MEMBRANE PEPTIDASE EXPRESSION IN MAMMALIAN CELL LINES

By

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others

PULLOUT

ABSTRACT

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide which is produced by the cleavage of the inactive precursor, big ET-1 at the Trp^{21}-Val^{22} bond by endothelin converting enzyme (ECE). ECE has been found to be expressed by a variety of cell types and tissues.

Using the human endothelial cell line, EA.hy926, ECE has been demonstrated, by analogy to the related protein, E-24.11 to be an integral transmembrane protein which is not anchored by a G-PI moiety. The relationship between E-24.11 and ECE was further investigated by screening of antibodies raised to E-24.11 for cross-reactivity with the ECE protein. Immobilised GK4A9, a monoclonal antibody to E-24.11 was found to immunoprecipitate >80 % of the total E-24.11 and ECE activity from Triton X-100 solubilised membrane preparations of EA.hy926 cells. The immunoprecipitation was reproduced using a transfected cell line which expressed high levels of ECE activity but was shown not to express E-24.11. Immunoprecipitation of ECE from transfected cells was confirmed by enzyme assay and immunoblotting. The immunoreactivity of GK4A9 was shown to be specific for ECE and E-24.11 suggesting a shared epitope between the two proteins. These data were consistent with the hypothesis that E-24.11 and ECE originated from the same protein family, sharing significant structural and sequence homology.

The cell specific expression of ECE was demonstrated by radioimmunoassay in the neuroblastoma cell line, SH-SY5Y, and the Bu17, C6 and D384 glioma lines. The identity of the activity was confirmed by immunoblotting which revealed a polypeptide of approximately 120 kDa in each of these cell lines.

Pre-treatment of EA.hy926, Bu17 and SH-SY5Y cells with the zinc metalloprotease inhibitor phosphoramidon (PR) resulted in an accumulation of the ECE protein. This increase was reproduced by treatment of cells with thiorphan and suggests the possible involvement of a novel PR and thiorphan-sensitive metalloprotease in the turnover of ECE.

Other possible regulatory mechanisms involved in ECE production were investigated. The protein tyrosine phosphatase inhibitor vanadate was found to cause a 2-3-fold up-regulation of ECE in EA.hy926 and Bu17 cells and appeared to induce ECE expression in rat pheochromocytoma PC12 cells which had been stimulated to differentiate to a neuronal phenotype in response to vanadate treatment.

The identification of two distinct human isoforms of ECE-1, α and β (Schmidt *et al.*, 1994; Shimada *et al.*, 1995) which were found to differ only in N-terminal amino acid sequence, allowed the generation of antibodies specific for each isoform. Peptides corresponding to immunogenic regions of the N-terminal sequence in each isoform were synthesised, attached to a carrier protein and used to raise anti-peptide antibodies. The immunoreactivity of the antiserum was monitored using an E.L.I.S.A. with the peptide as the antigen. The most immunoreactive antiserum was affinity purified and the antibody characterised by detection of each ECE-1 isoform using transfected cell lines expressing high levels of each isoform. One antibody (ECE1a 1 was successfully raised to ECE-1 α and found to be specific for this isoform.

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ABBREVIATIONS

The abbreviations used are as defined in *Biochem. J.* (1996) **313,** 1-15.
Other abbreviations are as follows:

ACE	- Angiotensin converting enzyme
AP-N	- Aminopeptidase-N
BCA	- Bicinchoninic acid
CAPS	- 3-[Cyclohexylamino]-1-propanesulphonic acid
CMC	- Critical micellar concentration
CTF	- C-terminal fragment
DCI	- 3,4-dichloroisocoumarin
Dip-F	- Di-isopropylfluorophosphate
DMSO	- Dimethylsulfoxide
DPP-IV	- Dipeptidyl-peptidase-IV
DTT	- Dithiothreitol
E-24.11	- Endopeptidase-24.11
E64	- L-trans-epoxysuccinyl-leucylamido-[4-guanido]-butane
ECE	- Endothelin converting enzyme
ECL	- Enhanced Chemiluminescence
EDCF	- Endothelial derived constrictor factor
EDRF	- Endothelial derived relaxing factor
EDTA	- Ethylenediamine tetraacetic acid
E.L.I.S.A.	- Enzyme-linked immunosorbent assay
ET	- Endothelin
FBHE	- Foetal bovine heart endothelial cells
G-PI	- Glycosyl-phosphatidylinositol
HPLC	- High performance liquid chromatography
HUVEC	- Human umbilical vein endothelial cell
kDa	- kiloDalton
KLH	- Keyhole limpet haemocyanin
LDH	- Lactate dehydrogenase

MBS	- 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester
NEM	- N-ethylmaleimide
PBS	- Phosphate Buffered Saline
PEG	- Polyethylene glycol
PLC	- Phospholipase C
PKC	- Protein kinase C
PR	- Phosphoramidon
PVDF	- Polyvinylidene difluoride
RIA	- Radioimmunoassay
SDS	- Sodium dodecyl sulphate
TBST	- Tris buffered saline containing 0.1 % (v/v) Tween-20
TEMED	- N,N,N'N'-tetramethylethylene diamine
TRLEC-03	- Simian virus 40-transformed rat lung vascular endothelial cells

Any remaining abbreviations used in this thesis have been clearly defined in the text.

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PUBLICATIONS

Extracts of this work have been reported in the following publications:

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Greenhough, K., Walkden, B., Murphy, L., McLaren, H., Barnes, K. & Turner, A.J. (1994) *Biochem. Soc. Trans.* **22,** 454S

CHAPTER ONE

INTRODUCTION

1.1 Location, function and diversity of peptidases

This work has investigated the expression of a range of membrane bound peptidases in a number of mammalian cell lines. Recent research into the biochemistry of peptidases focusing primarily on the identification of specific inhibitors of these has resulted in the possibility of new treatments for a number of conditions including hypertension (Burris, 1995), cancer and metastasis (DeClerck & Imren, 1994; Mikulewicz *et al.*, 1993); Alzheimer's disease (Ichai *et al.*, 1994) and inflammation (Jochum *et al.*, 1993). Inhibitors of angiotensin converting enzyme (EC 3.4.15.1; ACE; dipeptidyl peptidase A) have proved highly successful in the treatment and control of hypertension and congestive heart failure (for review see Remme, 1995). It is this potential for the treatment of human pathophysiological conditions that has established peptidase research as a rapidly growing branch of biochemistry.

Due to the limited sophistication of the techniques available at the time, the first peptidases to be isolated and characterised were those involved in the digestion of dietary proteins since these were relatively abundant and extracellular in location. These included examples such as the serine peptidases found in leukocytes and mast cells and peptidases of renal and brush border membranes including serine and metalloproteases. The physiological roles played by peptidases are both many and diverse including the processing of peptide hormones and enzyme precursors and also the deactivation of many biologically active peptides.

1.2 Nomenclature of peptidases

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology has defined peptidases as hydrolases (class 3) which cleave peptide bonds (subclass 3.4). Within this classification there are 2 general definitions referring to the type of cleavage shown by the enzyme. Exopeptidases cleave peptide bonds close to a free N or C terminus whilst in contrast endopeptidases preferentially hydrolyse internal peptide bonds since the active site of these proteins cannot accommodate a free N or C terminus.

Peptidases are classified according to one of four catalytic mechanisms (Hartley, 1960) based on the major catalytic amino acid residues involved: serine, cysteine and aspartic peptidases or the presence of an important metal ion (metalloproteases). The classification system of identification of catalytic mechanisms has been used to expand the carboxypeptidase class of peptidases into 3 sub-subclasses and endopeptidases into 4 sub-subclasses and endopeptidases of unknown catalytic mechanism into a fifth sub-subclass (EC. 3.4.99).

1.3 The mechanisms of peptidase association with membranes

1.3.1 Peripheral membrane proteins

Peripheral membrane proteins do not interact structurally with cellular membranes but associate with them by way of non-covalent interactions. They are often hydrophilic and can generally be dissociated from membranes by washing with buffers containing high salt concentrations or with sodium carbonate, pH 10.0.

1.3.2 Integral membrane proteins

An integral membrane protein is, by nature, an amphipathic molecule consisting of a hydrophilic, globular glycoprotein domain and, usually, a sequence of hydrophobic membrane spanning residues. Release of the protein can only result from the removal of the hydrophobic anchor or by shielding the anchor in a detergent micelle. Integral proteins are classified according to their orientation and the number of membrane spanning sequences. Type I integral membrane proteins are synthesised with an N-terminal signal sequence which is removed in the early stages of biosynthesis. The protein is retained in the membrane by the presence of a hydrophobic sequence of amino acids close to the C-terminus. In contrast, type II integral membrane proteins such as endopeptidase E-24.11 (neprilysin; EC 3.4.24.11) (Malfroy *et al.*, 1987; Devault *et al.*, 1987) have a signal peptide which remains intact throughout the process of biosynthesis eventually forming the hydrophobic membrane anchor. These proteins have a short hydrophilic, cytoplasmic N-terminal domain and an extracellular C-terminal domain. Proteins with multiple membrane spanning regions of hydrophobic amino acid residues are termed type III integral membrane proteins. The number of these sequences has been found to vary from 2 in subunit *c* of the F_o-ATPase complex up to 24 in the case of neuronal sodium channels.

1.4 Peptidases localised at the cell surface

Many mammalian enzymes are found bound to intracellular membranes where they serve a diverse range of functions. In addition, a second group of enzymes are found associated with the plasma membrane of the cell. To date, most of these enzymes have been characterised as ectoenzymes as defined by their orientation, with the bulk of the protein, including the catalytic site exposed at the extracellular surface.

The cell-surface peptidases identified to date associate with the membrane in one of two ways. This occurs either by way of a hydrophobic transmembrane anchor or by attachment to a glycosyl-phosphatidylinositol (G-PI) moiety. G-PI anchored proteins are attached to the G-PI structure at their C-termini and it is the G-PI structure which associates with the upper lipid leaflet of the membrane surface through fatty acids of the phospholipid component. Examples of G-PI anchored proteins include membrane dipeptidase (EC 3.4.13.19) (Hooper and Turner, 1988 a) and aminopeptidase P (EC 3.4.11.9) (Hooper *et al.*, 1987).

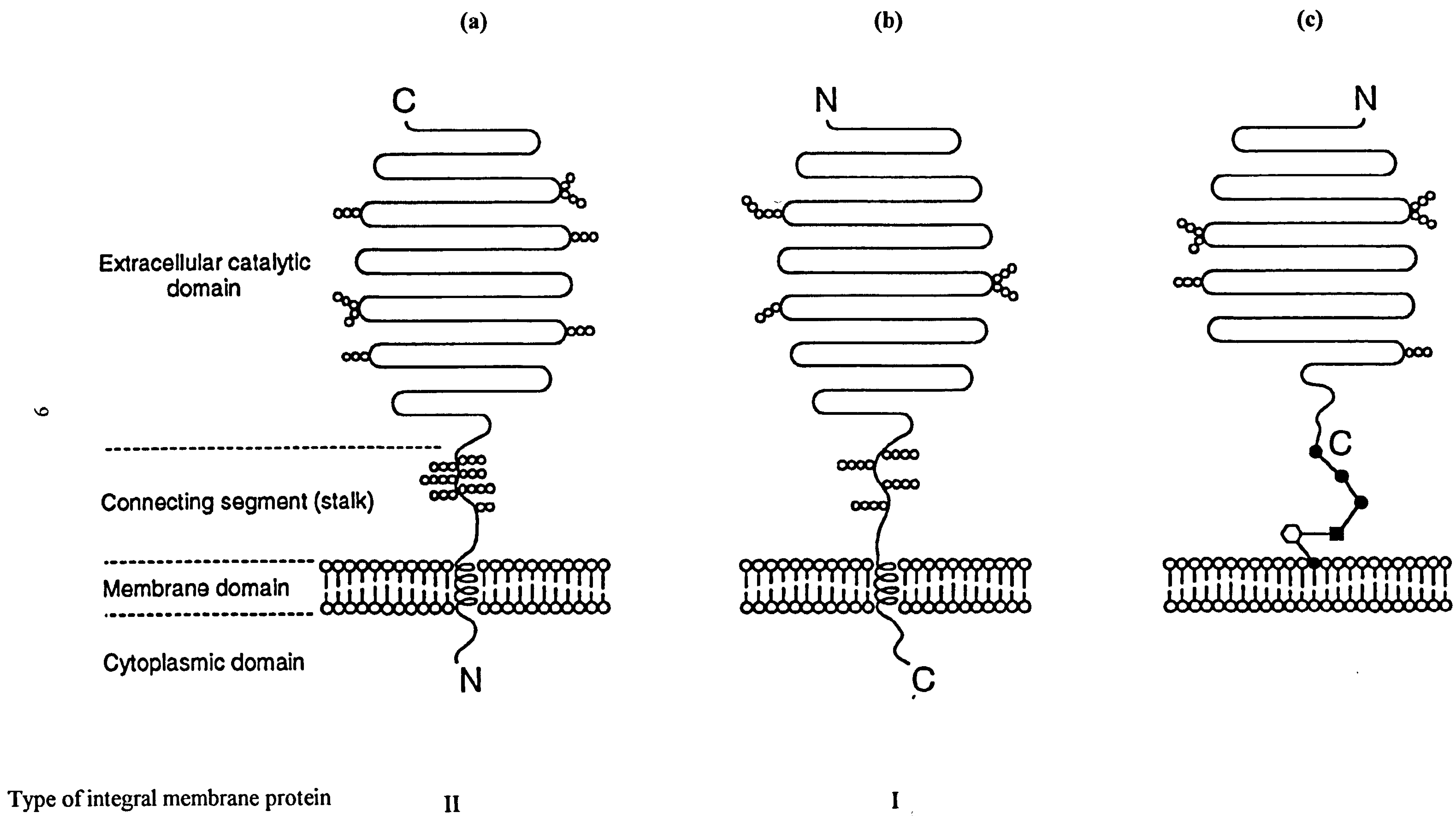
Peptidases which are G-PI anchored are generally identified by their release through the action of bacterial phospholipase C. A phospholipase C has been reported in rat liver membranes (Fox *et al.*, 1987) and a phospholipase D has been found in plasma (Davitz *et al.*, 1987; 1989). However, as yet the physiological significance of the endogenous release of G-PI anchored proteins by the action of phospholipases remains unclear.

Fig. 1.1 Membrane anchorage of cell surface hydrolases (see opposite)

Schematic representation of the mode of membrane attachment of cell surface hydrolases. (a) N-terminal transmembrane polypeptide anchor (uncleaved signal peptide) (e.g., endopeptidase-24.11, aminopeptidase-N and dipeptidyl peptidase-IV), (b) C-terminal transmembrane polypeptide anchor (e.g., angiotensin converting enzyme) and (c) C-terminal G-PI anchor (e.g., alkaline phosphatase, aminopeptidase P).

Predicted glycosylation sites are represented by °°° symbols.

(Modified from Kenny & Hooper, 1991).


(a)
(b)
(c)
C
N
N
Extracellular catalytic domain
Connecting segment (stalk)
Membrane domain
Cytoplasmic domain
N
C
C
Type of integral membrane protein
II
I

Cell surface peptidases are found in abundance in the brush border membranes of renal and intestinal tissues (Kenny *et al.,* 1987). The majority of these were first isolated from kidney microvillar membranes and have since been found to be metalloproteases though one significant exception is the serine protease dipeptidyl peptidase IV (DPP-IV). Cell-surface peptidases have been found in a wide range of tissues hydrolysing a vast array of substrates. The majority are not substrate specific but act together to metabolise a wide range of peptide substrates. The action of such cell-surface peptidases is crucial for hydrolysis of dietary constituents, hormones, nucleotides and neurotransmitters. The substrates are often oligopeptides with little secondary or tertiary structure. One such example is the identification of E-24.11 as the synaptic peptidase involved in hydrolysis of the opioid peptides (Relton *et al,* 1983; Matsas *et al.,* 1983) acting effectively as an "off" switch (Turner *et al.,* 1985).

1.4.1 Cell surface endopeptidases

The majority of cell surface peptidases identified to date have been shown to be exopeptidases however, one such cell-surface peptidase which has been thoroughly characterised and shown to be an endopeptidase is E-24.11.

E-24.11 was first purified and characterised from rabbit kidney microvillar membranes (Kerr and Kenny, 1974 a,b). Subsequently, E-24.11 was found to be located on the surfaces of cells throughout the mammalian body including intestine, lung and brain (Gee *et al.,* 1985). Whilst E-24.11 has a broad tissue distribution the enzyme is not ubiquitous and levels of tissue-specific expression vary substantially (Howell *et al.,* 1991; Gee *et al.,* 1985). It is has been found to be constitutively expressed in tissues such as brain and kidney but is developmentally regulated in other cell types (e.g. lymphocytes). Isolation of E-24.11 from different source tissues has shown the molecular weight to vary from 85 kDa to 100 kDa, the variations being attributable to differences in glycosylation (Relton *et al.,* 1983; Malfroy *et al.,* 1987). Molecular cloning of E-24.11 and subsequent sequencing of the cDNAs revealed rabbit (Devault *et al.,* 1987) and rat (Malfroy *et al.,* 1987) to consist of 750 and 742 amino acids respectively. A year later, Malfroy *et al.*, (1988) revealed human E-24.11 to have 742 amino acids, in common with the rat enzyme. Further differences in glycosylation were identified, the rabbit enzyme having 5 N-linked glycosylation sites compared with 6 in rat and human forms.

Structural characterisation of E-24.11 by biochemical analysis and study of the cDNA sequence revealed the enzyme to have a short N-terminal cytoplasmic domain (27 residues in rabbit; 20 in human and rat), a single hydrophobic membrane spanning sequence of 23 residues and a major extracellular C-terminal domain (Devault *et al.*, 1987; Malfroy *et al.*, 1987; Malfroy *et al.*, 1988). The topology of E-24.11 has been shown to be typical of a type II transmembrane anchored ectoenzyme which exists in its native form in rat and human as a non-covalently attached dimer (Kerr and Kenny, 1974 b; Fulcher & Kenny, 1983). Of the 12 cysteine residues found in the whole protein, 4 are found clustered within a 32 amino acid sequence on the C-terminal side immediately following the transmembrane anchor. Examination of the C-terminal sequence revealed an HExxH motif consistent with many zinc binding proteins, and accordingly E-24.11 is inhibited by metal chelating agents such as EDTA and 1,10-phenanthroline. E-24.11 is also inhibitable by phosphoramidon (PR), originally described as a potent inhibitor of bacterial thermolysin (Suda *et al.*, 1973) and subsequently found to be an equally potent (Ki = 2 nM) inhibitor of E-24.11 (Kenny, 1977). PR is not however a general inhibitor of zinc peptidases and has been shown to be ineffective against ACE or meprin.

