells expressing tACE-g12, -g13 and -g23 and tACE-g1 and -g3. Comparatively low levels of tACE-g1 are present in the medium, indicating the processing and solubilisation of this mutant may not be as efficient compared to tACE-g3. Soluble tACE-g2 was not evident in Western blots of harvested medium from cells expressing this mutant. These data, together with the low amounts observed in cells, indicate that this mutant is not processed normally. The ~70 kDa band observed in medium from tACE-g123, -g1, -g2 and -g3, which has a greater mobility than the mutants, appears consistently in medium harvested from untransfected CHO cells, indicating that it is a non-specific product.

3.3.1.4 The minimal glycosylation requirements for the expression of active tACE

Combined results from ACE activity assays and Western blot analysis show that all tACE glycoforms except tACE-g2 are enzymatically active, expressed and processed (Table 3.2). tACE-g2 is expressed in CHO cells, but is inactive and does not appear in the medium, which indicates that it is most likely degraded intracellularly. Removal of all sites except the three N-terminal sites produces active tACE (as with tACE-g123) and the presence of any two of these sites is sufficient for the expression of enzymatically active protein (as with tACE-g12, -g13 and -g23). When one site is present, activity is maintained when either the first or third site is glycosylated (as tACE-g1 and -g3 are active and tACE-g2 is inactive). Thus the minimal glycosylation requirements for the expression and proper processing of correctly folded enzymatically active tACE in CHO cells is the presence of oligosaccharides at a single site in the N-terminus with a preference for the first (Asn72) or third (Asn109) site.

These results are consistent with the findings that glycosylation at a single N-terminal site is sufficient for the expression of underglycosylated rabbit tACE mutants in HeLa cells (Sadhukhan & Sen, 1996). tACE was produced when glycosylation occurred at either the

Table 3.2. Activity, expression and processing of tACE mutants in CHO cells

	Cellular		Solubilised	
	Activity	Expression	Activity	Expression
untransfected CHO	N	N	N	N
tACE-wt	Y	Y	Y	Y
tACE-g1	Y	Y	Y	Y
tACE-g2	N	Y	N	N
tACE-g3	Y	Y	Y	Y
tACE-g12	Y	Y	Y	Y
tACE-g13	Y	Y	Y	Y
tACE-g23	Y	Y	Y	Y
tACE-g123	Y	Y	Y	Y
tACE-g1234	Y	Y	Y	Y

Y: ACE activity or expression observed, N: no ACE activity or expression observed

first or second site and the presence of glycosylation at the third site alone was not sufficient for tACE expression in HeLa cells. This mutant was expressed and processed in yeast cells, however. The differential glycosylation requirements of rabbit tACE in HeLa cells and yeast are defined by the glycosylation pathways within these cells. Proteins expressed in yeast contain N-linked oligosaccharides principally of the high mannose type, whereas the N-linked sugars attached to proteins expressed in mammalian cells fall within all three subgroups: hybrid and complex sugars and high mannose sugars, from which the former arise. Yeast lacks the machinery present in the Golgi apparatus of mammalian cells to process N-linked sugars and as a result may not have as stringent criteria for the maturation and processing of proteins. Not only does the glycosylation machinery differ between yeast cells and mammalian cells, but between different mammalian cells lines due to the profile of glycosidases which are expressed in the Golgi apparatus. This is the origin of the different glycosylation patterns and oligosaccharide sequences observed between proteins isolated from different species and from different tissues within the same species (Baenziger & Green, 1988; Bliithe, 1990). Human tACE was expressed in CHO cells, whereas rabbit tACE was expressed in HeLa cells. The different glycosylation requirements observed between the expression of human tACE and rabbit tACE may be the result of the specific requirements of the tissues in which they were expressed.

In both human and rabbit tACE removal of all but the first three sites closest to the N-terminus does not impair the expression, maturation and processing of both proteins. These three N-terminal sites are clustered over 40 residues within the N-terminus and are fully glycosylated in human tACE, resulting in a highly glycosylated region of the protein. Of the 6 glycosylated sequons identified from sequencing tryptic fragments of purified sACE,

four are clustered at the N-terminus spanning 100 residues (Fig. 1.5) (Yu et al, 1997). What is striking is that in rabbit and human tACE and human sACE the N-terminal sites appear to be heavily glycosylated with N-linked sugars. Furthermore, glycosylation at these N-terminal sites are important for the expression, maturation and processing of active tACE in both species.

In the absence of the crucial sites (i.e the first and second sites), rabbit tACE is rapidly degraded in the RER of HeLa cells (Sadhukhan & Sen, 1996). Results show that tACE-g2 expressed in CHO cells is inactive and degraded intracellularly. Western blotting data does not allow for localisation of this event. Interestingly, the rabbit tACE-g3 mutant was not glycosylated whereas SDS-PAGE indicates that tACE-g2 migrates as an underglycosylated protein, the same size as tACE-g1 (Fig. 3.2). This indicates that the production of tACE-g2 is most likely hampered at an access point further along the pathway, after tACE has been glycosylated. The lack of enzyme activity with tACE-g2 implies that the conformation of the active site has been misfolded, as a result of the removal of the first and third sites. Thus, the presence of glycosylation at these two sites appears to influence protein folding, whereas the second site in human tACE can not compensate for the absence of glycosylation at the other sites.

The role of these sites and the three partially glycosylated sites may extend to other events during protein maturation, cell surface expression and processing, and enzyme activity; which was not determined in this study. The different levels observed between tACE-g1 and -g3 in harvested medium (Fig. 3.3) suggest that glycosylation at the third site may aid solubilisation. The fifth site (Asn337) is conserved in both human and rabbit tACE and the equivalent site in human sACE is glycosylated (Asn913) (Yu et al, 1997). All three sites are within 50 residues of the active site and may interact with this region in some way (Fig 1.5), although the effect of removal of this site on the enzyme activity of human tACE was not quantitatively determined.

Further quantitative data would need to be collected to understand the effect each site has on protein expression and activity. This would include pulse-chase experiments to monitor the maturation of each mutant and determination of the catalytic activity by calculating the k_{cat} and K_m of purified protein to quantitate what effects the removal of sugars has on ACE activity.

Table 3.3. Mass analysis of purified glycosylation mutants

Glycosylation site	Lys-C peptide (residue no.)	tACE-g1		tACE-g13		tACE-g1234	
		observed	[M+H] ⁺ expected	observed	[M+H] ⁺ expected	observed	[M+H] ⁺ expected
1	47 – 79	-	(4004.2)	-	(4004.2)	-	(4004.2)
2	85 – 94	-	(1184.4)	-	(1184.4)	-	(1170.4)
3	102 – 113	1437.1	(1435.6)	-	(1421.6)	-	(1421.6)
4	138 – 174	4069.6	(4067.7)	4069.4	(4067.7)	-	(4053.7)
5	318 – 338	2436.6	(2436.7)	2436.3	(2436.7)	2436.8	(2436.7)
6	568 – 597	-	(3375.0)	3372.9	(3375.0)	3374.1	(3375.0)

All mass values are calculated for protonated molecular weights m/z . The calculated masses are indicated in parentheses, those containing Asn are indicated in bold.