E-24.11 was found to hydrolyse a broad range of substrates on the N-terminal side of hydrophobic residues. In this respect, peptides considered to be possible physiological substrates for E-24.11 are thought to include natriuretic peptides, tachykinins including substance P and chemotactic peptides such as fMet-Leu-Phe (for review see Turner & Tanzawa, 1997). The sensitivity of E-24.11 to PR was to prove crucial in the identification of E-24.11 as the synaptic peptidase involved in cleavage of the opioid peptides at the membranes of the synaptic cleft (Relton *et al.*, 1983; Matsas *et al.*, 1983) and further research has shown E-24.11 to play a variety of physiological roles cleaving other neuropeptides and regulatory peptides involved in inflammation and cardiovascular regulation.

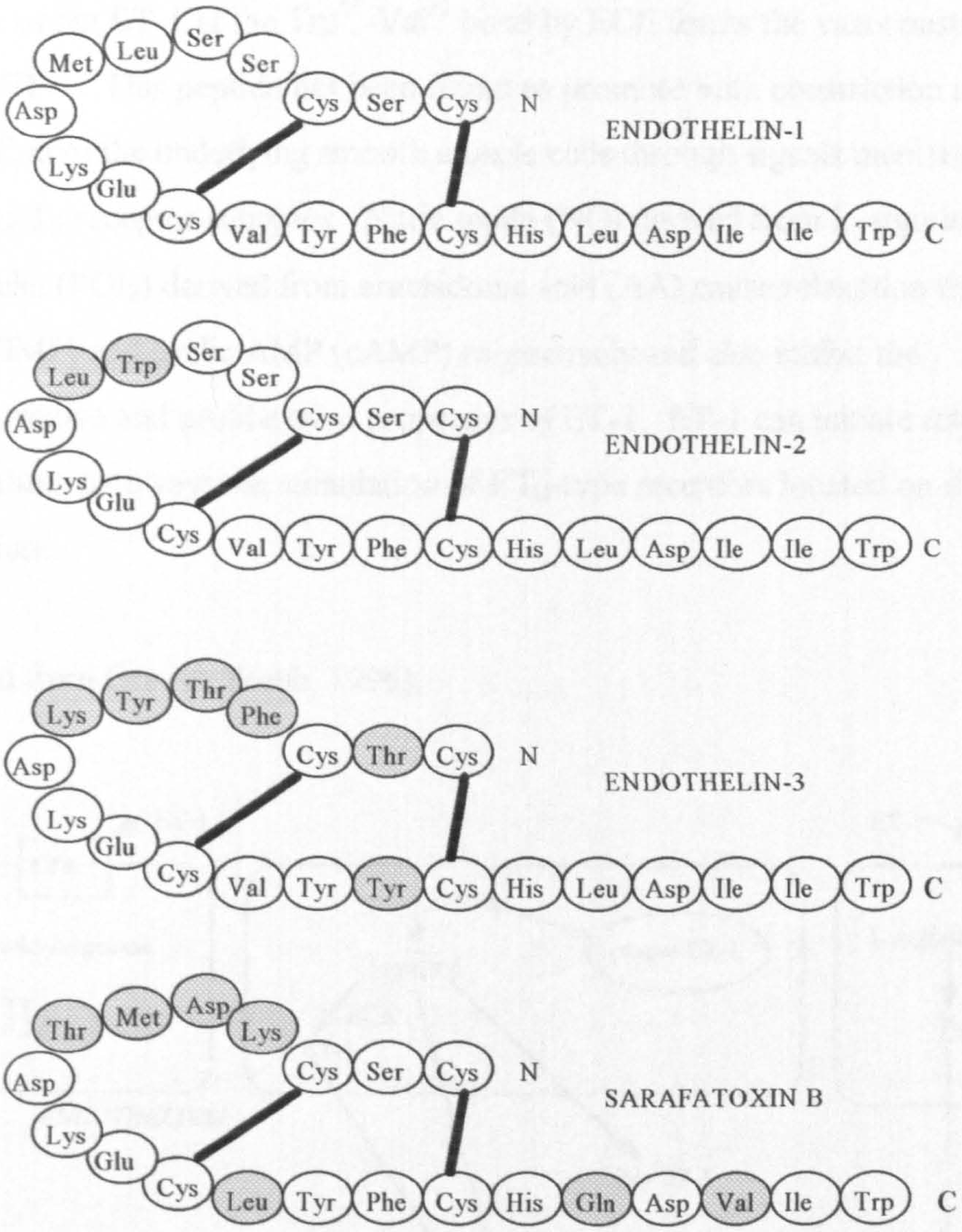
1.5 The biology of endothelial cells (EC)

The endothelial cell monolayer lines the inner surface of all blood vessels and forms a structurally and functionally heterogeneous population of cells occupying a surface area of > 1000 m^2 in a 70 kg adult (Jaffe, 1987). The endothelium was originally thought to be a passive, blood-compatible lining functioning as a diffusion barrier but is now regarded as crucial to maintaining vascular homeostasis (Vane *et al.*, 1990). Amongst the functions served by the endothelium are coagulation, thrombolysis, antigen presentation and control of vasotone, accomplished by locally acting endothelium-derived hormones. EC dysfunction is now recognised as a major causative event in the pathophysiology of cardiovascular disease (Luscher, 1992). The first indication that the endothelium played a role in the control of vasotone was the discovery of prostaglandin (PGI_2) (Moncada *et al.*, 1976) which was first described as an inhibitor of platelet aggregation but later was shown to act as a potent vasodilator. The endothelium was later shown to mediate vascular smooth muscle relaxation in response to acetylcholine (Furchgott & Zawadzki, 1980) and shown to produce other vasoactive agents including endothelium-derived constricting factors (EDCF) (De Mey & Vanhoutte, 1983; Hickey *et al.*, 1985; Gillespie *et al.*, 1986; O'Brien *et al.*, 1987) and nitric oxide which was found to be a potent vasodilator (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). The discovery of these vasoactive factors, apparently crucial to cardiovascular regulation gave hope that EC, which are advantageously situated to communicate chemical signals in the blood to the vascular smooth muscle cells, could be targeted for the development of novel therapeutic compounds with the aim of treating pathophysiological conditions. Fig. 1.3

1.6 The endothelin isopeptides

In 1988, Yanagisawa *et al.*, working on the supernatant of porcine aortic endothelial cells, isolated, cloned and sequenced one EDCF as a 21 amino acid bicyclic peptide with 2 intramolecular disulphide bonds. The peptide was named endothelin and had an

Fig. 1.2 The endothelin isopeptides and sarafatoxin b

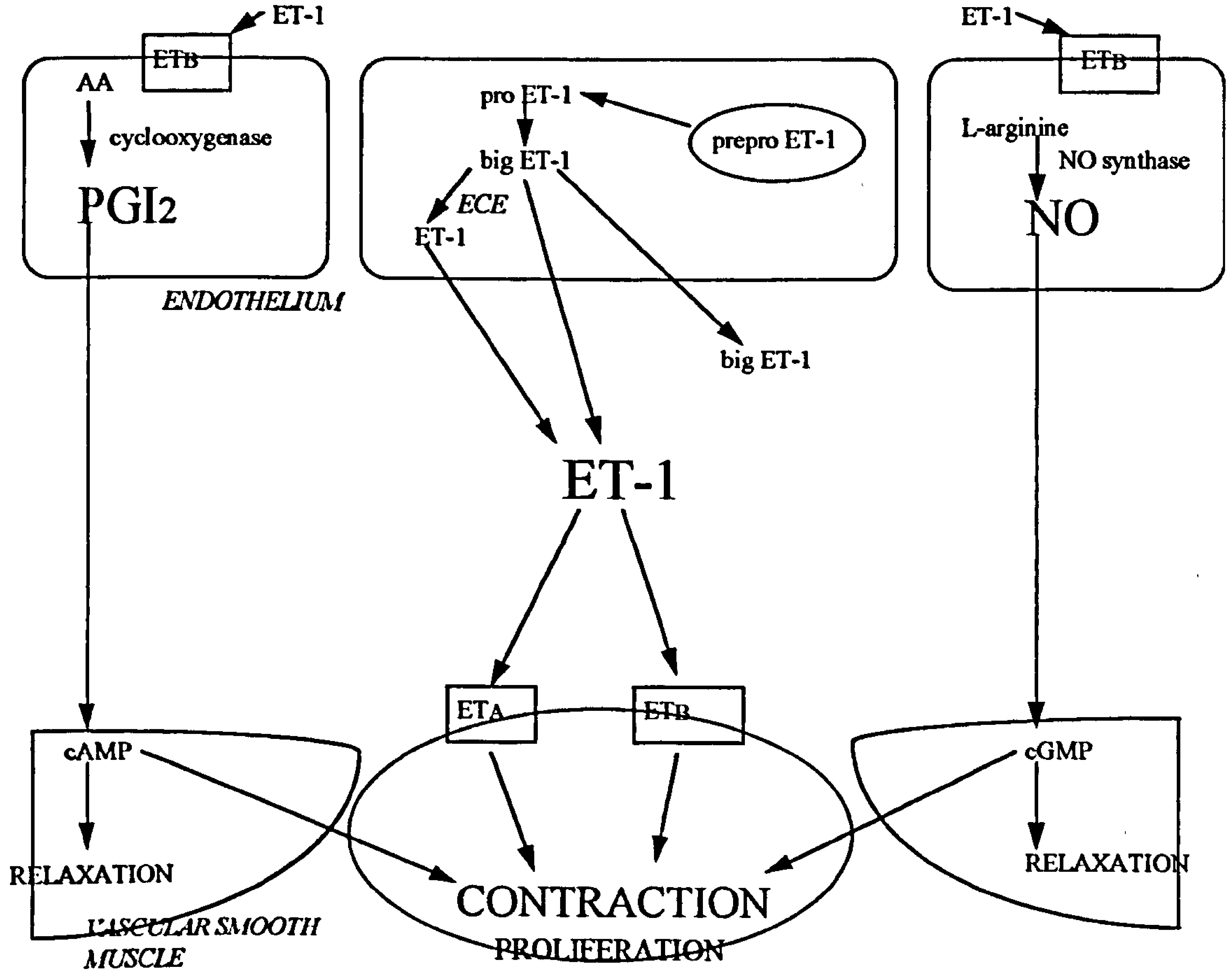


Three distinct endothelin isopeptides have been discovered, each the product of a separate gene (Inoue *et al.*, 1989). The ET isopeptide sequences and structure revealed significant homology to sarafatoxin b, the snake venom toxin produced by *A. engaddensis*. All of these peptides are bicyclic with 2 disulphide peptide bonds (Cys^1 - Cys^{15} and Cys^3 - Cys^{11}), a cluster of polar, charged side-chains within the hairpin loop and a predominantly hydrophobic C-terminus. The shaded residues in the figure highlight the amino acids which differ from the corresponding amino acid in the ET-1 sequence.

Fig. 1.3 Endothelium-derived factors involved in the regulation of smooth muscle tone

Cleavage of big ET-1 at the Trp^{21}-Val^{22} bond by ECE forms the vasoconstrictive peptide, ET-1. This peptide has been found to promote both constriction and proliferation of the underlying smooth muscle cells through signals mediated by both ET_A and ET_B receptor subtypes. Nitric oxide (NO) derived from L-arginine and prostacyclin (PGI_2) derived from arachidonic acid (AA) cause relaxation through cyclic GMP (cGMP) and cyclic AMP (cAMP) respectively and also inhibit the vasoconstrictive and proliferative properties of ET-1. ET-1 can initiate relaxation through these pathways via stimulation of ET_B-type receptors located on the vascular endothelium.

(Modified from Gray & Webb, 1996).



development, Kurihara *et al.*, (1994) found ET-1 $^{+/-}$ heterozygotes which, due to their impaired production of endothelin, would be expected to have lower blood pressure, were actually found to have a transient increase compared with the ET-1 $^{+/+}$ wild type, challenging the long held view that endothelin functioned only as a vasoconstrictor in the physiological state. Another approach involving targeted and natural (piebald-lethal) mutations of the endothelin-B receptor gene. Hosoda *et al.* (1994) and Puffenberger *et al.* (1994) revealed homozygous mutants deficient in endothelin-B receptors exhibited a phenotype reminiscent of that found in patients with Hirschprung's disease. In addition to exhibiting megacolon development, typical of Hirschprung's disease, these mice exhibited coat colour abnormalities of both myenteric ganglion neurones and epidermal melanocytes; two tissue types derived from the neural crest. Targeted mutations of the endothelin-3 gene resulted in a similar, though milder phenotype suggesting endothelin-3 is the natural ligand for the endothelin-B receptor during development (Baynash *et al.*,1994). Two naturally occurring recessive mutant mice (piebald-lethal and lethal spotting) were found to display the same phenotype to that exhibited by mice deficient in endothelin-3 and endothelin-B receptor. Genetic analysis of the two naturally occurring mutants revealed piebald-lethal mutants to have a deletion of the entire endothelin-B receptor gene whilst the lethal spotting mice had a point mutation in the endothelin-3 gene sequence. The physiological significance of these findings was shown by genetic analysis of the Mennonite religious sect in the U.S.A., a highly inbred population with a relatively high occurrence of hereditary Hirschprung's disease. The analysis revealed this population to express a mis-sense mutation in the endothelin-B gene and in addition, defects in pigmentation were often found in the Mennonites, a feature found consistently in the mice mutants. This evidence for a role in tissue development added to a growing list of disease models in which endothelin and its biosynthesis was thought to play a major role (see Table 1.1).

Since the production of endothelin is regulated at the mRNA level and secreted through the constitutive pathway, no further regulation is thought to occur during exocytosis. It is therefore essential that the production of this potent vasoactive

Table 1.1 Disease models in which ET production has been implicated as a possible causative factor

Disease	References
Systemic hypertension	Clozel *et al.*, 1993 Stein *et al.*, 1994 Ohlstein *et al.*, 1994
Ischaemic neuronal cell death	Ohlstein *et al.*, 1994
Hirschprung's disease	Puffenberger *et al.*, 1994 Hosoda *et al.*, 1994
Human endometrial cancer	Pekonen *et al.*, 1995
Acute myocardial infarction	Grover *et al.*, 1993
Progressive proliferative glomerulonephropathy	Benigni *et al.*, 1993
Cerebrovascular spasm	Clozel *et al.*, 1993 Itoh *et al.*, 1993
Craniofacial development abnormalities	Kurihara *et al.*, 1994
Prostate Cancer	Nelson *et al.*, 1995
Human coronary atherosclerosis	Bacon *et al.*, 1996

peptide be tightly regulated and it is the mode by which this process is controlled that has become a major focus for research.

1.6.2 Endothelin biosynthesis

Endothelins are synthesised constitutively by smooth muscle or vascular endothelial cells and function in the manner of a locally acting hormone secreted in a paracrine fashion (Kennedy *et al.*, 1993). Like many other biologically active peptides, endothelin is initially synthesised in the form of a much larger, inactive precursor molecule, termed pre-pro endothelin which is approximately 200 amino acids long (Yanagisawa *et al.*, 1988). Big ET was proposed to be a 38 or 39 residue processing intermediate resulting from cleavage of the prepro-peptide early in the biosynthetic pathway. The resultant pro-peptide is then cleaved at pairs of basic amino acids by a dibasic-pair specific endopeptidase to liberate big ET-1. The vasoconstrictive potency of big ET-1 is 100-fold less than that of correctly processed mature endothelin (Kashiwabara *et al.*, 1989, Kimura *et al.*, 1989), indicating processing of big ET-1 is necessary to induce the potent vasoactive effects of this system. The activation of many biologically active proteins and almost all peptide hormones occurs following endoproteolysis which is catalysed by cleavage at the carboxyl side of an evolutionary conserved site, typically a doublet or cluster of basic amino acids (usually Arg-Arg or Lys-Arg) (see Sossin *et al.*, 1989 for review). In the case of ET production, current opinion suggests that the cleavage of the pro-peptide is carried out by furin, a prohormone convertase of the constitutive secretory pathway (Denault *et al.*, 1995; Kido *et al.*, 1997) followed by trimming by exopeptidases. The processing step which processes big ET-1 to ET-1 and the C-terminal fragment (CTF) occurs by what is thought to be a unique step in peptide hormone processing involving cleavage of the Trp^{21}-Val^{22} bond by what is now termed endothelin converting enzyme (processing of big ET-2 to ET-2 involves cleavage of the Trp^{21}-Val^{22} bond whilst cleavage of big ET-3 occurs at the Trp^{21}-Ile^{22} bond). This hypothesis concerning endothelin production gained support when the substrate and end products of the reaction (big ET-1; ET-1 and CTF) were all detected in the culture supernatant of porcine and bovine endothelial cells (Emori *et al.*, 1989, Sawamura *et al.*, 1989 and Matsumura *et al.*, 1990 a). A further clue as to the nature of the putative ECE activity was found when it was shown that phosphoramidon, an inhibitor of certain metalloproteases, suppressed secretion of ET-1 and CTF from cultured endothelial cells (Ikegawa *et al.*, 1990 a). At this point in time it was still undetermined if the processing of big ET-1 occurred at the cell surface or was processed intracellularly. However, exogenously applied big ET-1 was rapidly converted to ET-1 and CTF consistent with a cell surface location for the processing step.

1.6.3 Endothelin receptors and signal transduction

Following the discovery of three distinct ET gene products it was suggested that each of these may exert their physiological effects through multiple receptor subtypes. The first indications that more than one receptor type existed came from the results of studies of the potency and activities of the three naturally occurring ET peptides (Inoue *et al.*, 1989; Warner *et al.*, 1989 a,b). This was supported by receptor binding assays suggested the expression of receptor subtypes in a variety of tissues (Watanabe *et al.*, 1989; Masuda *et al.*, 1989). Identification of these receptor subtypes was established with the cloning and expression of two different receptors designated ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990). Both were found to be G-protein coupled receptors with 7 transmembrane domains belonging to the rhodopsin type superfamily of receptors. ET_A was found to have much greater affinity for ET-1 exhibiting an affinity profile of ET-1 $\geq$ ET-2 >ET-3 (Arai *et al.*, 1990). In contrast the ET_B receptor was found to have equal affinity for all 3 ET peptides (Sakurai *et al.*, 1990). The development of novel receptor agonists and antagonists provided further understanding as to the role of each receptor subtype and was also instrumental in providing evidence for the existence of yet further ET receptor subtypes. Initial studies suggested ET-1 preferring receptors were located on vascular smooth muscle and responsible for the vasoconstrictive effects whilst ET isoform non-selective receptors were located in endothelial cells. Further work showed this concept to be flawed, for example ET-3 proved to be a less potent vasoconstrictive agent in guinea pig pulmonary artery whilst studies using the trachea revealed all 3 isopeptides were equipotent in promoting constriction (Cardell *et al.*, 1992). These data suggested different ET receptors were responsible for the mediation of the contractile activity of ET peptides in these tissues. It has now been shown that both ET_A and ET_B receptors mediate smooth muscle contraction whilst only ET_B receptors localised on endothelial cells mediate nitric oxide release resulting in endothelium-derived vasodilation (Sumner *et al.*, 1992; Clozel *et al.*, 1992; Warner *et al.*, 1993 a).