3.3.2 Further characterisation of tACE-g1, -g13 and -g1234

3.3.2.1 Determination of Glycosylation of tACE-g1, -g13 and -g1234

Purified tACE-g1, -g13 and -g1234 were further characterised using MALDI-TOF mass spectrometry of endoproteinase Lys-C digested peptide fractions. The mass spectra of selected fractions were determined to identify peptides containing the mutated glycosylation sequons, to show whether glycosylation had been removed.

tACE-g1, -g13 and -g1234 were purified from harvested culture medium. Purification of solubilised tACE-g1234 from 300 ml of medium yielded approximately 2 mg protein, with an apparent specific activity of 2.5 U/mg. Purification of solubilised tACE-g1 and -g13 gave a total of 0.34 mg and 0.5 mg, respectively, from 150 ml medium, each with an apparent specific activity of approximately 5.3 U/mg and 4.6 U/mg. Although these specific activities were similar to tACE-g1234, the yields for tACE-g1 and -g13 were 17% and 25% of the yield obtained for tACE-g1234.

3.3.2.1.1 tACE-g1234

The mass spectra of endoproteinase Lys-C digested peptides of tACE-g1234 revealed [M+H]⁺ ions at m/z 2436.8 and 3374.1, which correspond closely to the theoretical masses of peptides containing the fifth (calculated m/z 2436.7) and sixth (calculated m/z 3375.0) sequons, in which Asn was changed to Gln (Table 3.3). Peptides containing the Asn to Gln transition at the six glycosylated sites were identified according to peaks observed corresponding to the calculated masses, where peptide masses increased by m/z 14 when

Gln was present. Those retaining an Asn residue remained glycosylated resulting in different glycoforms, making the identification of a specific glycopeptide by MALDI-TOF mass spectrometry difficult. Thus, the removal of these two recognition sequons has successfully prevented glycosylation at the fifth and sixth sites. Peaks corresponding to peptides containing the first four sequons were not identified – as masses with either an Asn or Gln residue. This indicates that these peptides are glycosylated and elute as different glycoforms. The glycosylation profile of tACE-g1234 obtained from the mass spectra data corroborated the conclusions drawn from restriction enzyme digestion profiles of recombinant DNA (Fig. 2.14), namely that the fifth and sixth sites had been removed and that glycosylation occurs at the first four sites.

3.3.2.1.2 tACE-g13

With tACE-g13 the second, fourth, fifth and sixth sites were mutated. The mass spectra identified three $[M+H]^+$ ions at m/z 4069.4, 2436.3 and 3372.9, which are in agreement with the theoretical masses of three peptides containing the fourth (calculated m/z 4067.7), fifth (calculated m/z 2436.7) and sixth (calculated m/z 3375.0) sites where the Asn has been replaced by a Gln (Table 3.3). Peptide peaks of masses relating to unglycosylated protein at the second site were not isolated. This was the case for both the native and mutated sequon. As a result, the removal of glycosylation at g2 was not confirmed with mass spectra data. Restriction analysis of recombinant tACE-g13 DNA (Fig. 2.14) and the increased mobility with SDS-PAGE of tACE-g13 compared to tACE-g123 detected with Western blots (Fig. 3.3) suggest that this site is not glycosylated. It appears the first and third sites are glycosylated, as unglycosylated peptides both in the native and mutated form containing these two sites were not identified. Thus, mass spectra data indicates that the mutations at the fourth, fifth and sixth sites are present and that glycosylation has been successfully abolished from these sites.

3.3.2.1.3 tACE-g1

All sequons bar the first and seventh were mutated to obtain tACE-g1. Three $[M+H]^+$ ions of m/z 1437.1, 4069.6 and 2436.6 were identified that corresponded closely to the theoretical masses of peptides which contain the mutated third (calculated m/z 1435.6), fourth (calculated m/z 4067.7) and fifth (calculated m/z 2436.7) sites (Table 3.3). Peptides containing the second and sixth sites, both in the native and mutated unglycosylated form

were not identified. As with tACE-g13, this is more likely the result of a failure to isolate the unglycosylated peptide. Unlike tACE-g1234, the protein yield for tACE-g1 and -g13 was lower; thus peptides exist at much lower concentrations. It is likely that the unidentified peptides were present in amounts too low to detect. Thus, the presence of the mutation and the lack of glycosylation at these sites could not be confirmed with mass spectra data. However, restriction enzyme digests of tACE-g1 DNA indicate that these sites have been mutated (Fig. 2.14). The increased mobility of tACE-g1 on SDS-PAGE, compared to tACE-g12, -g13 and -g23 corroborates that this protein is glycosylated at a single site (Fig. 3.3). Masses corresponding to the unglycosylated peptides containing the first site in both the native and mutated form were not identified. Restriction enzyme digests show that this recognition sequon is intact, indicating that the failure to isolate a single peak is due the presence of numerous peptides containing the first site as different glycoforms, as a result of glycosylation at the first site. Thus, mass spectra data showed that mutations at the third, fourth and fifth sites of tACE-g1 has successfully abolished glycosylation. This could not be confirmed for the second and fifth sites.

3.3.2.2 Identification of the C-terminal endoproteinase Lys-C peptide

Further protein analysis was performed to identify the C-terminal peptide to 1) determine whether cleavage of tACE-g1, -g13 and -g1234 occurred at the same residues as wild-type and 2) identify the seventh recognition sequon which lies seven residues proximal to the wild-type cleavage site at Arg627. Identification of the C-terminal peptide serves to confirm that solubilisation occurs normally and that the presence of this peptide as a single peak corroborates that the seventh site is unglycosylated. The combination of limited proteolysis and MALDI-TOF mass spectrometry provides an effective approach to identifying the cleavage sites of solubilised proteins, a technique that has been successful with both isoforms of ACE and various tACE mutants (Ehlers et al, 1996; Schwager et al, 1999). This is achieved by identifying the C-terminal peptide of the solubilised protein after enzyme digestion with a specific endopeptidase. The C-terminal peptide appears as a peak corresponding to a calculated mass within a peptide ladder generated from the peptide that lies proximal to the transmembrane region (Fig. 3.4). Because the sequence of the peptide ladder is known, the terminal residue can be determined. Thus, with wild-type tACE, after endoproteinase Lys-C digestion this peptide has a calculated m/z 1690.8, corresponding to the peptide from Leu614 to Arg627.