More recent work has suggested receptor differences within the ET_B receptor classification (Warner *et al.*, 1993 a; Gray & Clozel, 1994). The ET_B receptor which is present on the endothelium mediating vasodilation through NO production has been found to be functionally different from the ET_B receptor of vascular and non-vascular smooth muscle which mediates vasoconstriction (Warner *et al.*, 1993 b). Tentative nomenclature of these ET_B subtypes has been proposed as ET_{B1} present on endothelium and ET_{B2} present on smooth muscle.

Further evidence supporting the existence of ET_B receptor subtypes has been reported by Elshourbagy *et al.* (1996) who reported the molecular characterisation of a novel human endothelin receptor splice variant (ET_B-SVR) which northern blotting revealed to be expressed in placenta, lung, kidney and skeletal muscle. Examination of the effects of ligand binding resulted in accumulation of inositol phosphate in COS cells transfected with ET_B receptor whereas no increase in inositol phosphates was observed in cells transfected with ET_B-SVR. The receptors were found to have 91 % sequence identity suggesting ET_B-SVR to be functionally distinct from the ET_B-receptor with the amino acid sequence differences determining functional coupling.

Evidence for a receptor selective for ET-3 over ET-1 would suggest at least one more receptor subtype. A receptor which has greater affinity for ET-3>ET-1 was discovered in bovine endothelial cells and tentatively designated an ET_C receptor (Sakurai *et al.*, 1992). Confirmation of this has not yet been possible since it has not yet been cloned. Further ET-3 receptors have been identified in rat brain and atria (Nambi *et al.*, 1990; Sokolovsky *et al.*, 1992) and anterior pituitary cells (Samson *et al.*, 1990).

The signal transduction mechanism whereby the interaction of an ET peptide with its receptor causes a physiological change is not yet understood in detail. The best characterised effect is that of vasoconstriction triggered by the binding of ET-1 to ET_A receptors in vascular smooth muscle cells. Initially several possible signal transduction mechanisms were suggested to be involved with the contractile mechanism mediated by ET-1. It is now known that the binding of ET-1 to the receptor leads to the formation of an activated receptor agonist complex. This initiates the stimulation of two different signal transduction cascades. These are the activation of the dihydropyridine-sensitive, voltage dependent Ca^{2+} channel causing an increase in intracellular free Ca^{2+} levels (Goto *et al.*, 1989; Kasuya *et al.*, 1989) and secondly, the G-protein mediated activation of phospholipase C leading to phosphatidylinositol hydrolysis and rapid formation of IP_3 and sustained diacylglycerol accumulation (Resink *et al.*, 1988; Takuwa *et al.*, 1990; Gardner *et al.*, 1992). Both pathways are G-protein dependent and the outcome is a biphasic increase in intracellular free Ca^{2+} ion concentration which then reduces to a lower level which is still significantly higher than baseline concentration. The initial transient increase in Ca^{2+}, in contrast to the sustained elevation of Ca^{2+}, is not sensitive to the removal of extracellular calcium (Danthuluri & Brock, 1990; Kai *et al.*, 1989; Takuwa *et al.*, 1991) but can be reduced by treatment of cells with caffeine in the absence of extracellular calcium (Kai *et al.*, 1989; Wagner-Mann & Sturek, 1991) or by ryanodine, a specific inhibitor of Ca^{2+} release from the sarcoplasmic reticulum (Wagner-Mann *et al.*, 1991). It is now accepted that the rapid, transient elevation of free Ca^{2+} is the result of Ca^{2+} release from the sarcoplasmic reticulum in response to stimulation by inositol phosphate. The sustained latter part of the elevation of calcium is thought to be due to the influx of extracellular calcium.

1.6.4 Synthesis of endothelin in brain cells

Until recently there was conflicting information about the possible expression of ECE in brain although a wealth of evidence for ET production and receptor expression had been acquired. RNA blot hybridisation had revealed ET gene expression in a variety of regions in the human brain (Lee *et al.*, 1990) and spinal cord (Giaid *et al.*, 1991).

Prepro-ET-1 mRNA was detected at the highest density in cells of the hypothalamus and striatum (Lee *et al.,* 1990) and ET-3 was shown to be produced in magnocellular neurons of the hypothalamoneurohypophyseal system (Samson *et al.,* 1991 a,b,c). Further evidence came from the examination of the location of ET binding sites which showed receptors for ET present in a variety of cell types in human brain (Jones *et al.,*1989; Kurihara *et al.,* 1990) and ET-1 and ET-3 binding sites to have a similar distribution profile in rat brain (Nambi, 1990). High affinity ET-1 binding was observed in cerebellar granule cells and astrocytes (MacCumber *et al.,* 1990) whilst subsequent research revealed the presence of ET-1 and ET-3 in glial and neuronal cells (MacCumber *et al.,* 1990 ; Ehrenreich *et al.,* 1991 ; Fuxe *et al.,* 1991; Giaid *et al.,* 1991).

Recent studies of astrocytoma cell lines have further characterised the expression of the ET receptors in this cell type. Wu-Wong *et al.,* (1995) working on human astrocytoma U373 MG cells concluded that the ET receptor predominantly expressed by this cell line was ET_B, with a molecular mass of 45 kDa whilst a later report (Wu-Wong *et al.,* 1996) demonstrated that a different astrocytoma cell line (U138 MG) expressed predominantly the ET_A receptor. They concluded that the major function of ET_B receptors was to remove ET from the neuronal environment though in response to certain pathological conditions, ET_A may become the dominant form resulting in impaired ability to clear ET.

These findings were supported by Lazarini *et al.,* (1996) who demonstrated that rat astrocytes preferentially expressed the ET_B receptor which is coupled to several intracellular signal transduction pathways via pertussis toxin-sensitive and insensitive G-proteins. These pathways were found to include inhibition of adenylyl cyclase / cAMP-dependent protein kinase and stimulation of the mitogen-activated protein kinase and DNA synthesis, suggesting the ET_B receptor may mediate several biological effects of ET in astrocytes *in vivo,* including proliferation during brain development and tissue repair. In view of this evidence suggesting ET production in brain it has been proposed that the ET peptides may play important roles in the regulation of cerebral blood flow and astrocytic function (Barone *et al.,* 1995).

It is possible that the effects of the ET peptides in brain may extend beyond those involving the control of vascular tone. Natural or induced disruption of the ET gene resulting in impaired ET production has shown ET to act as a morphogen involved in development of tissues derived from the neural crest (Baynash *et al.*, 1994; Kurihara *et al.*, 1994 ; Puffenberger *et al.*, 1994). The presence of ET and ET receptors in brain has led to interest in the expression of ECE by some brain cell types but, until recently, little was known about production of ECE in brain and much of the evidence was conflicting. Warner *et al.*, (1992 a) first detected a phosphoramidon inhibitable ECE activity in rat brain then subsequently in human brain (Warner *et al.*, 1992 b). More recent work by Deschepper *et al.*, (1995) has revealed ECE expression in highly purified cultures of rat brain astrocytes. During the course of this work, several cultured brain cell types were examined by western blotting and enzyme assay to determine possible ECE expression in brain.

1.7 Endothelin converting enzymes

Following the discovery of endothelin in 1988 by Yanagisawa *et al.* there was an explosion of interest in the biosynthetic mechanism that resulted in the production of this potent vasoactive peptide in the belief that this may provide a useful tool in the treatment of some human pathophysiological conditions. Initially a variety of enzyme activities were proposed as possible candidates for the physiological ECE activity. It was first thought that the primary specificity of ECE was due to a tryptophanyl residue in the P_1 position which would have implicated a chymotrypsin-like cleavage in the processing mechanism (Yanagisawa *et al.*, 1988). McMahon *et al.* (1989) demonstrated that the serine proteinase chymotrypsin was able to metabolise big ET to form a product which elicited similar responses as mature ET both *in vitro* and *in vivo.* However chymotrypsin was discounted as the physiological ECE since it was discovered that in addition to cleaving the Trp^{21}-Val^{22} bond, big ET was initially degraded at the Tyr^{31}-Gly^{32} bond therefore producing a truncated CTF (Takaoka *et al.*, 1990 a). In addition Wypij *et al.*, (1992) identified a chymostatin-sensitive serine protease from rat lung able to process extracellular big ET-1 to ET-1. The activity was identified as mast cell chymase I which significantly increased big ET-1 in perfused rat lung preparations by inducing degranulation of mast cells. However this has now been discounted as the activity responsible for physiological ET processing.

For a period, aspartic proteases were considered candidate ECE activities. Takaoka *et al.*, (1990 b) reported that the aspartic peptidase pepsin degraded big ET producing a product which was demonstrated to have potent vasoconstrictive activity in isolated rat aorta. Reverse-phase HPLC separation of the products confirmed that big ET was degraded at the Trp^{21}-Val^{22} bond to produce ET and CTF. The first reported big ET converting activity isolated from endothelial cells was reported by Matsumura *et al.*, (1990 a). They found an ET immunoreactive product was formed when an extract from cultured porcine aortic endothelial cells was incubated with porcine big ET. The degradation was found to be optimal at pH 4.0 and potently inhibited by the aspartic peptidase inhibitor, pepstatin A. This was soon followed by several reports describing both membrane and cytosolic aspartic protease converting activities in bovine and porcine endothelial cells, adrenal medulla and rat lung (Sawamura *et al.*, 1990 a,b; Ikegawa *et al.*, 1990 b; Wu-Wong *et al.*, 1990). Two of these reports (Sawamura *et al.*, 1990 a,b) implicated a cathepsin D-like enzyme. Bovine cathepsin D itself was shown to cleave big ET in the Trp^{21}-Val^{22} position but was also found to cleave at the Asp^{18}-Ile^{19} position forming a truncated inactive form of ET (Sawamura *et al.*, 1990 c; Takaoka *et al.*, 1990 c; Lees *et al.*, 1990). However, work by Wu-Wong *et al.*,1991) reported an ECE-like activity from rat lung which was inhibitable by pepstatin A but appeared to be distinct from cathepsin D.

This led to investigation of cathepsin E which was shown to cleave big ET exclusively at the Trp^{21}-Val^{22} bond to form mature ET and CTF (Robinson *et al.*, 1992). Kinetic studies of this activity revealed cathepsin E was able to bind the three ET precursors efficiently and generate the mature products of hydrolysis, ET-1, ET-2 and ET-3. The relevance of this enzyme in terms of physiological big ET breakdown was investigated *in vivo* by the use of specific inhibitors of cathepsin E which failed to provide evidence to support cathepsin E as the activity responsible for the physiological ECE (Bird *et al.*, 1992). Further evidence to discount cathepsin E as the true ECE activity was provided by Shields *et al.* (1991) who showed that the incubation of vascular endothelial cells with pepstatin A did not alter the ratio of big ET-1 to ET-1 nor the secretion of either peptide.

Ohnaka *et al.*, (1990) reported an ECE with an activity optimal at neutral pH. They identified two ECE-like activities in the homogenate of cultured bovine endothelial cells with pH optima of 3.0 and 7.0. Immunoreactive ET-1 was exclusively detected at pH 7.0 but several ET-like immunoreactive species were detected at pH 3.0. Pepstatin A was capable of complete inhibition of the activity at pH 3.0 whilst in contrast, the activity found at pH 7.0 was found to be insensitive to a wide range of inhibitors although it was found to be inhibited by the metal chelating agents EDTA and EGTA, suggestive of a metal-dependent neutral endopeptidase. Further evidence supporting this as the physiological ECE activity was reported by Okada *et al.* (1990) who found that bovine carotid artery endothelial cells converted human big ET-1 to ET-1 by a cleavage which was inhibitable by the metal chelating agents EDTA and *o*-phenanthroline and also by phosphoramidon. The activity was found to be insensitive to thiorphan and the ACE inhibitor captopril. The breakdown of big ET-1 was optimal between pH 6.6 and 7.6 suggesting the putative ECE to be a membrane bound neutral metallo-endopeptidase.

Activities matching the apparent criteria for the physiological ECE (optimal at neutral pH, insensitive to thiorphan, sensitive to metal chelators and phosphoramidon) derived from endothelial cells were reported by several groups (Matsumura *et al.*, 1990 b, 1991 a; Takada *et al.*, 1991; Devine *et al.*, 1991; Hioki *et al.*, 1991). Neutral ECE-like activities were isolated from cytosolic fractions of cultured endothelial cells (Okada *et al.*, 1990; Matsumura *et al.*, 1990 b, 1991 a,b; Takada *et al.*, 1991) and in vascular smooth muscle cells (Hioki *et al.*, 1991; Matsumura *et al.*, 1991 b). Whilst these were championed as possible candidate ECE enzymes these neutral pH activities were not identical. Matsumura *et al.*, (1990 b; 1991 a) discovered both membrane associated and cytosolic ECE-like activities in cultured endothelial cells originally isolated from fresh porcine thoracic aortas. Both activities were found to be inhibited by metal chelating agents though only the membranous form was found to be sensitive to phosphoramidon. Further differentiation of the two enzyme forms revealed that the sulphydryl blocking agent N-ethylmaleimide (NEM) markedly enhanced the apparent converting activity of the membrane fraction by inhibiting the degradation of ET-1. In contrast NEM completely inhibited the activity of the cytosolic fraction. These results were reproduced using porcine vascular smooth muscle cells (Matsumura, 1991 b). Further claims to have identified ECE were made by Deng *et al.* (1992) who found that a soluble extract of primary porcine endothelial cells contained an apparent ECE with a sharp pH optimum of 7.0-7.5 that was inhibitable by thiol peptidase inhibitors including Z-Phe-Phe-CHN_2 and p-hydroxymercuribenzoate but was found to be insensitive to serine or metallopeptidase inhibitors. No activity was detectable at pH 4.0. Takada *et al.* (1991) found a cytosolic ECE activity in bovine endothelial cells which was found to be phosphoramidon sensitive. However, the purified enzyme had an apparent molecular mass of 540 kDa, approximately five times greater than that previously recorded for solubilised ECE activity from membrane fractions.

Several characteristics of the physiological ECE activity had now been established and it is possible that many of the reports published claiming to describe ECE might have been avoided with more thorough investigation regarding their cell biology and assessment of their sensitivity to key inhibitor compounds. It was now apparent that the physiological ECE must be located in the correct sub-cellular location with the correct topology in order to hydrolyse big ET-1 efficiently.

The key to identification of a metalloprotease ECE was phosphoramidon. The co-inhibition of ECE and E-24.11 by this compound was indicative that ECE was related to the well characterised E-24.11 enzyme. Several key studies using phosphoramidon suggested a neutral PR-sensitive metalloprotease ECE activity was responsible for production of mature ET. Addition of micromolar concentrations of PR to cell culture media prevented the release of ET-1 from endothelial cells and was found to alter the ratio of ET-1 and big ET-1 in a manner consistent with the inhibition of hydrolysis of big ET-1 by the putative ECE (Ikegawa *et al.*, 1990 b; Shields *et al.*, 1991; Budzik *et al.*, 1991). In addition, effects consistent with PR inhibition of ECE were seen *in vivo* which showed inhibition of big ET-1 hydrolysis and the big ET-1 pressor response whilst the pressor response of mature ET-1 remained unaffected (Matsumura *et al.*, 1990 c; Shinyama *et al.*, 1991; McMahon *et al.*, 1991; Bird *et al.*, 1992;). Other inhibitors of E-24.11, including thiorphan appeared to show no effect on big ET conversion *in vivo* or *in vitro* (Bird *et al.*, 1992; Pollock & Opgenorth, 1991). McMahon *et al.*(1991) did report big ET-1 pressor inhibition by thiorphan though these apparent discrepancies in response were probably a reflection of dosage differences. All of these observations prompted many research groups to focus their search on an ECE activity distinct from, though structurally and catalytically related to E-24.11 by virtue of ECE being less sensitive to phosphoramidon and insensitive to thiorphan. The sensitivity of ECE to PR gave a further insight into the probable location of ECE. Since PR is a phosphorylated sugar derivative and would not be expected to enter cells at any appreciable rate, it implied that big ET-1 hydrolysis occurred on the extracellular face of the plasma membrane suggesting ECE to be a membrane bound or secreted protein. The different sensitivities of ECE and E-24.11 to PR and thiorphan were eventually to prove crucial in identification and assay of the true physiological ECE.

1.7.1 Endopeptidase 24.11 as a model for the characterisation of ECE

As described in section 1.4.1, E-24.11 is known to hydrolyse peptide bonds on the amino side of hydrophobic residues and therefore has many potential substrates including the Trp^{21}-Val^{22} bond of big ET-1. In this respect E-24.11 was thought to be a possible ECE and the sensitivity of big ET-1 breakdown to PR both *in vivo* and *in vitro* suggested at least catalytic similarities between E-24.11 and ECE. Further investigation of E-24.11 suggested this activity was capable of converting big ET-1 to ET-1 efficiently (Murphy *et al.*, 1994). However previous reports had revealed E-24.11 further degraded mature ET (Sokolovsky *et al.*, 1990; Vijayaraghavan *et al.*, 1990) and so E-24.11 was discounted as the true ECE. Nevertheless significant steady state levels of ET remain, implying that any PR-sensitive ECE activity could be due, at least in part, to big ET-1 hydrolysis by E-24.11. For this reason the ECE assay system used during this work, outlined in section 2.3.7 includes, amongst a range of inhibitors, the compound thiorphan which selectively inhibits E-24.11 ensuring any big ET-1 hydrolysis is due exclusively to physiological ECE activity. ECE has been successfully separated from E-24.11 by isoelectric focusing (Corder *et al.*, 1995).

1.7.2 Purification of ECE

Following the initial reports characterising ECE as a membrane bound PR sensitive/ thiorphan insensitive metalloprotease distinct from E-24.11 (Okada *et al.*, (1990), it was a further 3 years before the first published reports of purification of ECE to homogeneity. This delay was a consequence of several factors including the low specific activity of ECE in endothelial cells and other cell lines and tissue preparations and the lack of a suitably sensitive and reproducible assay system. The first reports of successful ECE purification came from the use of rat lung (Takahashi *et al.*, 1993) and porcine aortic endothelium (Ohnaka *et al.*, 1993) as source material.

The rat enzyme was purified a reported 6300-fold from a lung microsomal preparation following solubilisation with Triton X-100 (Takahashi *et al.*, 1993) and utilised the relatively high degree of glycosylation of the ECE protein to aid purification using a column of lectin (wheat germ agglutinin). The purification was enhanced further by using zinc-chelating Sepharose and Blue-B agarose. SDS-PAGE showed the protein to have an apparent monomeric weight of 130 kDa under reducing conditions.

Purification of ECE from porcine aortic endothelium was achieved some 12,000 fold to homogeneity (Ohnaka *et al.*, 1993). In this case the apparent monomeric weight of ECE was found to be 120 kDa by SDS-PAGE.

1.7.3 Molecular cloning of ECE

Following the purification of ECE by several groups in 1993 the ECE cDNA was subsequently cloned and sequenced. Partial amino acid sequence data for the rat lung enzyme was used to synthesise probes for the screening of a vascular endothelial cell cDNA library (Shimada *et al.*, 1994). This strategy was also adopted for the cloning of the bovine ECE cDNA following the purification of this from adrenal cortex (Xu *et* *al.*, 1994). Subsequently ECE was cloned from bovine aortic endothelial cells (Ikura *et al.*, 1994; Schmidt *et al.*, 1994) and human placental cells (Schmidt *et al.*, 1994). The 5 predicted amino acid sequences reveal a high degree of homology with some differences in the N-terminal region in 2 of the 3 bovine sequences (Ikura *et al.*, 1994; Schmidt *et al.*, 1994). From the experiments outlined in chapter 3 and the subsequent publishing of the sequence data for ECE-1, the ECE protein was determined to be an integral membrane protein with a single hydrophobic transmembrane sequence and a large catalytic domain containing the characteristic motif HExxH found in many zinc peptidases. It was also apparent that ECE was a highly glycosylated protein with 10 putative N-linked glycosylation sites compared with 5 or 6 found in E-24.11. Comparison of the predicted amino acid sequence with that of E-24.11 and Kell blood group protein revealed a high degree of sequence homology, particularly in the C-terminal region. In addition to sequence homology there were also similarities in predicted protein structure. All three proteins were found to have a cluster of 4 conserved cysteine residues within a 32 amino acid residue sequence immediately following the transmembrane hydrophobic anchor on the C-terminal side, a feature also found in brush border membrane hydrolases, sucrase isomaltase and γ-glutamyl transpeptidase (Hunziker *et al.*, 1986; Laperche *et al.*, 1986). A comparison of the major features of E-24.11 and ECE-1 can be found in Table 1.2.

All the ECE sequences cloned to date show > 95% homology in the C-terminal extracellular domain, however there are notable differences in the N-terminal regions. The bovine sequences cloned by Xu *et al.*, (1994) and Ikura *et al.*, (1994) were seen to be different from the bovine ECE sequence cloned by Schmidt *et al.*, (1994) in the N-terminal 28 amino acid residues. The bovine ECE-1 cDNA sequence cloned by Schmidt *et al.*, (1994) had a greater degree of homology in comparison with the rat ECE-1 sequence than the other cloned bovine sequences. In addition the cloned, sequenced human ECE-1 cDNAs (Schmidt *et al.*, 1994; Yorimitsu *et al.*, 1995) were also found to have a higher degree of homology to the rat sequence. The human cDNA sequence cloned by Shimada *et al.* (1994) was different to that cloned by Schmidt *et al.* (1994) at the 5` terminus. It was suggested by Turner & Murphy,

(1996) that the short form (cytoplasmic N-terminal domain of 51-52 residues) reported by Shimada *et al.*, (1994) and later by Schmidt *et al.*, (1994) be designated the α form and the longer form (cytoplasmic N-terminal domain of 56 residues) reported by Xu *et al.*, (1994); Ikura *et al.*, (1994); Shimada *et al.*, (1995) be designated the β form. This is the nomenclature adhered to throughout this work. To date no reason for the production of these isoforms has been identified though current opinion is that they are products of alternative splicing (Valdenaire *et al.*, 1995). An alternative system of nomenclature has been described by Valdenaire *et al.* (1995).

The tissue distribution of ECE mRNA has been examined by Northern blot analysis which has revealed abundant expression in lung, pancreas, placenta, adrenal gland, ovary and testis (Shimada *et al.*, 1994, 1995; Xu *et al.*, 1994; Ikura *et al.*, 1994; Schmidt *et al.*, 1994). Shimada *et al.* (1995) examined the relative tissue distribution of ECE-1 α and β isoform mRNAs in a variety of cells and tissues and found the α form to be between 5 and 10 fold more abundant in every case. These data suggested ECE-1 α to be the physiologically relevant form of the enzyme under normal conditions.

1.7.4 Tissue distribution of ECE-1

In situ hybridisation analysis confirmed the expression of ECE-1 mRNA in vascular endothelial cells derived from tissues including heart, lung, liver, brain, pancreas, adrenal cortex (Xu *et al.*, 1994). This represents a comparatively wide tissue distribution and is consistent with the wide distribution of prepro-endothelin and endothelin but contrasts with the distribution of E-24.11 which has been found to be expressed in renal and intestinal brush border membranes and is constitutively expressed by neutrophils (Connelly *et al.*, 1985) and is also expressed in the majority of acute lymphoblastic leukaemias where it is referred to as common acute lymphoblastic leukaemia antigen (CALLA) or CD 10 (Letarte, 1988; Shipp *et al.*, 1989). However E-24.11 is expressed at much lower levels in other tissues (Howell *et al.*, 1991).

1.7.5 Cloning and expression of ECE-2

In 1995 Emoto and Yanagisawa reported the molecular cloning and expression of a novel big ET-1 converting activity, ECE-2 which was shown to cleave big ET-1 at the Trp^{21}-Val^{22} bond to produce mature ET-1 both *in vitro* and in transfected Chinese Hamster Ovary (CHO) cells. The cDNA sequence predicted bovine ECE-2 to be a metalloprotease structurally related to ECE-1. ECE-2 had 59 % sequence homology to ECE-1 and was shown to be potently inhibited by phosphoramidon having apparently 250-fold greater degree of sensitivity compared with ECE-1. ECE-2 was found to have an acidic pH optimum of 5.5 and was also found to be virtually inactive at neutral pH. The same researchers predicted ECE-2 to act as an intracellular enzyme since CHO cells which lack detectable levels of endogenous ECE activity were found to secrete mature ET-1 into the culture medium following double transfection with ECE-2 and pre-pro endothelin cDNAs. In addition it was reported that ECE-2 transfected CHO cells do not efficiently produce mature ET-1 when cells were cultured in media supplemented with big ET-1 through coculture with prepro ET-1 transfected CHO cells. This evidence was consistent with the endogenous synthesis of big ET-1 at the trans-Golgi network where the vesicular fluid would be acidified providing optimal conditions for the activity of ECE-2. Since the work by Emoto and Yanagisawa (1995) showed ECE-2 to have virtually no activity at neutral pH it has been assumed through the course of this work that ECE-2 does not contribute to any significant big ET-1 breakdown in the assay system used and therefore all quoted ECE activities relate to ECE-1 α and/or ECE-1 β and not ECE-2.

1.7.6 The sub-cellular location of ECE

Until recently, ECE-1 has been regarded exclusively as an ectoenzyme converting big ET-1 to ET-1 at the extracellular face of the plasma membrane. This has been supported by a number of studies including immunohistochemical staining of ECE-1 in endothelial cells (Takahashi *et al.*, 1995) and the ability of PR to inhibit the formation of ET-1 from exogenous big ET-1 (McMahon *et al.*, 1991; Auguet *et al.*, 1992) consistent with the localisation of ECE-1 at an accessible plasma membrane site.

Table 1.2 Comparison of E-24.11, ECE-1 and ECE-2

	E-24.11	**ECE-1**	**ECE-2**
Total amino acids	742 or 750	754 or 758	787
Molecular weight (kDa)	90-100	120	120
N-linked glycosylation sites	5 / 6	10	10
Zn binding motif	H-E-I-T-H	H-E-L-T-H	H-E-L-T-H
Cleavage site	Broad range N-terminal side of hydrophobic residues	Trp^{21}-Val^{22} bond of big ET	Trp^{21}-Val^{22} bond of big ET
pH optimum	7.0	6.8	5.4
Localisation	Kidney, lung Small intestine Striatum Lymph nodes	Lung, Heart, Pancreas, Placenta Endothelial cells, Brain	Brain Uterus Testis
Phosphoramidon sensitivity (Ki)	3.5 nM	3.5 μM	4 nM
Thiorphan sensitivity	5 nM	200 μM	> 200 μM

Studies by Xu *et al.* (1994) did show PR inhibitable conversion of exogenous big ET-1 by ECE-1 transfected COS cells though this conversion was found to be relatively inefficient with only 5-10 % of big ET-1 being converted. The I_{50} for PR inhibition of

endogenous big ET-1 hydrolysis was found to be only 100 μM which is consistent with entry of 1 % of PR into cells, compared with 1 μM for exogenous conversion. Since PR is a phosphorylated sugar derivative it would not be expected to enter the cells at any appreciable rate and would therefore have limited access to the intracellular compartment consistent with these data. Double transfection studies in conjunction with inhibitor profiles indicated that some of the expressed ECE-1 protein is located as an ectoenzyme on the plasma membrane of the cell whilst the majority of ECE activity was located intracellularly in a membrane bound compartment, possibly within the Golgi apparatus. This hypothesis has been found to be consistent with immunohistochemical studies which have shown mature ET-1 in the cytoplasm of endothelial cells and the reported ability of a low density intracellular fraction to convert big ET-1 to the mature ET-1 peptide (Gui *et al.*, 1993; Harrison *et al.*, 1993). More recent evidence for an intracellularly localised ECE-1 is the accumulation of ECE-1 when cells are grown in the presence of PR concentrations suggesting this increase is mediated by inhibition of intracellular as opposed to cell-surface ECE (Schmidt *et al.*, 1994). Whilst it is possible that ECE-1 mediates both cell-surface and some intracellular processing it is likely that ECE-2, with an acidic pH optimum of 5.5, is responsible exclusively for intravesicular conversion of big ET-1 (Emoto & Yanagisawa, 1995). Current opinion is that the intracellular location of ECE-1 is in the Golgi (Xu *et al.*, 1994), though this has not yet been confirmed.

1.8 The existence of a metalloprotease sub-family

Characterisation of the genes encoding E-24.11 (D'Adamio *et al.*, 1989) and ECE-1 Valdenaire *et al.* (1995), allowed comparison of these genes encoding with those encoding other zinc metalloproteases. Analysis of gene structure, organisation, phylogenetic studies and comparisons of biochemical characteristics revealed the existence of a sub-family of proteins which is now thought to include ECE-1 (α and β isoforms), ECE-2, and lactococcal endopeptidase. Two other mammalian gene products have been shown to exhibit strong homology with E-24.11: the Kell blood group antigen of erythrocytes and the putative product of the PEX gene.

Molecular cloning of the Kell cDNA predicted a protein of 732 amino acids which showed both structural and sequence homology with E-24.11 (Lee *et al.,* 1991; 1995). In common with E-24.11, the Kell protein was found to have a short N-terminal cytoplasmic sequence, a single hydrophobic transmembrane-spanning sequence, a cluster of cysteine residues on the extracellular side immediately following the transmembrane sequence and a HExxH zinc-binding motif. In addition there is conservation of two residues (Glu^{646} and His^{711}) which are critical for catalytic activity in E-24.11 though in common with ECE, Kell lacks a residue equivalent to Arg^{102} of E-24.11 essential for dipeptidylcarboxypeptidase activity suggesting these enzymes act predominantly as endopeptidases. To date the physiological substrate of Kell remains unidentified.

The PEX gene product has been implicated in patients suffering from X-linked hypophosphataemic rickets (The HYP consortium, 1995). It was found that the PEX amino acid sequence shared a high degree of homology to the corresponding sequences of E-24.11, ECE-1 and Kell suggesting PEX to be a membrane bound zinc metallopeptidase and a member of this important sub-family of proteins.

1.9 Aims of the project

At the time of the start of this work, there was limited knowledge regarding the biochemistry of ECE. Research had shown phosphoramidon inhibitable ECE activity distinct from E-24.11 though the 2 activities were thought to be related. In view of this one of the initial aims of the project was to establish a sensitive radio-immunoassay procedure for detection of ECE activity in a human endothelial cell line which was known to express both ECE and E-24.11. Once these activities had been distinguished using the assay, a variety of methods were used to establish ECE as a type II integral membrane protein. This was later confirmed to be the case when ECE-1 amino acid sequence was determined from the cloning of ECE cDNA. Following the cloning of the ECE-1 α and β isoforms by Schmidt *et al.* (1994) and Shimada *et al.* (1995), it became possible to raise antibodies specific for each isoform due to N-terminal differences in the signal sequence. The procedure of antibody production is outlined in chapter 6. The reported expression of ECE-1 α mRNA in TRLEC-03 cells has led some researchers to believe that this is the predominant form.

A further aim of the project was to study possible regulatory mechanisms of ECE production. In the course of this work two key compounds were examined to study regulatory effects in a variety of cell lines. Phosphoramidon was thought to possibly have an up-regulatory effect on ECE production either by increasing the rate of biosynthesis or inhibiting a protein responsible for its breakdown (Schmidt *et al.*, 1994). Cytokine action on endothelial cells had been implicated in the regulation of ET-1 expression (Kurihara *et al.*, 1989; Yoshizumi *et al.*, 1990; Shimizu, 1994), and this was investigated further using the phosphotyrosine phosphatase inhibitor vanadate. The differing actions of each of these treatments aimed to show ECE up-regulation in neural and endothelial cell lines. Additional aims of this work were to show that the up-regulatory effects were specific for ECE and did not extend to other membrane peptidases including the related E-24.11 activity. By examining the effects on ECE expression of these compounds, which exert their effects in very different ways, it was hoped to gain an insight into the complex mechanisms by which the cell is able to regulate ECE activity.

1.9.1 The use of cell culture as a basis for the investigation of ECE

Cell culture has become a convenient and powerful research tool for the study of a wide and diverse range of proteins. Whilst the technique offers many advantages, it is not without some problems. To establish the most effective research system it is necessary to consider these and how effective cell culture will address the aims of the project. A major advantage of using cell culture is that the general state of the cells can be monitored throughout the experiment. It is important that healthy, viable cells are used throughout the experiment, to create a standard population of cells. This is particularly important when cells are treated with specific reagents (in the case of this work phosphoramidon, thiorphan and sodium orthovanadate) which are cytotoxic at higher concentrations. The administration of such compounds to cells and also the environment in which they are cultured (media composition, pH, temperature and O_2 / CO_2 levels) can be controlled very precisely. Much less of the compound is required and there is no problem with the compound being metabolised by the liver, stored in muscles or excreted by the kidney as there would be if animals were used. Cells grown in culture remain viable for a much longer period of time, whilst organ cultures or primary cell cultures are only viable for hours or days and this allowed sufficient cells to be grown to perform all necessary assays. A typical membrane preparation was found to yield approximately 5 mg protein and this, coupled with the use of the radioimmunoassay procedure sensitive to picomolar levels, provided sufficient cellular material, with all the advantages of cell culture outlined above.

1.9.2 The use of EA.hy926 as a model system for the study of ECE

The EA.hy926 cell line is a permanent human endothelial cell line resulting from the fusion of human umbilical vein cells with the human lung cell carcinoma cell line A549. Previous work with this cell line has shown EA.hy926 cells to sustain expression of many differentiated functions associated with the vascular endothelium, (Edgell *et al.*, 1983). Prior to this work, the cell line had previously been shown to express ET-1 mRNA and to secrete big ET-1 and ET-1 into the culture media, (Saijonmaa *et al.*, 1991). This evidence suggested that the cell line expressed a specific big ET-1 processing enzyme and this was a possible candidate for the putative physiological endothelin converting enzyme. For this reason the EA.hy926 cell line was used in the initial part of this study as a model for the study of ECE biochemistry and regulation of expression. Whilst in many ways this cell line is a convenient model for the study of ECE (relatively high ratio of ECE activity : E-24.11 activity), any work using cell lines is not without problems. Cells which undergo repeat passaging are prone to genetic shifts, often changing the phenotype of the cell line. The original work by Edgell *et al.*, (1983) tested these cells for the expression of one highly specific function of the vascular endothelium; the expression of factor VIII-related antigen (VIIIR:Ag). This protein is an endothelial cell product (Jaffe *et al.*, 1973) maintained in normal human plasma at approximately 10 μg/ml. This protein is also known as Von Willebrand factor as decreased levels are found in patients suffering from Von Willebrands disease. The expression of VIIIR:Ag by EA.hy926 cells was found to be consistent through 50 passages and 3 cloning steps (Edgell *et al.*, 1983), indicating EA.hy926 to be a permanent cell line. Whilst during the course of this work expression of VIIIR:Ag was not monitored there are several indicators that the genotype of the cell line was maintained. Repeat passaging of the cells did not result in any morphological change in the cells or affect growth characteristics and media requirements. In addition the specific activities of several proteases were assayed during the course of this work and were found to be consistent throughout, indicating no significant changes to the cell line characteristics had occurred. Whilst the EA.hy926 cell line has been used as a model system for the study of the physiologically relevant ECE, it cannot be said that this truly reflects the conditions of cells *in vivo*. The transformation of any cell line is likely to alter the genotype and therefore enzyme expression by the cell and in addition the lack of organised tissue structure in a cultured environment may also contribute to promoting changes to the expression of enzymes by the cell. Examples of this include E-24.11 expression *in vivo* on neuronal cells (Barnes *et al.*, 1988) an activity which is not expressed on some neuroblastoma cell lines (Medeiros *et al.*, 1991). Such differences in peptidase expression were also found in the EA.hy926 cell line which was found not to express ACE or DPP-IV activity (chapter 3) although these activities have previously been considered to be markers for endothelial cells (Caldwell *et al.*, 1976; Walsh *et al.*, 1993). ACE activity has also been found in human umbilical vein endothelial cells (HUVEC) (Watanabe *et al.*, 1991 and 1993). Since EA.hy926 cells did not express these marker activities this cell line cannot be said to reflect the true physiological situation of endothelial cells, however since they do show limited expression of other membrane peptidases including ECE, they have been used here and elsewhere as a model for the study of ECE biochemistry.