Endoproteinase Lys-C C-terminal peptide:

614 LGWPQYNWTPNSARSEGPLPDSGRV 638

N-terminal ladder:

Peptide no.	Mass (M ⁺ H ⁺)	Residues	AA Sequence
1	2786.04	614-638	LGWPQYNWTPNSARSEGPLPDSGRV
2	2686.90	614-637	LGWPQYNWTPNSARSEGPLPDSGR
3	2530.72	614-636	LGWPQYNWTPNSARSEGPLPDSG
4	2473.66	614-635	LGWPQYNWTPNSARSEGPLPDS
5	2386.59	614-634	LGWPQYNWTPNSARSEGPLPD
6	2271.50	614-633	LGWPQYNWTPNSARSEGPLP
7	2174.38	614-632	LGWPQYNWTPNSARSEGPL
8	2061.22	614-631	LGWPQYNWTPNSARSEGP
9	1964.10	614-630	LGWPQYNWTPNSARSEG
10	1907.05	614-629	LGWPQYNWTPNSARSE
11	1777.94	614-628	LGWPQYNWTPNSARS
12	1690.86	614-627	LGWPQYNWTPNSAR
13	1534.67	614-626	LGWPQYNWTPNSA
14	1463.59	614-625	LGWPQYNWTPNS
15	1376.51	614-624	LGWPQYNWTPN
16	1262.41	614-623	LGWPQYNWTP
17	1165.29	614-622	LGWPQYNWT
18	1064.19	614-621	LGWPQYNW
19	877.97	614-620	LGWPQYN
20	763.87	614-619	LGWPQY

Figure 3.4. Isolation of the C-terminal peptide. An N-terminal digestion ladder was generated from the Lys-C peptide closest to the transmembrane domain. The calculated peptide masses are indicated and the wild-type tACE C-terminal peptide identified is highlighted in bold.

Table 3.4. Mass spectra of the C-terminal endoproteinase Lys-C peptide

Peptide (residue no.)	[M+H] ⁺ observed			
	wild-type tACE	tACE-g1	tACE-g13	tACE-g1234
614 - 627	1689.6*	1689.7	1690.5	1690.9

All mass values are calculated for protonated molecular weights *m/z*. The calculated mass for all samples is *m/z* 1690.8. * Determined by Schwager et al (1999)

The mass spectra of endoproteinase Lys-C digested peptides of tACE-g1, -g13 and -g1234 revealed three [M+H]⁺ ions of *m/z* 1689.7, 1690.5 and 1690.9, respectively (Table 3.4), which are in close agreement with the theoretical mass of the C-terminal peptide (calculated *m/z* 1690.8). Thus, for tACE-g1, -g13 and -g1234 proteolysis occurs between Arg627 and Ser628, which is at the same site as wild-type tACE. Furthermore, the absence of glycosylation at specific sites within these proteins has not hampered proteolysis and all three are processed normally.

In the construction of the glycosylation mutants, the seventh site was not targeted as it had been shown to be unglycosylated (Yu et al, 1997). With the removal of the glycosylated sequons, it is possible that this site may be glycosylated in order to compensate for the loss of oligosaccharide chains. MALDI-TOF mass spectra data indicates that this does not occur. After endoproteinase Lys-C digestion of tACE-g1234, the C-terminal peptide containing the seventh site was isolated as a homogeneous peak, indicating that the removal of two partially glycosylated sites at Asn337 and Asn586 does not result in glycosylation at the seventh site. Furthermore, the C-terminal peptides isolated from digested tACE-g1 and -g13 confirm that glycosylation does not occur at the seventh site, even in the absence of most of the N-linked sugars at the other sites.

There is a preference for oligosaccharides to be attached to sequons containing threonine residues rather than serine residues. This was observed in human tACE, where all sequons with a serine in the third position were partially glycosylated and those with threonine were fully glycosylated, except for the seventh site, which has a threonine in the third position (Fig. 2.1) (Yu et al, 1997). The attachment of oligosaccharides, mediated by oligosaccharyl transferase (OST), occurs when the sequon adopts an Asx-turn (Fig. 3.5a). The tryptophan in the second position of the seventh sequon, which has a bulky aromatic ring in the side chain, may occlude the formation of the Asx-turn, thereby preventing glycosylation at this site (Fig. 3.5b). The fact that glycosylation at g7 appears to be occluded by the