1.9.3 The selection and use of other cell lines

Whilst the EA.hy926 cell line was to prove an invaluable and convenient source of ECE, the expression of ECE in brain remained a controversial issue (section 1.6.4). In order to further understand tissue specific expression of ECE within the brain, several brain cell types were examined for the possible expression of ECE and other membrane peptidases. The cell lines were selected primarily on the basis of their origin, with the intention of attempting to define ECE expression in certain tissues. The cell lines selected included glial lines, an astrocytoma clone, a neuroblastoma line and a pheochromocytoma with characteristics of cells of the adrenal medulla.

Through the course of this work, a range of cell types have been investigated for ECE expression and possible induction of expression by various agents. Further explanation regarding their use is given where they are mentioned. Culture details are given in section 2.4.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Peptidase inhibitors

Amastatin, Di-isopropylfluorophosphate (Dip-F), 3,4 Di-isochlorocoumarin (DCI), ethylenediaminetetra-acetic acid (EDTA), trans-epoxysuccinyl-leucylamido-(4-guanido) butane (E-64), enalaprilat and thiorphan ([DL-3-mercapto-2-benzylpropanoyl]-glycine) were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). Phosphoramidon (N-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp) was obtained from Peninsula Laboratories Europe Ltd. (St. Helens, Merseyside, U.K.). Pepstatin A was purchased from the Peptide Institute, (Osaka, Japan).

2.1.2 Peptides

Porcine big ET-1 (1-39) and human ET-1 (1-21) were purchased from the Peptide Institute, (Osaka, Japan). Succinyl-Ala-Ala-Leu-nitroanilide and *Streptomyces griseus* aminopeptidase (SGAP) were kind gifts from Dr. S. Blumberg (University of Tel Aviv, Tel Aviv, Israel).

BzGly-His-Leu (Hip-His-Leu), L-ala *p*-nitroanilide and Gly-Pro *p*-nitroanilide were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). [D-Ala^2, Leu^5] enkephalin was obtained from Cambridge Research Biochemicals Ltd. (Cambridge, U.K.). Radioiodinated endothelin (I^{125}-ET-1) used for RIA determination of ET-1 was purchased from Amersham International Plc. (Amersham, Bucks, U.K.).

Synthetic peptides (>80 % purity) used for the production of antibodies to ECE-1 α and β isoforms, (NH_3^+CTYKRATLDEED-NH_2 and NH_3^+CQRNPFLQGKRG-NH_2) respectively, were purchased from Zinsser Analytic (U.K.) Ltd. (Maidenhead, Berkshire, U.K.). Mass spectrometry of these peptides (performed by Dr. J. Keen, University of Leeds, Leeds, U.K.) confirmed both peptides to be > 85 % pure.

2.1.3 Antibodies

The polyclonal antibody RP161 was generated in rabbits immunised with porcine kidney E-24.11 and affinity purified as described in Barnes *et al.*, (1991). Monoclonal antibodies, GK4A9 and 3C7 were raised to porcine E-24.11 as described in (Gee *et al.*, 1983). The polyclonal antibody (E5) recognising the C-terminal ET-1 (16-21) sequence was a gift from Dr. R. Corder (The William Harvey Research Institute, London, U.K.).

Sheep anti-(rabbit IgG Fc) and normal rabbit serum were obtained from ImmunoGen International Ltd. (Gateshead, Tyne and Wear, U.K.). The monoclonal antibody, AEC 32-236, raised to rat ECE-1 was a gift from Dr. Tanzawa (Sankyo Co. Ltd., Tokyo, Japan). Peroxidase-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG used as second antibodies for protein detection were from Amersham International Plc. (Amersham, Bucks., U.K.).

2.1.4 Enzymes

Phospholipase C (PLC) from *B. cereus* was from Fluka Chemicals (Gillingham, Dorset, U.K.). This commercial preparation of PLC is known to contain phosphatidylinositol-specific PLC activity (Ikezawa & Taguchi, 1981; Ferguson *et al.*, 1985). Endopeptidase E-24.11 was immunoaffinity purified from porcine kidney cortex (Gee *et al.*, 1983) by using the monoclonal antibody GK4A9 immobilised to Sepharose 4B. Purification of the enzyme to homogeneity was confirmed by SDS-PAGE.

2.1.5 Antibody Production

All reagents were from the Sigma Chemical Co. (Poole, Dorset, U.K.), except the sulpholink affinity gel which was purchased from the Pierce Chemical Co. (Chester, Cheshire, U.K.).

2.1.6 Electrophoresis and western blotting

Acrylamide/ bis acrylamide stock solution (30% w/v acrylamide- 0.8% w/v bis acrylamide was from Severn Biotech Ltd. (Kidderminster, Worcs., U.K.). Sodium dodecyl sulphate, 2-mercaptoethanol, Coomassie blue R250 and bromophenol blue were from BDH Chemicals, (Poole, Dorset, U.K.). Ammonium persulphate, CAPS (3- [cyclohexylamino]-1-propanesulfonic acid), TEMED (N,N,N'N'-tetramethylethylene diamine) and high molecular weight markers were from the Sigma Chemical Co. (Poole, Dorset, U.K.). Immobilon-P PVDF transfer membranes were from the Millipore Corporation, (Bedford, MA, U.S.A.). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham International Plc. (Amersham, Bucks., U.K.). Additional chemicals were of analytical grade and supplied by BDH Chemicals Ltd. (Poole, Dorset, U.K.).

2.1.7 Cell culture

Cell culture reagents and flasks were supplied by Gibco BRL, Life Technologies Ltd. (Paisley, Scotland, U.K.). Additional reagents used for studies in membrane peptidase expression (sodium orthovanadate, thiorphan, tunicamycin and phenylarsine oxide) were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). Phosphoramidon was purchased from the Peptide Institute (Osaka, Japan).

2.1.8 Detergents

Octyl glucopyranoside and t-octylphenoxypolyethoxyethanol (Triton X-100) were obtained from ICN Biochemicals Ltd. (Thame, Oxfordshire, U.K.) and BDH Chemicals (Poole, Dorset, U.K.) respectively. Triton X-114, polyoxyethylene-sorbitan monolaurate (Tween 20) and Nonidet P-40 were from the Sigma Chemical Co. (Poole, Dorset, U.K.).

2.2 Protein assays

2.2.1 BCA protein assay

Protein concentrations were determined by the BCA protein assay (Smith *et al.*, 1985). The working reagent was prepared by addition of 4% w/v copper sulphate solution to the BCA solution at a ratio of 1:50. A standard curve using bovine serum albumin (BSA) was set using dilutions ranging from 2 μg to 10 μg in 10 μl total volume. Unknown samples (10 μl) and standards were mixed with 200 μl of BCA working reagent. The plate was briefly shaken and incubated at 37°C for 30 min. The absorbance was then read at 570 nm using an Anthos plate reader, model 2001 (Anthos Labtech Instruments, Salzburg, Austria), and sample protein concentrations were calculated by comparison of absorbance readings with those recorded for a standard curve, linear up to 10 μg of protein.

2.3 Enzyme assays

2.3.1 Endopeptidase-24.11 (EC 3.4.24.11)

Endopeptidase-24.11 (EC 3.4.24.11; neprilysin) was assayed using [D-Ala^2, Leu^5] enkephalin (0.5 mM) as substrate and the specificity of breakdown was checked with phosphoramidon (10 μM). The Tyr-D-Ala-Gly product was resolved by reverse phase HPLC and quantified by measuring absorbance at 214 nm (Turner *et al.*, 1989). Endopeptidase-24.11 was also assayed using the colorimetric method described by Indig *et al.*, (1989) using Succinyl-Ala-Ala-Leu-nitroanilide (0.4 mM) as substrate, and adapted for use on a microtitre plate. Specificity was checked with phosphoramidon (10 μM). Endopeptidase-24.11 activity was determined using the rapid colorimetric assay unless otherwise stated.

2.3.2 Dipeptidyl peptidase IV (EC 3.4.15.5) and Aminopeptidase-N (EC 3.4.11.2)

Dipeptidyl peptidase-IV (DPP-IV) and aminopeptidase-N (AP-N) assays were performed on microtitre plates with Gly-Pro *p*-nitroanilide (1 mM) and L-Ala *p*-nitroanilide (1 mM) as substrates respectively. The *p*-nitroanilide product was determined spectrophotometrically at 405 nm. Specificity of substrate breakdown was checked using Dip-F (1 mM) and amastatin (100 μM) respectively.

2.3.3 Angiotensin converting enzyme (EC 3.4.15.1)

Angiotensin converting enzyme (ACE) was assayed using Hip-His-Leu (5 mM) as substrate and specificity was checked with enalaprilat (10 μM). The Hip product was resolved by reverse phase HPLC and quantified by absorbance at 214 nm (Turner *et al.*, 1989).

2.3.4 Alkaline phosphatase (EC 3.1. 3.1)

Alkaline phosphatase was assayed on microtitre plates using *p*-nitrophenyl phosphate as substrate at pH 10.5. The *p*-nitrophenol product was quantified by absorbance at 405 nm (Bessey *et al.*, 1946).

2.3.5 Lactate dehydrogenase (EC1.1.1.27) and 5'-nucleotidase

Lactate dehydrogenase (LDH) was assayed on microtitre plates and determined spectrophotometrically according to the method of Schwartz & Bodansky (1966).

The activity of 5'-nucleotidase was determined by the measuring the release of inorganic phosphate from 5'-Adenosine monophosphate (Aronson & Touster, 1974). The reaction was carried out in a microtitre plate in a volume of 50 μl and terminated by the addition of 50 μl of 12 % SDS. Phosphate was then estimated colorimetrically (Ames, 1966).

2.3.6 Endothelin converting enzyme

Endothelin converting enzyme (ECE) was assayed in 50mM Tris/HCl (pH 7.0) containing 0.1% w/v BSA and 0.1% w/v Triton X-100 in the presence and absence of 100 μM phosphoramidon. Membranes (10-20 μg of protein) were incubated with inhibitors (pepstatin A, 10 μM; amastatin, 10 μM; E-64, 10 μM; 3,4-DCI, 100 μM; Dip-F, 1 mM; thiorphan, 10 μM) and incubated at 37°C for 1 h. Reactions were started by the addition of big ET-1 to a final concentration of 1 μM (in a total volume of 100 μl) and incubated at 37°C for 90 min unless stated otherwise. All reactions were stopped by heating at 100°C for 4 min followed by centrifugation (12,000 g, 10 min) at 4°C. The endothelin produced was then quantified by RIA (2.3.7.1).

2.3.7 Radioimmunoassay for ET-1

Endothelin-1 was quantified using a RIA specific for the C-terminal ET-1 (16-21) sequence. The E5 antibody used in this method was a kind gift from Dr. R. Corder (William Harvey Research Institute, St Bartholomew's Hospital, London, U.K.). This antibody had previously been shown to have < 0.015 % cross reactivity with big ET-1 (Corder *et al.*, 1993) and was raised by injecting rabbits with His-Leu-Asp-Ile-Ile-Trp conjugated to bovine thyroglobulin with glutaraldehyde. The antibody as supplied was used at a final concentration of 1 : 2000. Radioiodinated ET-1 was used as a tracer and serial dilutions (7.8 fmol to 4000 fmol) of human ET-1 were used as standards. The standard curve is shown in Fig. 2.1.

2.3.7.1 Radioimmunoassay procedure

Standards or unknowns (200 μl) were diluted in an appropriate volume of RIA buffer (50 mM sodium phosphate containing 0.15 % w/v BSA, 0.005 % v/v Triton X-100 and 0.01 % w/v sodium azide) and were incubated for 16 h at 4°C with 50 μl antibody and 50 μl tracer (8000 c.p.m.). Sheep anti-(rabbit IgG Fc region) serum (diluted 1 : 10 in RIA buffer, 100 μl) and 100 μl of normal rabbit serum (diluted 1 : 70 in RIA buffer), were added to each tube, mixed briefly then incubated for 3 h at 4°C. Following incubation, 4 % w/v polyethylene glycol (500 μl) was added to each tube and bound ligand separated from free by centrifugation at 3000 g for 30 min. Scintillation counting of the bound ligand complex was used to calculate the relative amounts of ET-1 present in the samples. For this a Wallac 1261 Multigamma counter was used with RIAcalc software.

2.3.8 Reverse phase HPLC protocols

Reverse-phase HPLC was used to monitor angiotensin converting enzyme and endopeptidase-24.11 activity. In both cases assay products were separated with a μBondapak C_{18} column (3.9 mm x 300 mm) (Millipore Corporation, Milford, MA, U.S.A.) using a Waters HPLC system. Samples were injected on to the column via a Waters 712 WISP and elution was at a flow rate of 1.5 ml/min with a gradient of 4.5 %-30 % v/v acetonitrile in 0.08 % H_3PO_4 at pH 2.5 followed by 5 minutes elution at the final conditions. The column effluent was monitored at 214 nm using a Waters tunable absorbance detector.

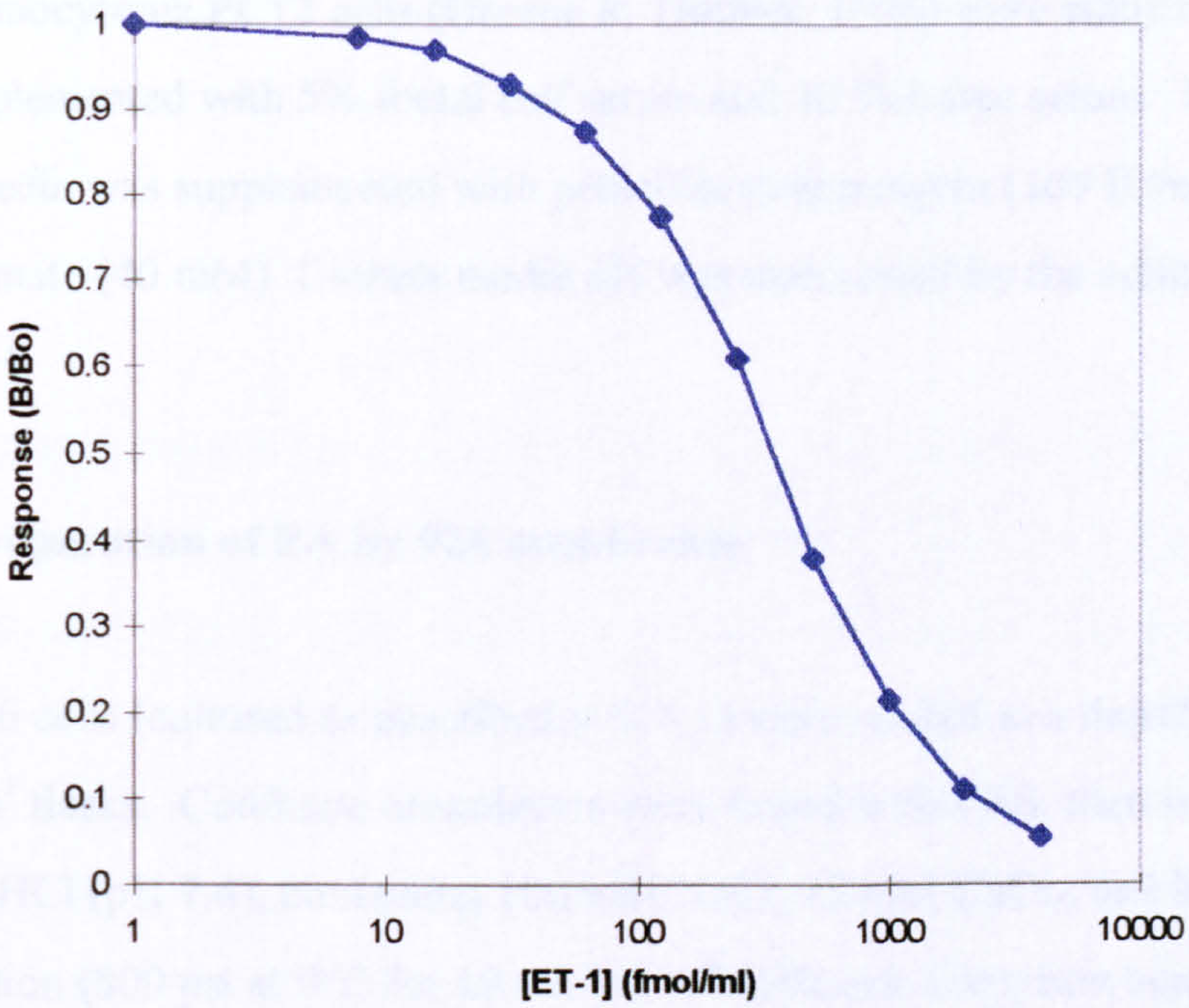
2.4 Cell culture

2.4.1 EA.hy 926 cell culture

EA.hy926 cells were obtained from Dr. C.-J.S.Edgell (North Carolina, U.S.A.). The EA.hy926 cell line was routinely grown in Dulbecco's modified eagle medium supplemented with HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), 40 mM glutamine, 10 % foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in 5 % CO_2 in air. Confluent monolayers were passaged using trypsin-EDTA (0.05 % : 0.02 % w/v) and seeded at a density of 0.75×10^6 cells per 75 cm^2 flask.

Fig. 2.1 Radioimmunoassay standard curve for ET-1

The curve was prepared using increasing concentrations of ET-1 in RIA buffer (7.8 - 4000 fmol/ml). Standards (200 µl of each) were assayed in duplicate as described in section 2.3.7.1. Standard curve data were fitted by a four parameter logistic equation using the RiaCalc programme (LKB) on line to a multi-head gamma counter (Wallac 1261 Multigamma). Samples for analysis were diluted appropriately in RIA buffer to yield a response (B/Bo) in the range of 0.25 - 0.8.