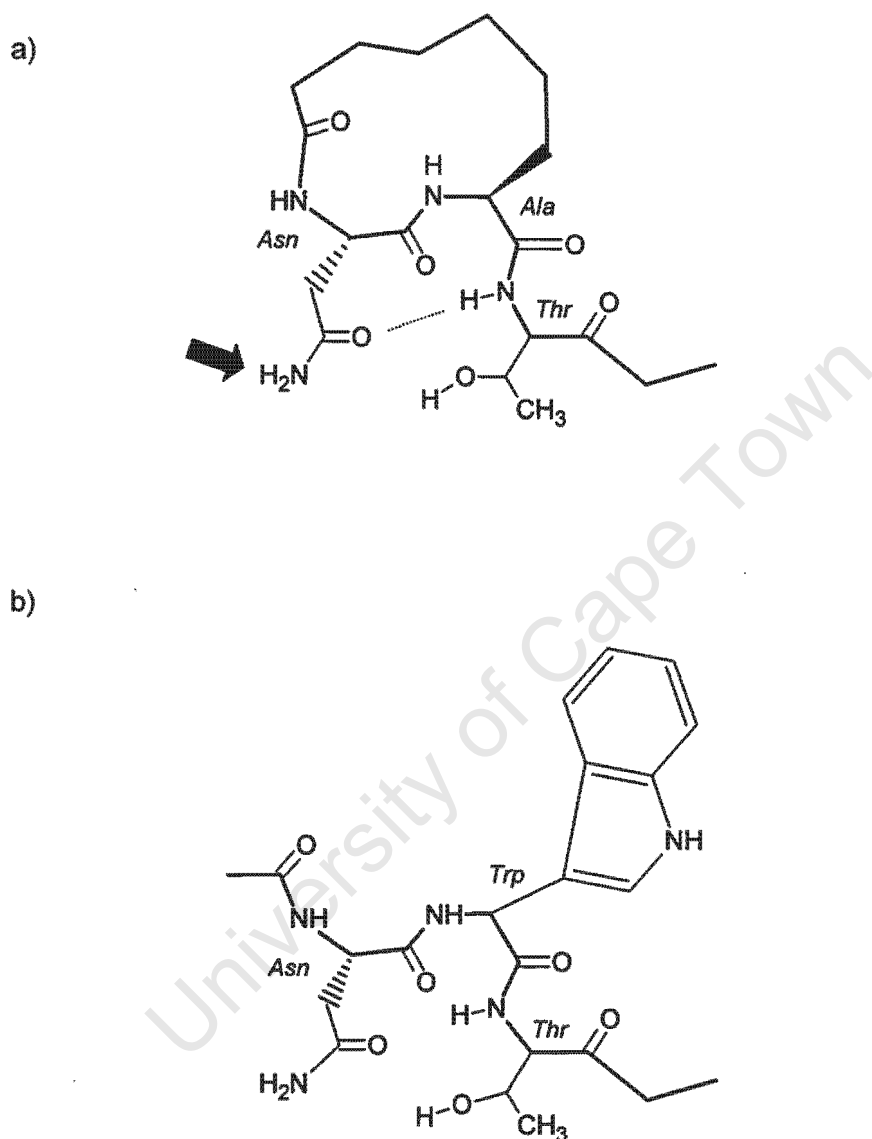


Figure 3.5. Occlusion of the Asx-turn. a) The Asx-turn recognised by oligosaccharyl transferase (OST) with Asn-Ala-Thr, the arrow indicates the amine to which the oligosaccharide is attached. b) The presence of the aromatic ring in the Trp side chain may occlude the formation of the Asx-turn and subsequent interaction with OST.

Figure adapted from Imperiali et al, 1999.

presence of the tryptophan, rather than the structure of the juxtamembrane region, is supported by the findings that a tACE deletion mutant, tACE- $\Delta 6$, which generates a novel Asn-Arg-Ser sequon adjacent to cleavage site, is glycosylated (Schwager et al, 1999)

A tryptic fragment containing the equivalent sequon in human sACE was not glycosylated and this site is not present in rabbit tACE (Sadhukhan & Sen, 1996; Yu et al, 1997) (Fig. 1.5). The extended conformation of oligosaccharides may prevent access of the secretase at this point, as was demonstrated with a tACE- $\Delta 6$, where the glycan adjacent to the wild-type cleavage site did not abolish solubilisation, but resulted in displacement of the cleavage site 13-residues proximal to the glycan (Schwager et al, 1999).

3.3.3 Inhibition of tACE glycosylation

3.3.3.1 Treatment of CHO cells with tunicamycin

CHO cells expressing wild-type tACE were exposed to tunicamycin, which inhibits N-linked glycosylation, to determine the effect of complete deglycosylation on the expression and activity of tACE over time. Samples were compared to those from cells not exposed to the drug.

Cellular ACE activity in cells treated with tunicamycin is approximately three-fold lower than that of untreated cells (Fig. 3.6a). Analysis of Western blots of cellular tACE revealed a 105 kDa protein. In the presence of tunicamycin an additional 85 kDa protein was seen (Fig. 3.6b). These results are consistent with the interpretation that the smaller 85 kDa protein is the deglycosylated form and the 105 kDa protein the mature glycosylated form (Yu et al, 1997). The removal of sugars results in a sharp band on SDS-PAGE consistent with a more homogeneous protein, unlike the broad band of mature glycosylated tACE. Thus, tunicamycin inhibits the formation of mature glycosylated tACE to a certain degree resulting in the accumulation of unglycosylated tACE in the cell, without a significant decrease in the total amount of protein compared to untreated cells. The loss of activity observed with cells treated with tunicamycin is inconsistent with the amount of protein present in the Western blots. There is a three-fold decrease in the ACE activity in cells treated with tunicamycin (from 40 mU/well to 10 mU/well), indicating that unglycosylated tACE is inactive. The ACE activity present in cells treated with tunicamycin is most likely the product of a pool of glycosylated tACE, which was either produced prior to tunicamycin treatment or is resistant to tunicamycin.

Over 18 hours, enzyme assays of harvested medium from untreated cells shows an accumulation of ACE activity (from 10 mU/well to 25 mU/well), which is due to continuous solubilisation of cellular tACE (Fig. 3.6c). In medium harvested from cells treated with tunicamycin, ACE activity is 8 times lower and the accumulation of soluble ACE is not as evident (Fig. 3.6c). This is consistent with the interpretation that unglycosylated tACE is not solubilised. ACE activity present in the medium of treated cells is most likely due to the solubilisation of the pool of mature glycosylated tACE. It seems that the inactive deglycosylated tACE is degraded before it can be solubilised. Thus, it appears that tACE requires the presence of N-glycans for proper expression, maturation and processing.

3.3.3.2 Previous observations regarding the inhibition of tACE glycosylation by tunicamycin

Kasturi et al observed the same effect with tunicamycin treatment of HeLa cells expressing rabbit tACE. Pulse-chase experiments and subsequent immunoprecipitation of tACE detected a deglycosylated protein of 76 kDa that was degraded intracellularly within three hours. No soluble tACE was detected in treated cells. (Kasturi et al, 1994).

Treatment of CHO cells expressing human tACE with tunicamycin, had a significant effect on the maturation of the protein but did not result in complete deglycosylation. The different effects observed between the two cell lines may be the result of the cell type used, which has been observed with other cells lines. Originally rabbit tACE was expressed in ACE89 cells to determine the extent of glycosylation (Kasturi et al, 1994). When these cells were exposed to tunicamycin, the drug had no effect on tACE glycosylation and expression. The ineffectiveness of tunicamycin in this cell line was demonstrated by its inability to inhibit glycosylation of vesicular stomatitis virus G protein (Kasturi et al, 1994). As a result, tACE was expressed in HeLa cells, which are known to be susceptible to the drug (Cox, 1981). Furthermore, tunicamycin treatment of a neuronal cell line (IMR-32 cells) expressing human tACE failed to completely deglycosylate the protein as mature tACE was secreted normally, as evidenced by its inhibition by batimastat, which prevents the action of the ACE secretase (Hooper, N.M.; personal communication). No evidence in the literature was found regarding the effects of tunicamycin treatment on tACE expression in CHO cells, although our findings suggest that inhibition of tACE with tunicamycin is not completely effective in CHO cells.

Tunicamycin treatment of temperature sensitive mutant CHO cell lines, which were defective in N-linked glycosylation, effectively prevented glycosylation as demonstrated by

the induction of apoptosis in treated cells (Walker et al, 1998). However, the drug was effective only at low concentrations (LD_{50} for wild-type CHO cells was $0.2 \mu\text{g/ml}$) and after prolonged exposure (up to 12 hours). An investigation into the role of glycans in the expression and activity of the thyrotropin receptor used tunicamycin to demonstrate the effects of complete deglycosylation of the receptor. In this case, the cells were exposed to tunicamycin for 3 days to ensure the removal of any glycosylated receptor (Nagayama et al, 1998). Thus, a longer incubation of CHO cells in the presence of tunicamycin may decrease the pool of mature tACE observed. Tunicamycin treatment of tACE expressed in CHO cells showed a slight decline in the amount of mature glycosylated tACE after 40 hours incubation, however cell growth had deteriorated, with a great number lifting from the dish (Fig. 3.6b). Thus, prolonged incubation of CHO cells with tunicamycin does not appear to be a useful means to increase the effectiveness of the drug, nor does increasing the concentration of tunicamycin, as the drug appears to be more effective at low concentrations (Walker et al, 1998). An approach that may improve the observed effects of tunicamycin on tACE glycosylation is pulse-chase labelling and immunoprecipitation. This technique was used to monitor the inhibition of glycosylation of rabbit tACE in HeLa cells (Kasturi et al, 1994). The advantage this technique has over immunoblotting is that the synthesis and maturation of tACE can be monitored by immunoprecipitation of radiolabelled protein over time, allowing the protein to progress through the cell. If pulse-chase labelling occurs whilst cells are exposed to tunicamycin, the effect of the drug on this pool of labelled tACE can be determined independently of the unlabelled glycosylated tACE that is present within the cell.

3.4 CONCLUSIONS

The minimal glycosylation requirements for the expression and processing of enzymatically active tACE were determined by progressive deglycosylation. To investigate which sugars are vital for tACE expression, a series of mutants were constructed that contain a limited number of sites for N-linked glycosylation. N-glycan recognition sequons within the protein sequence were disrupted by mutagenesis of the Asn to a Gln within the recognition sequon. These mutants were transfected into CHO cells to determine whether the sugars present were sufficient to produce active tACE. Their expression, processing and enzyme activity was determined by immunodetection and enzyme assays. These experiments showed that the removal of a large percentage of sugars did not hamper active tACE production and that the presence of sugars at two of the three N-terminal sites was sufficient to produce enzymatically active protein. Furthermore, the minimum N-linked glycosylation that allowed for enzymatically active tACE to be expressed is the presence of sugars at the first or third site. Glycosylation at these sites appears to be involved in directing protein folding, as the absence of glycosylation at both these sites resulted in rapid intracellular degradation, although the protein structure could have been disrupted by the amino acid substitution.

Attempts were made to express unglycosylated tACE by exposing CHO cells expressing wild-type tACE to tunicamycin, which inhibits N-linked glycosylation. Tunicamycin treatment of CHO cells affected the maturation and processing of tACE to some degree, although immunodetection and enzyme assays of treated cells showed that about 60 % of tACE was deglycosylated (compared to untreated cells), whereas a third remained fully glycosylated. This pool only appeared to be reduced after 40 hours of continuous exposure to tunicamycin. A decrease in enzyme activity between untreated and treated cells with the appearance of underglycosylated tACE was observed. This indicated that in the absence of N-linked glycosylation tACE is inactive and that some N-linked glycosylation is required for the expression of enzymatically active tACE.

This study has contributed to the understanding of the minimal glycosylation requirements for the production of structurally intact human tACE, as monitored by the preservation of ACE activity in underglycosylated mutants. These underglycosylated isoforms have been stably transfected into CHO cells to facilitate large-scale purification with

unglycosylated tACE for crystallisation through the use of tunicamycin was investigated and was found to be an ineffective approach for two reasons. First, unglycosylated tACE was inactive, indicating that it is misfolded; and second, tunicamycin treatment of CHO cells was not completely effective. tACE-g1 and -g3 have been discussed as potential candidates, although the expression levels of these proteins in CHO cells were low, which did not facilitate large-scale purification. A possible means to improve expression may be to transfect these mutants into CHO *Id/D* cells. Additionally, mutants which are more heavily glycosylated but show better expression in CHO cells could be expressed in yeast or CHO *Id/D* cells to reduce the heterogeneity of the oligosaccharides. This would facilitate enzymatic deglycosylation of purified protein.

After countless attempts at crystal formation of tACE and tACE- Δ 36sol, small crystals have been obtained from purified tACE-g123. Conditions are currently being optimised for the production of larger crystals for analysis. This preliminary success validates the role that the construction of these eight minimally glycosylated tACE mutants plays in the elucidation of the three-dimensional structure of ACE.

APPENDIX

A 1 DNA ISOLATION

A 1.1 Small-Scale Isolation of Plasmid DNA for Screening of Mutants

This alkaline lysis and phenol extraction protocol is an adaptation of the method described (Sambrook et al, 1989). Overnight cultures of transformed *E.coli* XL1-blue or JM109 cells were grown in 5 ml LB broth containing 50 µg/ml ampicillin. Cells were harvested by centrifugation at 12 000×g for 2 minutes, resuspended in 100 µl buffer (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0) and lysed by the addition of 200 µl 200 mM NaOH, 1 % (v/v) SDS, mixed by inversion and incubated at RT for 5 minutes. Addition of 150 µl of 3 M KAc (pH 4.8) neutralised the lysate and incubation for 5 minutes on ice precipitated chromosomal DNA. Cellular debris and chromosomal DNA was removed by centrifugation at 12 000×g for 10 minutes. The supernatant containing the plasmid DNA was transferred to a fresh tube. 1 volume of TE buffered phenol and 1 volume of chloroform:isoamyl alcohol (24:1) was added to the supernatant and mixed by vortexing. The phases were separated by centrifugation at 12 000×g for 5 minutes. The aqueous phase was removed from the non-aqueous and protein layers and 1 volume of chloroform:isoamyl alcohol was again added. The phases were mixed by vortexing and re-centrifuged. The aqueous phase was removed and plasmid DNA was precipitated with 2.5 volumes of 100 % ethanol at -70°C for 30 minutes. After centrifugation for 30 minutes at 12 000×g, the DNA pellet was washed with 70 % ethanol, air dried and dissolved in 40 µl double-distilled H₂O and 100 ng RNaseA.

A 1.2 Large-Scale Isolation of Plasmid DNA

Plasmid DNA containing the desired mutants was prepared in bulk with the QIAGEN Midi-preparation kit (DIAGEN GmbH, Germany). Overnight cultures of transformed *E.coli* XL1-blue or JM109 cells were grown in 50 ml LB broth containing 50 µg/ml ampicillin. Plasmid DNA was isolated per the manufacturer's instructions. Precipitated DNA was resuspended in 150-200 µl TE (final concentration between 0.5 and 1 mg/ml) and stored at

4°C. The DNA concentration was estimated from the absorbance at 260 nm with a Anthele Advanced spectrophotometer (SECOMAN, France).

A 2 RESTRICTION ENZYME DIGESTS AND AGAROSE GEL ELECTROPHORESIS

For diagnostic purposes, a 20 µl digestion cocktail containing:

- 1× Restriction Enzyme buffer
- 1-2 µl Plasmid DNA
- 5 Units Restriction Enzyme
- in dH₂O

was incubated at 37°C for an hour. The reaction was terminated by the addition of 0.1 volume 10× sucrose loading dye.