2.4.2 Culture of other cell lines

Cells were cultured in either plastic flasks or in 24 well plates. All cell lines, unless otherwise stated were cultured in media supplemented with 10 % foetal calf serum and maintained at 37°C in 5 % CO_2 in air. EA.hy926 cells were cultured as described in section 2.4.1 . C6 (Amberger *et al.*, 1994) and Bu17 (Kametani *et al.*, 1990) cells were grown in supplemented Dulbecco's modified Eagle's medium. D384 (Balmforth *et al.*, 1986) were maintained in a culture medium (1 : 1) of Ham's F10 and Dulbecco's modified Eagle's medium. The neuroblastoma cell line SH-SY5Y (Ross *et al.*, 1985) was cultured in a 1 : 1 mix of Ham's F12 and minimal essential medium supplemented with non-essential amino acids (Murphy *et al.*, 1991). Rat pheochromocytoma PC12 cells (Greene & Tischler, 1976) were cultured in RPMI 1640 supplemented with 5% foetal calf serum and 10 % horse serum. In all cases culture media was supplemented with penicillin/streptomycin (100 IU/ml : 10 μg/ml) and glutamate (40 mM). Correct media pH was maintained by the addition of $NaHCO_3$ and NaOH.

2.4.3 Preparation of EA.hy 926 membranes

EA.hy 926 cells (cultured as described in 2.4.1) were seeded at a density of 1.5×10^6 in 150 cm^2 flasks. Confluent monolayers were rinsed with PBS, then scraped into 50 mM Tris-HCl (pH 7.4), containing 100 mM NaCl, 18 mM $CaCl_2$, and homogenised by N_2 cavitation (800 psi at 0°C for 10 min) in a PARR cell disruption bomb. Homogenates were centrifuged at 1000 g for 10 min to remove nuclei and cell debris and the supernatants centrifuged again (100,000 g, 90 min) to yield a membrane pellet which was resuspended in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. Resuspended membranes were either assayed immediately or stored at -70°C.

2.4.4 Porcine lung membrane preparation

All operations were performed at 4°C. Fresh porcine lung (<2 h old) was washed in 10 mM Tris-HCl (pH 7.4) to remove excess blood and then cut into small pieces.

The washed porcine lung tissue (300 g) was homogenised in 3 vol. 10 mM Tris-HCl (pH 7.4) and centrifuged at 1000 g for 15 min. The supernatant was removed and filtered through a 1 mm pore nylon mesh and the filtrate retained as the post nuclear fraction. This fraction was then centrifuged at 150,000 g for 40 min to obtain the membrane fraction (pellet) and cytosolic fraction (supernatant). The membrane pellet was resuspended in 10 mM Tris-HCl (pH 7.4) to give a membrane preparation with a final concentration of approx. 10 mg/ml.

2.4.5 Preparation of kidney brush border membranes

Kidney brush border membranes were prepared according to the method of Booth & Kenny (1974), with the exception that all 12,000 g centrifugation steps were increased to 15,000 g.

2.4.6 Preparation of reagents used for regulation studies

Thiorphan, phosphoramidon and tunicamycin were dissolved in Millipore grade water to a final concentration of 10 mM, sterilised by passing through a 0.2 μm filter and aliquots stored frozen at -20°C. For treatment of cell cultures, aliquots were thawed and further diluted in the appropriate amount of cell culture media. Sodium orthovanadate was activated prior to use according to the method of Mahadevan & Bell, (1989). Briefly, a 10 mM solution of Na_3VO_4 was adjusted to pH 10.0 using either NaOH or HCl. The solution was boiled then cooled to room temperature and the pH re-adjusted to 10.0. This procedure was repeated until the solution remained colourless at room temperature. Aliquots were stored frozen at -20°C. Phenylarsine oxide was dissolved in acidified DMSO and added to cell culture media immediately.

2.4.6.1 Application of reagents used for expression regulation studies

Frozen aliquots of thiorphan, phosphoramidon, tunicamycin or sodium orthovanadate were thawed and passed through a 0.2 μm filter then diluted with cell culture media to give a final concentration of 1 mM. Appropriate volumes of this solution were added to cultured flasks or plates of cells to give the desired final concentration.

2.5 Solubilisation of cell membranes

2.5.1 Detergent solubilisation

Triton X-100 detergent solubilisation of EA.hy926 membranes was performed in detergent solubilisation experiments and in preparation for immunoadsorption experiments using the GK4A9 immunoaffinity column. All operations were performed at 4°C. Triton X-100 in 10 mM Tris-HCl (pH 7.4) was added to EA.hy926 membranes to give a final protein concentration of 1mg/ml of protein and a final Triton X-100 concentration of 7 mg/ml (i.e. a ratio of 7 : 1, detergent : protein). The detergent/membrane suspension was gently agitated for 1 h, then centrifuged at 100,000 g for 90 min to obtain the detergent-solubilised membrane preparation.

2.5.2 Precondensation of Triton X-114

Triton X-114 used for temperature induced phase separation was precondensed before use by the method of Bordier, (1981). Butylated hydroxytoluene (16 mg) was dissolved in 20 g of Triton X-114. This solution was added to 980 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 and incubated at 0°C for 4 h. After incubation the solution was transferred to a 30°C water bath and incubated overnight to allow condensation of the detergent. Following this the large aqueous phase was removed from above the smaller detergent enriched phase and replaced with an equal volume of 10 mM Tris-HCl, 150 mM NaCl, pH 7.4. The solution was mixed and the condensation repeated a further two times. Following the third condensation the upper aqueous phase was removed and discarded. The concentration of detergent in the final lower phase was determined by comparing the absorption at 280 nm before and after condensation. The precondensed Triton X-114 was then stored at room temperature.

2.5.3 Temperature-induced phase separation of proteins

Temperature-induced phase separation was carried out according to the method of Bordier (1981). EA.hy926 membranes were prepared as described in section 2.4.3. Cell membranes (50 µl, 0.5 mg/ml) were diluted to a total volume of 250 µl in 10 mM Tris-HCl, 0.15 M NaCl, 1% v/v Triton X-114, pH 7.4. Temperature-induced phase separation was performed by incubating on ice for 5 min and layering the solution on to a sucrose cushion (300 µl of 6 % w/v sucrose, 10 mM Tris-HCl, 0.15 M NaCl, 0.06 % v/v Triton X-114, pH 7.4). This was then incubated at 30°C for 3 min then centrifuged at 3000 g for 3 min using an MH2 Desaspeed centrifuge (Sarstedt, Leicester, U.K.). The upper aqueous phase was removed and fresh Triton X-114 was added to a final concentration of 0.5 % v/v. This sample was phase separated as described above and the aqueous phase removed. Triton X-114 was then added to a final concentration of 2 % (v/v) then again phase separated. The resultant supernatant (0.15 ml) was removed, this representing the aqueous phase and the remainder, representing the detergent-rich phase was made up to a total volume of 0.15 ml with 10 mM Tris-HCl , 0.15 M NaCl, pH 7.4. The aqueous and detergent-rich phases were then assayed for several membrane peptidase activities and protein concentration.

2.5.4 Detergent solubilisation profiling

EA.hy926 cell membranes (1 mg/ml), prepared as described in 2.4.3, were incubated with rotation at 4°C in 10 mM HEPES (pH 7.4). Enzyme activities were determined in the total incubation mixture and in the supernatant following centrifugation at 31,000 g for 90 min. Detergent solubilised activity was expressed as a percentage of the total activity in the original incubation sample. The detergents used with CMC data and the actual concentration used were octyl glucopyranoside (CMC 25 mM ; concentration used, 60 mM): Nonidet P-40 (0.29 mM; 6.1 mM) and Triton X-100 (0.24 mM; 5.9 mM).

2.5.5 Solubilisation of membrane proteins with phospholipase C

EA.hy926 cell membranes (1 mg/ml) prepared as described in 2.4.3 were incubated with rotation for 2 h at 37°C in 10 mM Tris-HCl (pH 7.4) with up to 2 units of *B.*

Cereus PLC in a total volume of 1 ml. Following incubation membranes were centrifuged (31,000 g, 90 min) and assayed for membrane peptidase activity. (1 unit corresponds to the amount of PLC needed to hydrolyse 1μmol of L-α-phosphatidylcholine per min at pH 7.5 and 37°C).

2.6 Separation and detection of proteins by PAGE

2.6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) on 10 % w/v polyacrylamide mini-slab gels, or 5%-20% w/v polyacrylamide gradient gels. Non-reducing SDS-PAGE samples were prepared by dilution with SDS dissociation buffer to give a final concentration of 62.5 mM Tris-HCl, pH 6.8 containing 3% w/v SDS, 10% v/v glycerol and 0.001% bromophenol blue. Samples for reducing SDS-PAGE were prepared with 2-mercaptoethanol to a final concentration of 5% v/v. In all cases samples, including molecular weight markers were heated to 100°C for 4 min then centrifuged at 10,000 g for 5 min prior to loading. All gels were run at constant voltage (80 V) for up to 2 h. High molecular weight standard markers were from Sigma and consisted of: myosin (*Mr* 205,000), β- galactoside (*Mr* 116,000), phosphorylase b (*Mr* 97,400), ovalbumin (*Mr* 45,000) and carbonic anhydrase (*Mr* 29,000). Medium weight molecular weight markers used to monitor antibody purification were from BDH Chemicals Ltd and consisted of: ovotransferrin (*Mr* 76-78,000), albumin (*Mr* 66,250), ovalbumin (*Mr* 42,500), carbonic anhydrase (*Mr* 29,000), myoglobin (*Mr* 17,200) and cytochrome c (*Mr* 12,300).

2.6.2 Staining of acrylamide gels

Acrylamide gels were stained using Coomassie blue (30 min), except in the monitoring of antibody purification for which the more sensitive technique of staining using silver nitrate was used (Oakley *et al.*, 1980).

2.6.3 Immunoelectrophoretic (Western) blotting.

Proteins separated by electrophoresis were electophoretically transferred (30 mA constant current for 80 min) in CAPS-NaOH (pH 11.0) containing 10% methanol to an Immobilon-P PVDF membrane (Millipore). The transferred marker lane was cut from the membrane and stained with a solution of 25% isopropanol, 10% acetic acid and 0.1% Coomassie blue R250 followed by destaining using a solution of 5% v/v isopropanol and 10% v/v acetic acid. The remainder of the membrane was washed in TBST (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.1% Tween 20) and incubated at room temperature in: blocking buffer (5% w/v low fat milk powder in TBST) for 1 h; ECE antibody (AEC32-236, raised to rat ECE) at a concentration of 20 μg /ml in TBS containing 0.02% sodium azide for 1 h and peroxidase-conjugated rabbit anti mouse IgG or goat anti rabbit IgG (Amersham), diluted 5000 fold in TBST for 1 h. Between each incubation, the membrane was washed liberally with TBST for 40 min. Protein bands were visualised using the ECL detection system (Amersham) according to the manufacturer's instructions.

2.7 Production of anti-peptide antibodies

2.7.1 Coupling of peptides to keyhole limpet haemocyanin (KLH)

Peptides 1 and 2, NH_3^+CTYKRATLDEED-NH_2 and NH_3^+CQRNPFLQGKRG-NH_2, respectively, were coupled to KLH through the cysteine residue of the peptide as described by Stephenson & Duggan (1991). KLH (20 mg/ml) was dissolved in 10 mM potassium phosphate (pH 7.2), dialysed overnight at 4°C against 10 mM potassium phosphate (pH 7.2) and then the concentration was adjusted to 16 mg/ml. This preparation (250 μl) was mixed with MBS (m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (3 mg/ml in dimethyl formamide) for 30 min at room temperature. Unbound MBS was removed by gel filtration through a column of Sephadex G-25 equilibrated with 50 mM potassium phosphate (pH 6.0). Fractions of 0.5 ml were collected and elution of the KLH-MBS complex was followed by measuring absorbance at 280 nm. KLH-MBS (3 ml) was mixed with 1ml of peptide solution (5 mg/ml in 10 mM potassium phosphate, pH 7.2), the pH adjusted to 7.4 and the solution mixed by gentle rotation for 3 h at room temperature. Solid NaCl was added to a final concentration of 0.09% (w/v). Aliquots of the peptide conjugate were stored at -20°C until required for injection. Efficiency of peptide coupling was monitored using the Ellman reaction (Ellman, 1959). 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB) (4 mg/ml) was freshly prepared in 0.1 M sodium phosphate buffer (pH 8.0) and 20 µl of this solution was mixed with 20 µl of samples taken before and after peptide coupling. Absorbance at 405 nm was measured to determine the relative concentration of free sulphydryl groups remaining and uses a molar extinction coefficient for the thionitrobenzoate anion of $\varepsilon = 14{,}100\ M^{-1}\ cm^{-1}$.

2.7.2 Antiserum production

Two male New Zealand White rabbits were injected per peptide. Rabbits were immunised by a series of sub-cutaneous injections of KLH-conjugated peptide (each containing 0.25 - 0.5 mg of peptide) at 3 week intervals (Widdows *et al.*, 1987). The first sample was mixed with an equal volume of Freund's complete adjuvant whilst subsequent booster injections were performed using samples mixed with an equal volume of Freund's incomplete adjuvant. Maintenance of the rabbits and all immunisation procedures were carried out by the staff of the University of Leeds animal house.

2.7.3 Coupling of peptide to resin

Synthetic peptide (3 mg) was dissolved in 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. DTT 0.5 M, (50µl) was added and the solution incubated under nitrogen for 1 h at room temperature. The reduced peptide was then separated from excess DTT on a Sephadex G-10 column equilibrated in 50 mM Tris-HCl, 5 mM, pH 8.5. Fractions of 1 ml were collected and monitored for absorbance at 280 nm. The peak void volume fractions were pooled and the second, larger peak containing only DTT was discarded. Sulpholink coupling gel (3 ml) was washed at 25°C in a sintered glass funnel with 50 mM Tris-HCl, 5 mM EDTA, pH 8.5 (20 ml). The gel was then centrifuged (400g, 2 min) and the supernatant removed. The peptide solution eluted from the G-10 column was incubated with the sulpholink gel for 1 h in the dark with gentle rotation, then centrifuged (400 g, 2 min) and the supernatant removed. Efficiency of peptide coupling was then assessed using the Ellman reaction (Ellman 1959) to compare the sulphydryl content of duplicate samples (100 µl) of the final supernatant with that of the peak fractions from the G-10 eluate. Excess peptide was removed by washing the gel with 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. This was repeated a further 4 times. The gel was then incubated for 1 h at 25°C with 50 mM cysteine in 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. Following transfer to a sintered filter funnel the gel was washed repeatedly with 1 M NaCl. The gel was equilibrated in PBS containing 0.02 % w/v sodium azide and stored at 4°C.

2.7.4 Affinity purification of antisera

The peptide-sulpholink affinity gel, synthesised as described in section 2.7.3 was washed with 250 ml of PBS. Antisera (5 ml) were passed through an 80 µm filter and incubated with the gel overnight at 4°C with gentle agitation. The gel was transferred to a column and washed at 10 ml/h with 10 mM sodium phosphate buffer containing 800 mM NaCl (pH 7.2). Eluted fractions of 1 ml were collected and the absorbance at 280 nm monitored. When the absorbance of the eluted fractions had decreased to <0.05, the antibody bound to the column was eluted by washing the column with 0.2M glycine-HCl, pH 2.4 and by collecting 1 ml fractions into tubes containing 1 M Tris-HCl buffer (pH 8.0). Absorbance at 280 nm was again monitored and peak fractions with an absorbance of >0.05 were combined and dialysed against 1 L PBS for 24 h at 4°C. Antibodies were concentrated by PEG dialysis followed by dialysis against 1 L PBS for 24 h at 4°C. Antibodies were then dialysed against PBS containing 0.02 % w/v sodium azide and stored at 4°C. Following elution, the peptide-sulpholink affinity gel was equilibrated with PBS until the pH returned to 7.5. The column was then stored in PBS containing 0.02 % sodium azide.

2.7.5 E.L.I.S.A. (Enzyme-linked immunosorbent assay)

Immunoreactivity of test bleeds taken from immunised rabbits was monitored by using an adaptation of the methods of Stephenson & Duggan (1991) and Howell *et al.*, (1991). Pro-bind 96-well plates were coated with 80 µl peptide per well (0.25 µg/ml in 50 mM $NaHCO_3$, 10 mM NaOH, pH 9.6) overnight at room temperature. The plates were then washed five times with phosphate buffered saline (PBS), 0.05 % v/v Tween-20 and each well then incubated with 200 µl of PBS containing 5 % (w/v) low-fat milk powder for 2 hours at 37°C. Washing was repeated and antisera added in serial dilutions in antibody buffer (PBS containing 1 % low-fat milk powder) and incubated overnight at 37°C. The plates were again washed and then incubated with 100 µl horseradish peroxidase linked IgG (goat anti-(rabbit IgG) IgG) diluted 1 : 1000 in antibody buffer, for 1 h at 37°C. The washing was repeated and 100 µl of substrate solution added to each well, (0.55 mg/ml 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) with 0.01 % v/v H_2O_2 in citrate buffer (2.85 ml of 0.1 M citric acid and 21.5 ml of 0.2 M sodium phosphate made up to a total volume of 50 ml with millipore grade H_2O). The plate was then incubated at room temperature in the dark for 30 min. After 30 min the reaction was terminated by the addition of 100 µl of 0.1 M citric acid to the wells and absorbance measured at 405 nm using an Anthos 2001 plate reader. Each sample was tested in quadruplicate using the complementary peptide as a control to detect non-specific antigen recognition.

2.7.6 Western blotting of transfected cell lines

The specific recognition of the ECE-1 α isoform by purified antibodies was monitored using western blotting of membranes prepared from transfected and non-transfected COS and chinese hamster ovary cells (CHO). These membranes were provided by M. V. Hoang and C. D. Brown (University of Leeds, Leeds, U.K.). Briefly, the expression vectors pcDL-SRα296/rECE-1α and pcDL-SRα296/rECE-1β were used to transfect COS and CHO cells. The cells were transfected at 50 - 80 % confluence in 175 ml^2 flasks. Plasmid DNA (88 µg) was added to 4.4 ml of OptiMem and 200 µl of Lipofectamine reagent was added. This mixture was incubated at room temp. (20 min) then added to the cells which were first washed with OptiMem. The cells were incubated with the DNA complexes for 3 h at 37°C, 5 % CO_2. An equal volume of growth medium was added without removing the transfection mixture. All media were replaced after 24 h. Cells were collected and membrane fractions prepared 48 h after transfection.

2.8 Immunoadsorption of peptidase activities

2.8.1 Coupling of antibody to cyanogen-bromide activated Sepharose 4B

Affinity purified GK4A9 antibody (5 mg/ml, 1ml), previously dialysed against 0.25 M $NaHCO_3$/ NaOH, 0.5 M NaCl, pH 8.7 (coupling buffer) was coupled to cyanogen bromide-activated Sepharose 4B beads (1 ml swollen beads) according to the manufacturer's instructions. The coupling procedure was performed at room temperature for 2 h after which the remaining active groups on the gel matrix were blocked by transferring the gel and resuspending in 0.2 M glycine, pH 8.0 for 2 h at room temperature. Excess glycine was removed by alternate washing with 1 M sodium acetate buffer (pH 4.0) and coupling buffer (pH 8.7). The gel matrix with coupled antibody was transferred to PBS containing 0.02 % w/v sodium azide and stored at 4°C.