DNA was separated by gel electrophoresis at 70 V in 6×10 cm agarose gels in 1×TBE buffer, containing ethidium bromide. The percentage gel was determined by the size of the fragments being separated.

DNA for subcloning was digested in two stages:

A 20 µl cocktail containing vector or insert DNA and the first enzyme was incubated for an hour at 37°C, whereupon 5 µl was removed and terminated in 1× sucrose loading dye. The volume of the remaining mix was made up to 30 µl by the addition of 3 µl 10× restriction enzyme buffer and 5 units of the second enzyme, in dH₂O. This was incubated for an hour at 37°C and terminated with 0.1 volume 10× sucrose loading dye. The desired fragments were separated in a 1 % (w/v) agarose gel, run alongside the 5 µl single digested DNA, to monitor the efficiency of digestion. DNA fragments were visualised with a UV luminometer.

A 3 PREPARATION OF INSERT AND VECTOR DNA FOR SUBCLONING

DNA was digested with the appropriate restriction enzymes to generate either vector or insert DNA with compatible sticky ends. These fragments were separated by gel electrophoresis in agarose gels and the appropriate bands of DNA were excised from the gel and extracted from the agarose with GenElute agarose spin columns (Sigma-Aldrich Chemie GmbH, Germany), according to the manufacturers' instructions. The flow-through was precipitated with 0.1 volumes 7.5 M ammonium acetate and 2.5 volumes absolute alcohol and left at -20°C overnight. The DNA was pelleted at 12 000×g for 30 minutes, the

supernatant discarded, and the pellets washed with 70 % ethanol to remove excess salt and spun again. The pellets were air dried and resuspended in 1-10 μ l TE, depending on the size of the pellet. After extraction, 1 μ l of insert and vector DNA was visualised by gel electrophoresis with DNA of known concentrations to estimate the amount of DNA isolated.

A 4 DNA LIGATIONS AND TRANSFORMATION

Vector and insert DNA were ligated in a 20 μ l cocktail containing 1 Unit T4 DNA ligase (Promega), 1 \times ligase buffer and the appropriate concentrations of DNA at a molar ratio of 4:1 insert to vector DNA. The ligation reaction occurred at room temperature for 2-3 hours.

A modification of the method described by (Hanahan, 1983) was used to prepare competent *E. coli* XL1-blue cells. This included the use of enriched solutions that contain RbCl and MOPS, which seem to increase the efficiency of DNA uptake by the cells. 5 ml of overnight culture was inoculated into 100 ml LB broth and grown at 37°C until cell growth had reached logarithmic phase ($OD_{600}=0.35$). Cells were chilled on ice for 15 minutes and pelleted by centrifugation at 4000 $\times g$ for 5 minutes at 4°C. The pellet was resuspended in 21 ml TFB1 (100 mM RbCl, 50 mM MnCl₂, 30 mM KOAc, 10 mM CaCl₂, 15 % glycerol) and incubated on ice for 90 minutes. Cells were harvested by centrifugation at 4000 $\times g$ for 5 minutes at 4°C and resuspended in TFB2 (10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂, 15 % glycerol). 100 μ l aliquots of cells were flash frozen with liquid nitrogen and stored at -70°C.

5 μ l of each ligation reaction was transformed into 100 μ l competent *E. coli* XL-Blue cells and incubated on ice for 30 minutes. DNA uptake occurred by heat shock at 42°C for 50 seconds and cooling on ice for 2 minutes. Amplification of DNA occurred after addition of 900 μ l LB broth for 1 hour at 37°C. Cells were pelleted by centrifugation and resuspended in 100 μ l fresh LB broth and plated on LB agar plates containing 50 μ g/ml ampicillin and incubated at 37°C overnight.

A 5 OLIGONUCLEOTIDES USED FOR SITE-DIRECTED MUTAGENESIS AND SEQUENCING

Primers and oligonucleotides used for site-directed mutagenesis and sequencing were designed using the Primer Designer for Windows software package (version 2.0) from Scientific and Educational Software. They were purchased from Integrated DNA Technologies, Inc. (USA) through Whitehead Scientific (SA).

Sequencing reactions were primed from sites within vector DNA in both orientations with a pair of universal sequencing primers

Forward primers:

T7 (22-mer): 5'-GTAATACGACTCACTATAGGGC-3'
 M13-F (24-mer): 5'-CGCCAGGGTTTTCCAGTCACGAC-3'

Reverse primers:

T3 (20-mer): 5'-AATTAACCCTCACTAAAGGG-3'
 SP6 (19-mer): 5'-TATTTAGGTGACACTATAG-3'
 M13-R (22-mer): 5'-TCACACAGGAAACAGCTATGAC-3'

These oligonucleotides were also used in PCR-based mutagenesis as outer primers.

For non-PCR based site-directed mutagenesis, the selection oligonucleotide used was provided with the GeneEditor *In Vitro* Site-Directed Mutagenesis kit. The bottom selection oligonucleotide was used in every mutagenesis reaction (mutations of the β -lactamase gene sequence are highlighted in bold):

5'-CCGCGAGACCCACCCTTGGAGGCTCCAGATTTATC-3' (35-mer)

The mutagenic oligonucleotides encode the Asn to Gln transition (underlined) at the glycosylation sites and a silent mutation that alters a restriction enzyme recognition site (highlighted in italics). Mutated residues are indicated by bold type:

Forward primers:	Restriction site:
g1-F (40-mer): 5'-CGAGGCCAAATTGGAACTACAACACCC <u>CAG</u> ATCACCACAGAG-3'	+ <i>MunI</i>
g2-F (36-mer): 5'-ATGCAAATAGCCC <u>CAG</u> CACACCCTTAAGTACGGCACC-3'	+ <i>AflII</i>
g3-F (40-mer): 5'-GAAGTTTGATG <u>TTA</u> ACCAGTTGCAG <u>CAG</u> ACCACTATCAAG-3'	+ <i>HpaI</i>
g4-F (30-mer): 5'-GTGTGCCACCCG <u>CA</u> AGGTAGCTGCCTGCAG-3'	- <i>PvuII</i>
g5-F (36-mer): 5'-CCGTGCCTCCTGAA <u>TTCTGG</u> CAGAAGTCGATGCTGG-3'	+ <i>EcoRI</i>
g6-F (36-mer): 5'-ACGGGCCAGCCC <u>CAG</u> ATGAGCGCTTCGGCC-3'	+ <i>Eco47III</i>

Reverse primers are complementary to the forward primers:

g1-R (40-mer): 5'-CTCTGTGGT**GATCT**GGGTGTTGTAGTTCCAATTGGCCTCG-3'
 g2-R (36-mer): 5'-GGTGCCGTACTTAAGGGTGTG**CTG**GGCTATTGCAT-3'
 g3-R (40-mer): 5'-CTTGATAGTGGT**CTG**CTGCAACTGGTTAACATCAAACCTC-3'

A 6 IN-VITRO SITE-DIRECTED MUTAGENESIS OF THE GLYCOSYLATION SITES

Site-directed mutagenesis was performed according to the methods of the Gene Editor *In-Vitro* Site-Directed Mutagenesis System (Promega). Each mutagenic oligonucleotide was phosphorylated and annealed to alkaline denatured template DNA at 56°C. Second strand synthesis was performed with T4 DNA polymerase and the samples were transformed into *E. coli* BMH71-18 *mutS* competent cells. DNA was prepared from the transformants according to the alkaline lysis method described. Samples were visualised in 1 % (w/v) agarose gels to estimate the concentration of the DNA. Approximately 10 ng of DNA was used to transform *E. coli* JM109 competent cells and these cells were plated on LB agar plates containing 5 µl/ml Antibiotic Selection Mix (Promega). DNA minipreps, from colonies grown at 37°C overnight, were screened for mutants by digestion with the appropriate restriction enzyme to detect the loss or gain of that site compared to the corresponding wild-type tACE fragment in pGEM.

A 7 PCR

A 7.1 PCR on a Gradient Block to Determine the Optimal Annealing Temperature of Mutagenic Oligonucleotides

DNA was amplified with the relevant mutagenic oligonucleotide (g1-F, g2-F, g3-F, g4-F, g5-F or g6-F) and M13-R. Ten 50 µl reaction cocktails of:

1×	Reaction buffer (with 1.5 mM MgCl ₂)
200 µM	each dNTP
1 µM	Mutagenic oligonucleotide
1 µM	M13-R
0.5 µg	Template DNA (FR1, FR2, FR3 or FR4)
2.5 units	<i>Taq</i> polymerase (Roche)
in dH ₂ O	

were amplified in a thermocycler (Hybaid Express, Hybaid Ltd, UK) on a gradient block, with a temperature range from 50°C to 65°C. The reaction proceeded as:

95°C for 3 minutes – template denaturing step
30 cycles of:
50°C-65°C for 30 seconds – primer annealing step
72°C for 30 seconds – extending step
95°C for 1 minute – denaturing step
followed by:
72°C for 10 minutes – final extending step

The products were separated by gel electrophoresis on a 1.5 % (w/v) agarose gel.

A 7.2 Megaprimer PCR-Based Site-Directed Mutagenesis

The megaprimer was amplified in a 50 µl cocktail of:

1× Reaction buffer (with 2 mM MgSO₄)
200 µM each dNTP
1 µM Forward primer
1 µM Reverse primer
0.5 ng DNA
1 Unit *Pfu* DNA polymerase (Promega)
in dH₂O

was amplified on a thermocycler (Hybaid Sprint) using this profile:

95°C for 2 minutes
30 cycles of:
55°C for 30 seconds
70°C for 30 seconds
95° C for 1 minute
followed by:
70°C for 10 minutes

The product was separated by electrophoresis on a 1 % (w/v) agarose gel, excised and eluted and resuspended in 10 μ l of dH₂O. This DNA was used to prime a second round of amplification with a flanking primer to generate FR1 containing the desired mutation.

A 50 μ l cocktail containing:

1 \times Reaction buffer (with 2 mM MgSO₄)

200 μ M each dNTP

1 μ M Flanking primer

5 μ l Megaprimer

0.5 ng DNA

1 Unit *Pfu* DNA polymerase (Promega)

in dH₂O

was amplified on a thermocycler using this profile:

95°C for 2 minutes

30 cycles of:

45°C for 30 seconds

70°C for 30 seconds

95° C for 1 minute

followed by:

70°C for 10 minutes

The product was separated by electrophoresis on a 1 % (w/v) agarose gel, excised and eluted and resuspended in 10 μ l of dH₂O. DNA was digested and cloned into a suitable vector for sequencing.

A 7.3 Overlap-Extension PCR Site-Directed Mutagenesis

Two overlapping products amplified from pGEM-FR1 were annealed and amplified to generate the entire sequence of FR1 containing the desired mutants. The overlapping fragments comprised the upstream and downstream regions of FR1 primed with the mutagenic oligonucleotides. The upstream was amplified in a 50 μ l cocktail of:

1× Reaction buffer (with 2 mM MgSO₄)
200 μM each dNTP
1 μM M13-F
1 μM g1-R, g2-R or g3-R
1 Unit *Pfu* DNA Polymerase (Promega)

The downstream region was amplified in a 50 μl reaction cocktail containing:

1× Reaction buffer (with 2 mM MgSO₄)
200 μM each dNTP
1 μM M13-R or SP6
1 μM g1-F, g2-F or g3-F
1 Unit *Pfu* DNA Polymerase (Promega)

The reaction profile for both reactions followed:

95°C for 2 minutes
30 cycles of:
56°C for 30 seconds
72°C for 30 seconds
95°C for 1 minute
and: 72°C for 10 minutes

PCR products were separated by gel electrophoresis in a 1 % (w/v) agarose gel, excised, eluted and resuspended in 10 μl dH₂O. Overlapping fragments were mixed in a 50 μl cocktail to amplify FR1 containing the desired mutants:

1× Reaction buffer (with 2 mM MgSO₄)
200 μM each dNTP
1 μM M13-F
1 μM M13-R, SP6 or T3
5 μl upstream product
5 μl downstream product
1 Unit *Pfu* DNA Polymerase

The reaction profile followed that of amplification of each overlapping fragment. PCR products were separated by gel electrophoresis in a 1 % (w/v) agarose gel, excised, eluted and resuspended in 10 μ l dH₂O. DNA was *Bam*HI/*Not*I digested and introduced into *Bam*HI/*Not*I digested pGEM or pBS.

A 8 DNA SEQUENCING OF GLYCOSYLATION MUTANTS

A 8.1 Automated Sequencing

DNA was cycle sequenced according to the chain termination method (Sanger et al, 1977), using the Thermo Sequenase Cy5 Dye Terminator Sequencing kit (Amersham Pharmacia Biotech) on a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems). Reactions were separated by polyacrylamide gel electrophoresis in 5 % gels, controlled by AFLwin 2.1 software with the ALFexpress DNA Automated Sequencer (AP Biotech).