2.8.2 Immunoadsorption of membrane peptidase activities

GK4A9, a monoclonal antibody was immobilised to Sepharose 4B beads as described in section 2.8.1. The immunoaffinity beads were stored in a 1 : 1 suspension of beads : buffer. Immunoaffinity column volumes were determined by measuring a known volume of this suspension and sedimenting beads by centrifugation at 10,000 g for 2 min. The supernatant was removed and discarded, the beads resuspended and incubated at 4°C with rotation in an equal volume of 0.1 M Tris-HCl (pH 7.5), containing 1M NaCl and 3.5 % w/v Triton X-100. Beads were again sedimented (10,000 g, 2 min.) and the supernatant removed. The precipitated beads were then incubated with rotation with a standard volume (500 μl) of detergent solubilised membrane sample (w/w ratio of 7:1, detergent : protein) for 16 h at 4°C. Samples were centrifuged (400 g, 2 min), supernatants collected and assayed for E-24.11, AP-N, ECE and protein, comparing activities found in the immunoadsorbed samples with those found in the original detergent solubilised sample. Beads were washed with 0.2 M glycine, then equilibrated in PBS (pH 7.4) containing 0.02 % w/v sodium azide. The beads were then stored at 4°C.

CHAPTER THREE

THE BIOCHEMISTRY OF ECE

3.1 AIMS

To characterise the human endothelial cell line, EA.hy926 with regard to peptidase content and to assess its suitability for the study of ECE.

To determine the type of membrane anchorage by which ECE associates with the membrane.

To screen mono and polyclonal antibodies against E-24.11 for cross reactivity with ECE.

3.2 Introduction

In the initial stages of this work there was limited knowledge regarding ECE activity and the biochemical nature and characteristics of this protein. The human endothelial cell line, EA.hy926, had previously been found to show sustained expression of many functions associated with the vascular endothelium (Edgell *et al.*, 1983) and had also been found to express big ET-1 mRNA and to secrete ET-1 into the culture medium (Saijonmaa *et al.*, 1991), suggesting this cell line did express ECE activity. For this reason, EA.hy926 was used as a model system for the study of ECE. The potential benefits and problems associated with tissue culture compared with the use of primary tissue types are discussed further in section 1.9.1.

The early work of this project involved the optimization of conditions for the preparation of cell membranes from these cells and the assay of enzyme activities in the membrane fraction. A number of membrane peptidases were assayed to produce a profile of peptidase expression in this cell line which was used later in comparison with other cell lines and to determine the effects on expression of peptidases in response to peptidase and tyrosine phosphatase inhibitors.

Having established EA.hy926 as a cell line suitable for the study of the physiological ECE protein, the work focused on the identification of the anchorage mechanism by which the ECE protein associates with cellular membranes. This work relied on detergent solubilisation techniques and involved comparisons with E-24.11 which was known to be a type II integral membrane protein.

At the time of this part of the work, no antibodies to ECE were available. As a method of overcoming this problem existing polyclonal and monoclonal antibodies raised to E-24.11 were examined for possible co-recognition of ECE. The primary objective of this part of the study was to investigate the possibility of a rapid purification of ECE from cell membrane preparations.

3.3 Membrane peptidase expression in EA.hy926 cells

3.3.1 Expression of E-24.11 by EA.hy926 cells

In order to characterize the levels of expression of E-24.11 by EA.hy926 cells and also to determine the suitability of the RP161 polyclonal antibody to E-24.11 for use in western blotting, EA.hy926 membrane preparations were prepared as described in section 2.4.1 and subjected to SDS-PAGE and western blotting (Fig. 3.1). Kidney brush border membranes, known to be a rich source of E-24.11 were examined in parallel with various amounts of EA.hy926 membrane preparation.

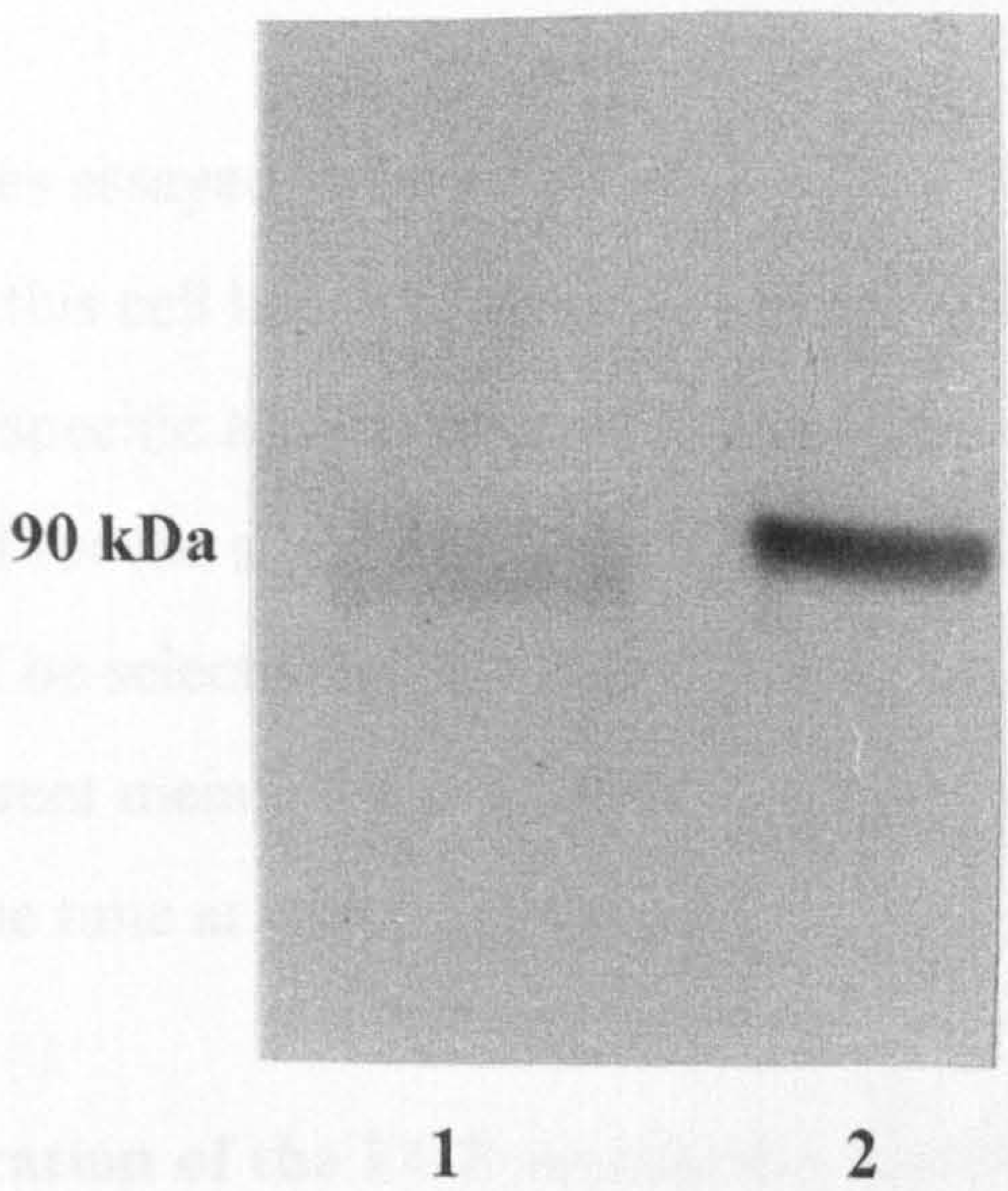
As expected, E-24.11 was strongly detected in the kidney brush border membrane preparation (*M*r 90 kDa) and the specificity of the antibody was supported by the fact that no other protein bands were detected. The expression of E-24.11 by EA.hy926 cells was also confirmed by immunoblotting (Fig. 3.1) and enzyme assay which showed E-24.11 levels to be <1 % of those found in kidney brush border membrane preparations.

Significantly, the RP161 polyclonal antibody did not reveal immunorecognition of any protein of molecular weight >90 kDa indicating this antibody had no immunoreactivity towards ECE.

3.3.2 Profile of membrane peptidase activities

Initial studies using the EA.hy926 cell line involved assaying membrane preparations for a range of peptidase activities. Six different membrane preparations were assayed for each activity. All assays were conducted in parallel with kidney brush border membranes except for ECE which, due to the high levels of E-24.11, precluded an accurate determination of ECE in these membranes.

Fig. 3.1 Detection of the E-24.11 protein expressed by EA.hy926 cells by western blotting of membrane preparations using the polyclonal antibody RP161



Flask cultured EA.hy926 cells were grown until confluent and then harvested and membranes prepared as described in section 2.4.1. Cell membranes prepared from EA.hy926 cells were loaded on lane 1 (15 μg protein) and membranes prepared from fresh pig kidney (5 μg) were loaded into lane 2. Proteins were separated by gel electrophoresis using a 10 % SDS-polyacrylamide gel followed by immunoblotting with the polyclonal E-24.11 antibody, RP161.

Enzyme assay of membranes prepared from EA.hy926 cells failed to detect ACE and DPP-IV activities, often considered to be markers of the endothelial cell phenotype. This finding may have been due to the limits of sensitivity of the assay but was supported by attempted immunostaining for both activities which showed neither to be present in this cell line (Dr. K. Barnes, unpublished data).

Other activities assayed included AP-N, E-24.11 and ECE which were all found to be expressed by this cell line, indicating EA.hy926 cells to be a suitable endothelial model system. The specific activity of E-24.11 activity relative to ECE showed this cell line could be used for the study of ECE since virtually all the E-24.11 big ET-1 degrading activity could be selectively inhibited by thiorphan. The reproducibility of these values over six different membrane preparations showed expression levels to be essentially constant at the time at which cells were harvested. Table 3.1.

3.4 Identification of the ECE membrane anchor

3.4.1 Temperature-induced phase separation of membrane proteins

Integral membrane proteins are characterised by the presence of a hydrophobic domain which interacts directly with the hydrophobic core of the lipid bilayer (Tanford & Reynolds, 1976). Triton X-114 is effective at solubilising membrane proteins at low temperatures forming mixed detergent-protein micelles (Egan *et al.,* 1976). Phase separation of Triton X-114 (Bordier, 1981) occurs at 30° C and results in the formation of two separate phases. The mixed micelles of amphiphilic proteins and detergent were found to aggregate with the remaining detergent while hydrophilic proteins, which have lost their hydrophobic membrane anchoring domain were found to remain in the aqueous phase. This allows the effective separation of membrane-bound from cytosolic and peripheral membrane proteins. Temperature-induced phase separation of proteins expressed by EA.hy926 cells was performed according to the method described in section 2.5.3. The partitioning of E-24.11, ECE and LDH was compared by determination of the percentage of the total activity found localised in the detergent-rich phase (Table 3.2).

Table 3.1 Profile of specific activities of membrane peptidases expressed in EA.hy926 endothelial cells

Membrane Peptidase	EA.hy926	Kidney Brush Border
E-24.11	4.9 ± 0.2	576.8 ± 30.1
AP-N	23.3 ± 4.8	232.2 ± 9.8
DPP-IV	N.D	284.1 ± 14.7
ACE	N.D	296.6 ± 15.6
ECE	1.5 ± 0.1	N.A

Specific activities are given in nmol/min/mg except the value for ECE which is given as pmol/min/mg.

N.D Activity not detected

N.A Activity not available. The high expression levels of E-24.11 found in kidney brush border membranes, coupled with the relatively low levels of ECE in this tissue type precluded determination of ECE activity.

Specific activities of a range of membrane peptidases were determined in membrane preparations from EA.hy926 cells and compared with the activities found in membranes prepared from kidney brush borders. E-24.11 and ACE were assayed using the HPLC methods described in section 2.3.1 and 2.3.3. DPP-IV and AP-N were assayed using the spectrophotometric methods described in sections 2.3.2. ECE was assayed using the radioimmunoassay protocol described in section 2.3.6 and 2.3.7.

Results are the mean of 6 experiments ± S.E.M.

Table 3.2 Temperature-induced phase separation of E-24.11, ECE and lactate dehydrogenase (LDH)

Activity	Total Activity Recovered in the Detergent-Rich Phase (%)
E-24.11	87.3 ± 6.7
ECE	78.2 ± 7.2
LDH	12.4 ± 7.8

Membranes prepared from EA.hy926 cells were subjected to temperature-induced phase separation using Triton X-114 as described in section 2.5.3. Following the recovery of the detergent-rich phase, enzyme assays were performed and the activities of E-24.11, ECE and LDH determined in this phase and compared to the total activity in untreated membrane preparations. Results are given as the mean ± S.D. for 6 separate experiments.

LDH a known cytosolic protein was found to partition predominantly in the detergent-depleted phase. In contrast, the majority of both ECE and E-24.11 activity was found to be located in the detergent-rich phase. These data suggested that ECE, like E-24.11 was an integral membrane protein.

3.4.2 Detergent solubilisation profiles of membrane peptidases in EA.hy926 Cells

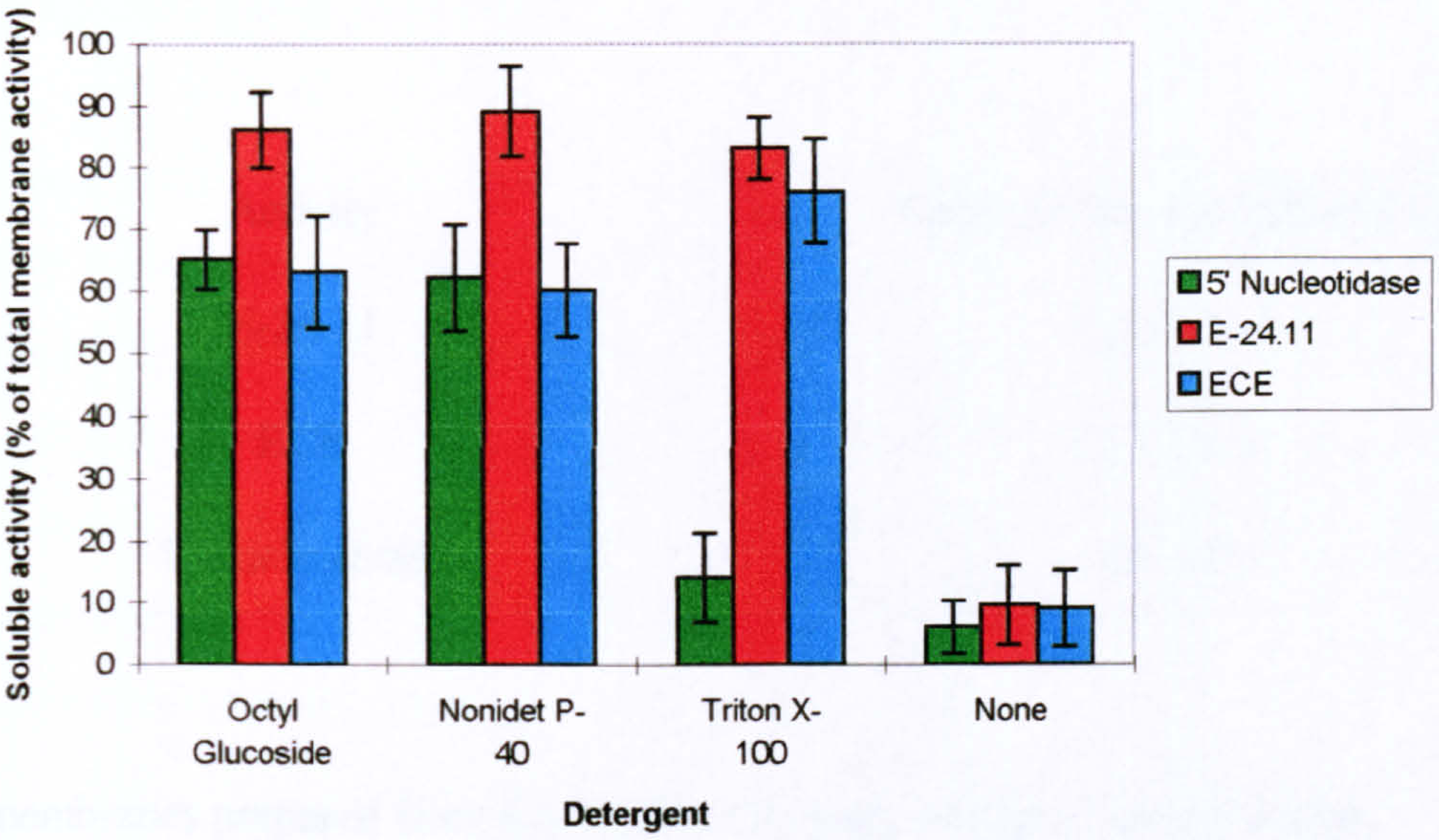
Detergent solubilisation profiling is a useful technique used for distinguishing between G-PI (glycosyl-phosphatidylinositol) anchored and transmembrane proteins (Hooper & Turner, 1988 a,b; Hooper, 1992). Differentiation of G-PI and non G-PI anchored proteins is accomplished on the basis of the ability of detergents of varying critical micellar concentrations (CMC) to solubilise proteins to differing extents. In this study the solubilisation profile of E-24.11 and ECE was compared with that of an established G-PI anchored protein, 5'-nucleotidase (Fig. 3.2). 5'-nucleotidase was assayed in preference to alkaline phosphatase (EC 3.1.3.1) (section 2.3.4) since the assay was less susceptible to interference due to the detergent content of the samples.

Three detergents of varying CMC were used to solubilise each activity. Whilst detergents of relatively high CMC, including octyl glucoside and Nonidet P-40, were adept at solubilising all three activities it can be seen that a detergent with a low CMC such as Triton X-100 is unable to solubilise the G-PI anchored protein. This pattern of solubilisation suggested neither ECE nor E-24.11 was G-PI anchored and the similar solubilisation profile of E-24.11 and ECE further suggested close topological similarities between the two peptidases.

3.4.3 Bacterial phospholipase C solubilisation of EA.hy926 membrane proteins

A property of G-PI anchored proteins is the ability of most of these to be released from cell membranes by the degradation of the G-PI moiety by bacterial phosphatidylinositol specific phospholipase C (PLC). It was the discovery of this property which gave the first indications of the existence of G-PI anchored proteins (Low, 1989, 1990).