A 8.2 Manual Sequencing

DNA was cycle sequenced according to the chain termination method (Sanger et al, 1977), using the Sequitherm EXCEL II DNA Sequencing kit (Epicentre Tech). DNA was amplified with a forward primer and a reverse primer, which flank the multiple cloning site of pGEM and pBS. For each oligonucleotide, four reaction tubes were prepared, containing template DNA, dNTP's, DNA polymerase, a subset of terminating dideoxynucleotides and α^{33} P[dATP]. These were amplified in a thermocycler (Hybaid Sprint) with the following profile:

95°C for 5 minutes

30 cycles of :

70°C for 1 minute

95°C for 30 seconds

ending with:

70°C for 10 minutes

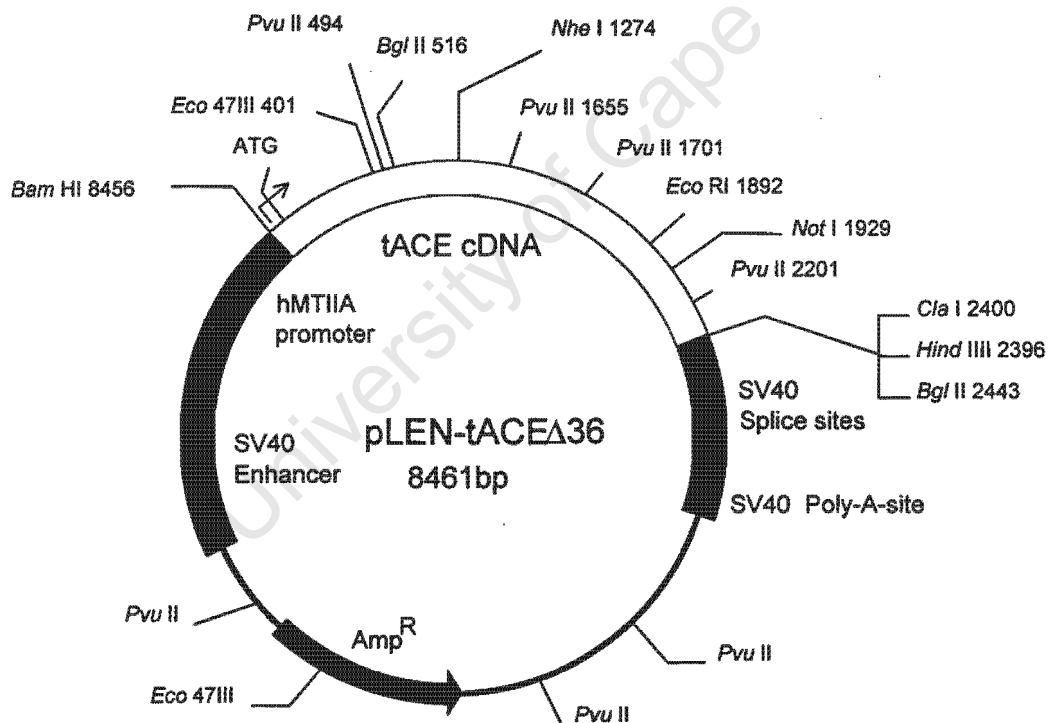
Samples were fractionated in a 6 % polyacrylamide gel in 1 \times TBE at 50 W. The gel was dried on blotting paper and exposed to X-ray film to detect radioactively labelled DNA fragments. The film was developed in an automated developer after 1 week exposure.

Human	MGQGWATAGLPSLLFLLLCYGHPLLVPVSOEAS SOQVTVTHGTSSQ ATTSSOTTTHQATAHQTSAQSPNL LVTDAAEASKFVEEYDR	84
Rabbit	-----ap-----l-----c-s-----rv-arr-nq-t-atttsk-----ira-----t-----r-----	89
Human	TSQVVWNEYAEANWNYNTN 72 ITTETS 108 KILLQKNMQIAN 90 HTLKYGTQARKFDVNQLQNT 109 TIKRIKKVQDLERAALPAQELEEYNKILLDME	174
Rabbit	sf-a-----a-----t-nw-r-snf-a-s-----q-v-vk-----q-----	179
Human	TTYSVATVCH 155 ENG 155 SCLOLEPDLTNVMATSRKYEDLLWAWEGWRDKAGRAILOFYPKYVELINQAARLNGYVDAGDSWRSMYETPSLEQDL	264
Rabbit	-i-----n-rv d -----l-----de-v-ts-v-----pyf-----ft-k-----t-----	269
Human	ERLFQELQPLYLNLHAYVRRALHRHYGAQHINLEGPIPAHLLGNMWAQTWSNIYDLVVPFSPAPSMDTTEAMLKQGWTPRRMFKEADDF	354
Rabbit	-----g-----a-----st-a-i-----e-k-----	359
Human	TSLGLLPVPPEFW 337 NKSMLEKPTDGREVVCHASAWDFYNGKDFRIKQCTTVNLEDLVVAH HEM GHIQYFMQYKDLPVALREGANPGFHEAI	444
Rabbit	i-----m-----v-----	449
Human	GDVLALSVS 373 TPKHLHSLNLLSSEGGSD EH DINFLMKMALDKIAFIPFSYLVDQWRWRVFDGSITKENYNQEWWSLRLKYQGLCPPVPRTO	534
Rabbit	-----i-----gy-----e-----a-s-----	539
Human	GDFDPGAKFHIPSSVPYIRYFVFSFIIQFQFHEALCQAAGHTGPLHKCDIYQSKEAGQRLATAMKLGFSRPWPEAMQLITGQ 586 ENMSASAML	624
Rabbit	-----k-----t-----k-d-y-k-kv----- 622 -----m-----	629
Human	SYFKPLLDWLRTENELHGEKLGWPOYN 620 WTTPNSARSEGPLPDSGRVSVFLGLDLDAQARVGO WLLFLGLIALLVATLGLS QRLFSIRHRSL	714
Rabbit	n-----m-----l-----gr----- t -----s-----n-mn-----v-----v-----l-s-----t-----yq-----	719
Human	HRHSHGPQFGSEVELRHS	732
Rabbit	rqph-----	737

Figure A1. Comparison of the amino acid sequence human and rabbit testis ACE. The amino acid sequence homology of wild-type human and rabbit tACE is shown. Residues identical to the human sequence are shown as a (-), changes are indicated in small caps. The $\Delta 36$ deletion and transmembrane region of human tACE are highlighted and the active site is indicated in bold type. The glycosylation recognition sequons are boxed. The sites are numbered according to Yu (1997) for human tACE and Sadhukhan (1996) for rabbit tACE.

A 9 pLEN-tACE- Δ 36 RESTRICTION MAP


This mammalian expression vector is derived from tACE cDNA and pJ4 Ω cloned into pLEN (Ehlers, 1989). It contains the simian virus 40 enhancer element, SV40 splice donor and acceptor sites and the SV40 tumour antigen poly-adenylation signal, allowing for integration of tACE into the host genome. The hMTIIA promoter activates transcription of tACE cDNA. Restriction sites used for construction and identification of human tACE glycosylation mutants are indicated. Because complete sequence data was not available, the position of sites lying outside tACE cDNA were estimated from fragmentation of digested DNA after gel electrophoresis.



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