Fig. 3.2 Detergent solubilisation profile of E-24.11, ECE and 5'-nucleotidase



Results are the mean ± S.D. for 4 separate experiments.

Cell membranes prepared from EA.hy926 cells (1 mg/ml) were solubilised with detergents of various critical micellar concentrations (section 2.5.4). Assays were carried out to determine the extent to which each detergent was able to solubilise E-24.11 and ECE. The HPLC protocol (section 2.3.1) was used to assay E-24.11 activity since this method was found to be less susceptible to interference by detergents. These data were compared with the solubilisation of a known G-PI anchored protein, 5'-nucleotidase assayed as described in section 2.3.5.

Table 3.3 Solubilisation of membrane proteins using bacterial phosphatidylinositol-specific phospholipase C

Activity	Total activity solubilised (%)
E-24.11	22.1 ± 9.1
ECE	13.7 ± 5.2
5'-nucleotidase	74.9 ± 7.6

Cell membranes prepared from EA.hy926 cells were incubated with bacterial phosphatidylinositol specific phospholipase C and the solubilised fractions retrieved (section 2.5.5). The results are the means ± S.D. for four separate experiments.

EA.hy926 membranes (1mg/ml) were incubated with a commercial preparation of PLC from *B. Cereus* for 2 h at 37°C and peptidase activities assayed in the supernatant to determine the extent of solubilisation. The results are shown in Table 3.3.

The percentage of ECE and E-24.11 activity solubilised by PLC was relatively low and probably due to contamination of the PLC preparation with protease activity. The inability of PLC to solubilise either activity to a major extent supported the evidence that neither ECE nor E-24.11 was anchored by a G-PI moiety and these two proteins shared the same membrane anchorage. This was ultimately confirmed when ECE was finally purified, cloned and sequenced (Xu *et al.*, 1994; Shimada *et al.*, 1994; Schmidt *et al.*, 1994; Ikura *et al.*, 1994; Yorimitsu *et al.*, 1995).

3.5 Recognition of ECE by antibodies raised to E-24.11

Prior to the purification, cloning and sequencing of ECE, no antibodies were yet available to the protein. Evidence was mounting that both E-24.11 and ECE shared many common biochemical characteristics and it was therefore considered likely that these proteins originated from the same peptidase family, suggesting the possibility of a degree of sequence homology between the two. The purpose of this work was to investigate whether monoclonal antibodies to E-24.11 might share a common epitope with ECE. Further studies investigated whether this approach might prove useful for rapid purification of ECE.

3.5.1 Immunoprecipitation of ECE and E-24.11 by GK4A9

A monoclonal antibody raised to E-24.11, GK4A9 (Gee & Kenny, 1985) was immobilised on Sepharose as described in section 2.8.1. Volumes of GK4A9 coated Sepharose beads (0µl to 300µl) were incubated with Triton X-100 solubilised EA.hy926 cell membranes (0.5 mg protein) and the beads precipitated by centrifugation. The specific activities of E-24.1l, ECE and AP-N and protein content were determined in the immunodepleted supernatants and given as a percentage of total activity in untreated membranes (Fig. 3.3).

These data showed ECE and E-24.11 to co-precipitate when incubated with GK4A9 coated Sepharose beads. The control incubation of CNBr activated Sepharose beads with no GK4A9 attached did not show any reduction in either of these activities suggesting specific immunorecognition by GK4A9 of both ECE and E-24.11. The specificity of this binding was further supported by the finding that there was no significant reduction in protein nor AP-N activity. The effectiveness of the immunodepletion of E-24.11 activity from EA.hy926 cells using this antibody is demonstrated by immunoblotting with the RP161 polyclonal antibody to E-24.11 (Fig. 3.4). This shows a significant reduction in levels of E-24.11 protein following immunodepletion.

3.5.2 Immunodepletion of ECE from transfected CHO cells using GK4A9

To examine whether GK4A9 immunoprecipitated ECE activity, volumes of GK4A9 labelled Sepharose beads (300 µl) were incubated with Triton X-100 solubilised membranes prepared from ECE transfected CHO cells which did not express E-24.11. Beads were precipitated and the immunoreactivity of the membrane preparations following immunoadsorption determined (Fig. 3.5). Bound proteins were eluted into the same volume using glycine (pH 2.3) and samples examined for ECE content by immunoblotting with the monoclonal antibody to ECE, AEC32-236.

Since this cell line did not express E-24.11 activity, any substrate breakdown could be attributed to ECE activity. Volumes applied to the column, the immunodepleted fraction and the eluted fractions were consistent in volume (100 µl). The total activity in the immunodepleted fraction was found to be approximately 10 % of the activity present in the same sample prior to incubation with the GK4A9 antibody. This is consistent with immunoblotting (Fig. 3.5) which showed a significant reduction in levels of ECE protein in the sample following immunodepletion. Following removal of bound protein from the column, there is evidence that ECE is detectable in the eluted fractions.

3.5.3 Assessment of the effectiveness of thiorphan and PR inhibition of E-24.11 activity

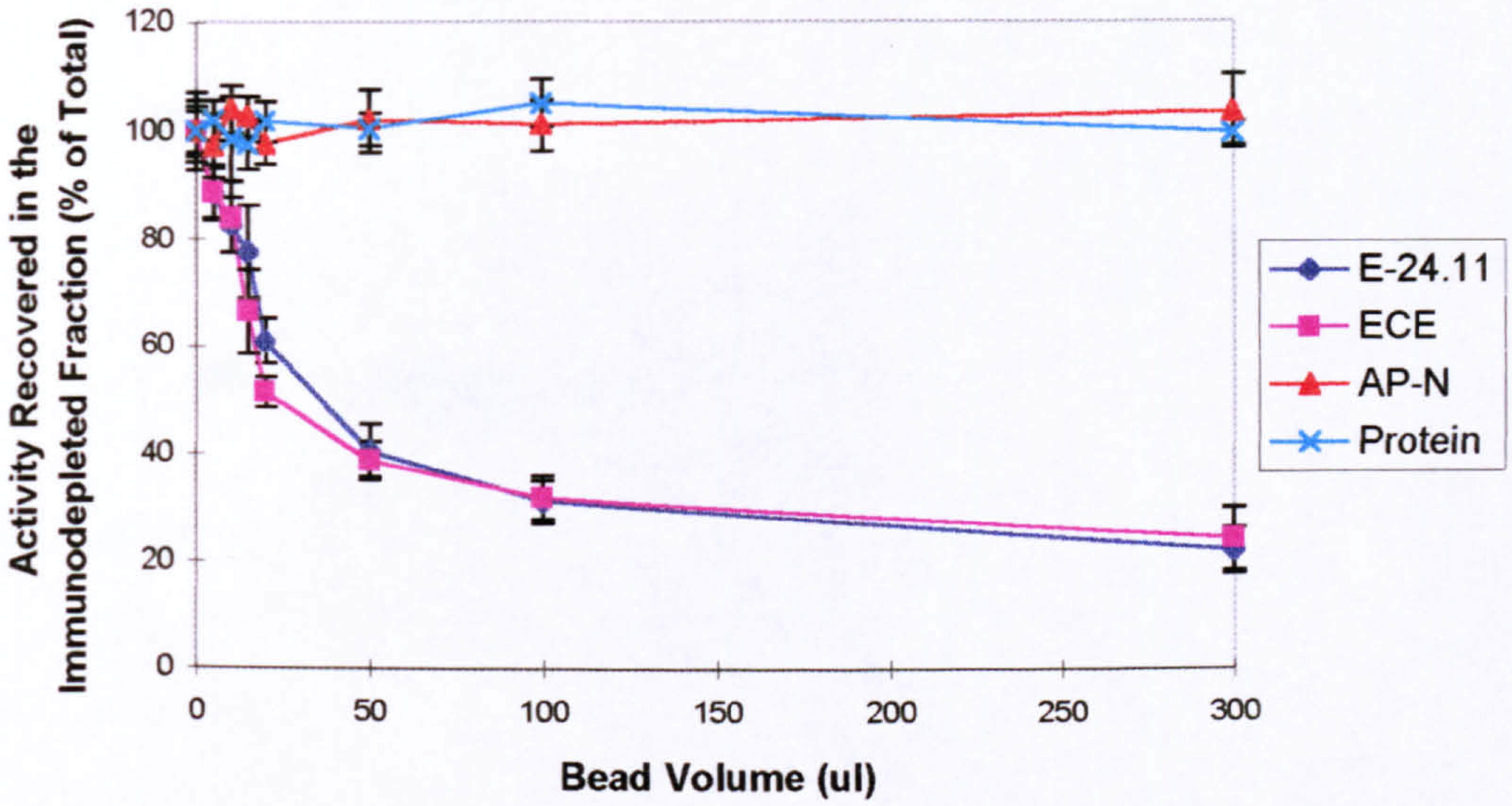
The assay of immunodepleted fractions and the apparent recognition of ECE was dependent on the distinction between ECE and E-24.11 activity. The compound thiorphan was routinely used to inhibit E-24.11 activity selectively ensuring all big ET-1 degrading activity was solely due to breakdown by ECE. Oxidation of the sulphydryl groups of thiorphan has been shown to reduce its potency which would allow cleavage of big ET-1 by E-24.11. In order to ensure this was not the case and that all big ET-1 breakdown was due exclusively to ECE activity, the inhibition of E-24.11 by both phosphoramidon and thiorphan was determined. These data demonstrated both thiorphan and phosphoramidon inhibited >90 % of total E-24.11 activity in membranes prepared from both EA.hy926 cells and pig lung.

3.5.4 Immunoprecipitation of peptidase activities by 3C7 monoclonal antibody to E-24.11

The apparent recognition of ECE by GK4A9 supported the hypothesis that ECE and E-24.11 may originate from the same protein family and therefore likely to share significant sequence homology. With this in mind, a second monoclonal antibody to E-24.11, 3C7 (Gee & Kenny, 1985), was tested for potential co-immunorecognition of ECE and E-24.11 using the same protocols used for GK4A9. Immunoadsorbed samples were assayed for E-24.11, ECE, AP-N and protein (Fig. 3.6).

These data showed 3C7 to be considerably less effective at precipitating E-24.11 protein from membrane preparations compared with the GK4A9 antibody. In addition, this antibody showed no specificity toward ECE since levels of immunoprecipitation of this protein mirrored that of protein binding in general and also the precipitation of AP-N. No further studies were carried out involving this antibody.

Fig. 3.3 Immunodepletion of EA.hy926 cell membranes using GK4A9 labelled Sepharose beads



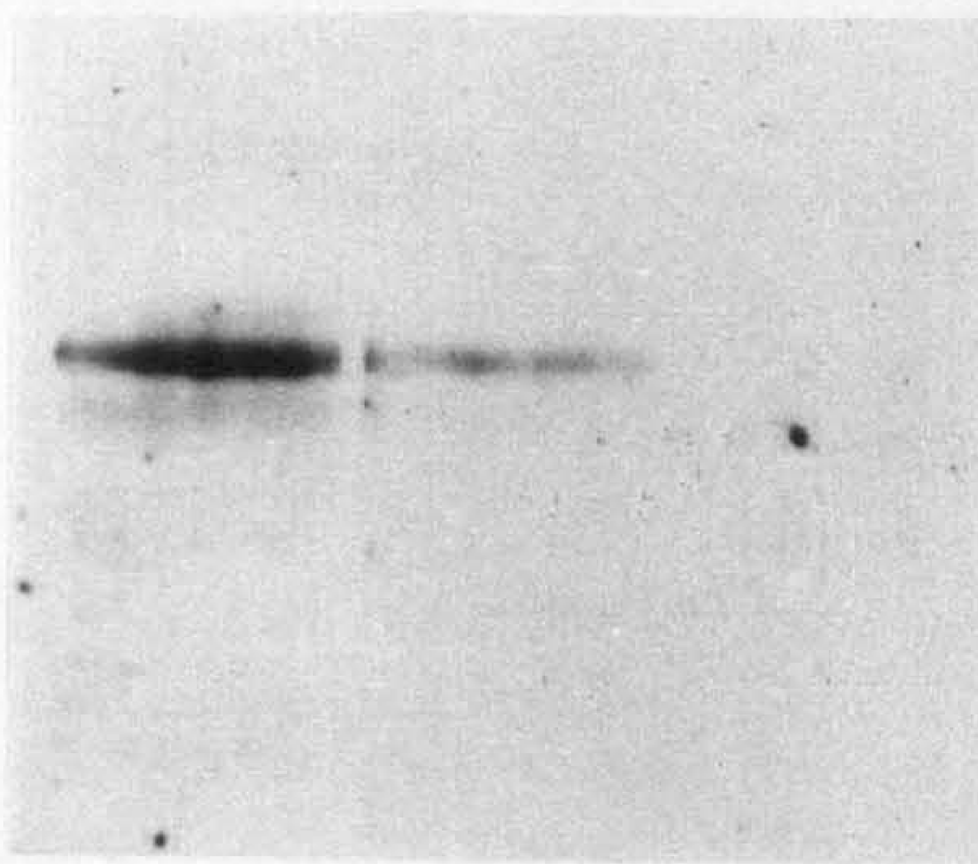
EA.hy926 cell membranes (1 mg/ml) were solubilised with Triton X-100 and incubated with 0 to 300 µl of GK4A9 labelled Sepharose beads for 24 h at 4°C. Beads were removed by centrifugation and the remaining immunodepleted fraction was assayed for E-24.11 (using the spectrophotometric protocol described in section 2.3.1), ECE, AP-N and protein. These data were compared with values for untreated membranes (shown here as 0 µl beads). Levels of enzyme activity in the immunodepleted fractions were then calculated as a percentage of the total in untreated membranes.

The results are shown as the mean ± S.D. for four experiments.

Fig. 3.4 Immunoprecipitation of E-24.11 from EA.hy926 cells detected by the polyclonal antibody to E-24.11, RP161

kDa

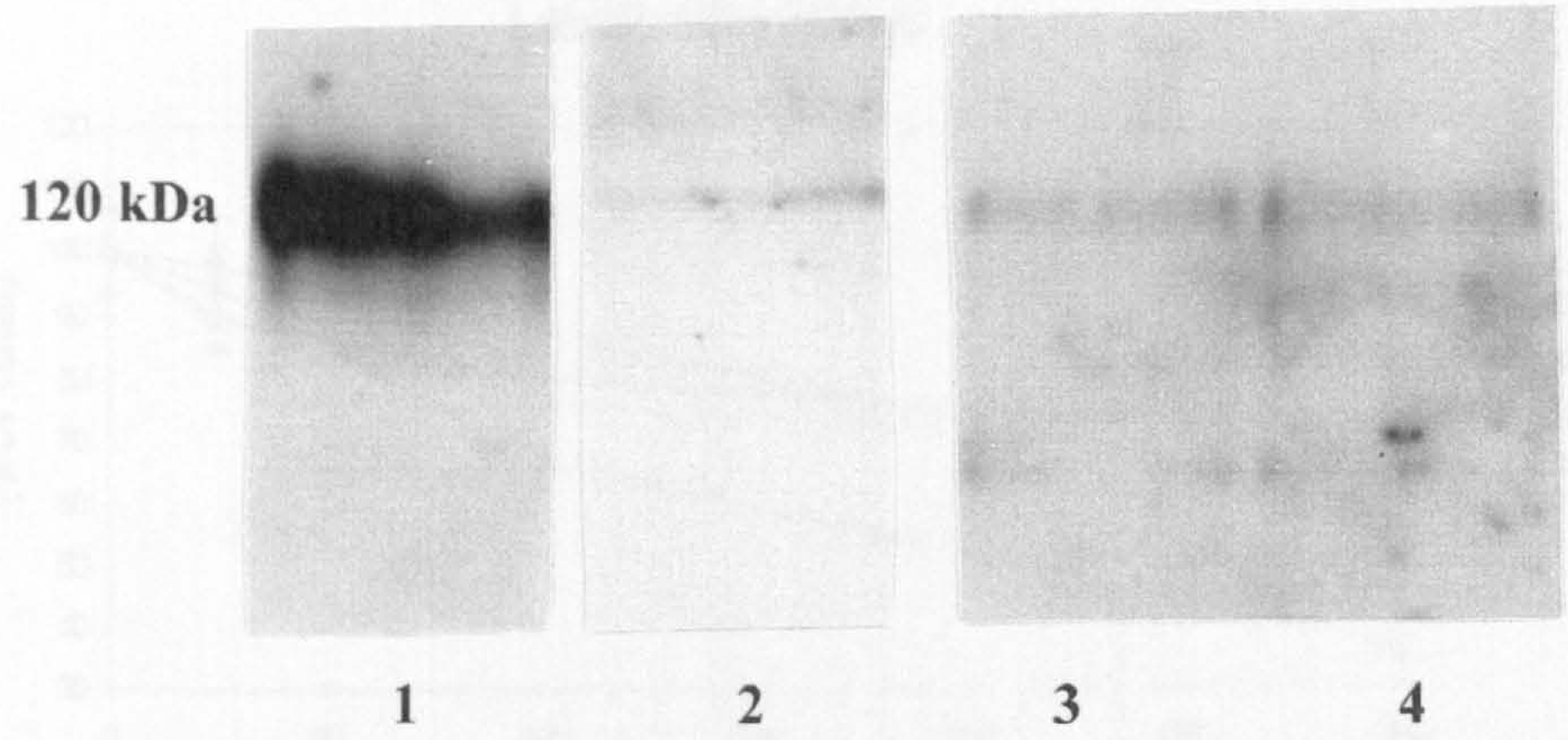
90



1 2 3

EA.hy926 cell membranes (1 mg/ml) were solubilised with Triton X-100 and incubated with GK4A9 labelled Sepharose beads (300 μl) for 24 h at 4°C. Beads were removed by centrifugation (10,000g, 2 min.) and bound proteins eluted from the beads. Samples (10 μg of protein) of EA.hy926 membranes prior to immunodepletion (lane 1), following immunodepletion (lane 2) and proteins precipitated by GK4A9 (lane 3) were separated on 10 % SDS-polyacrylamide gels under reducing conditions and immunoblotted, using the polyclonal antibody to E-24.11, RP161.

Fig. 3.5 Immunoprecipitation of ECE activity from CHO cells transfected with ECE detected by the monoclonal antibody AEC32-236



Total ECE activity (%)	**100**	**12.3**

1 Cell membranes before immunodepletion

2 Cell membranes after immunodepletion

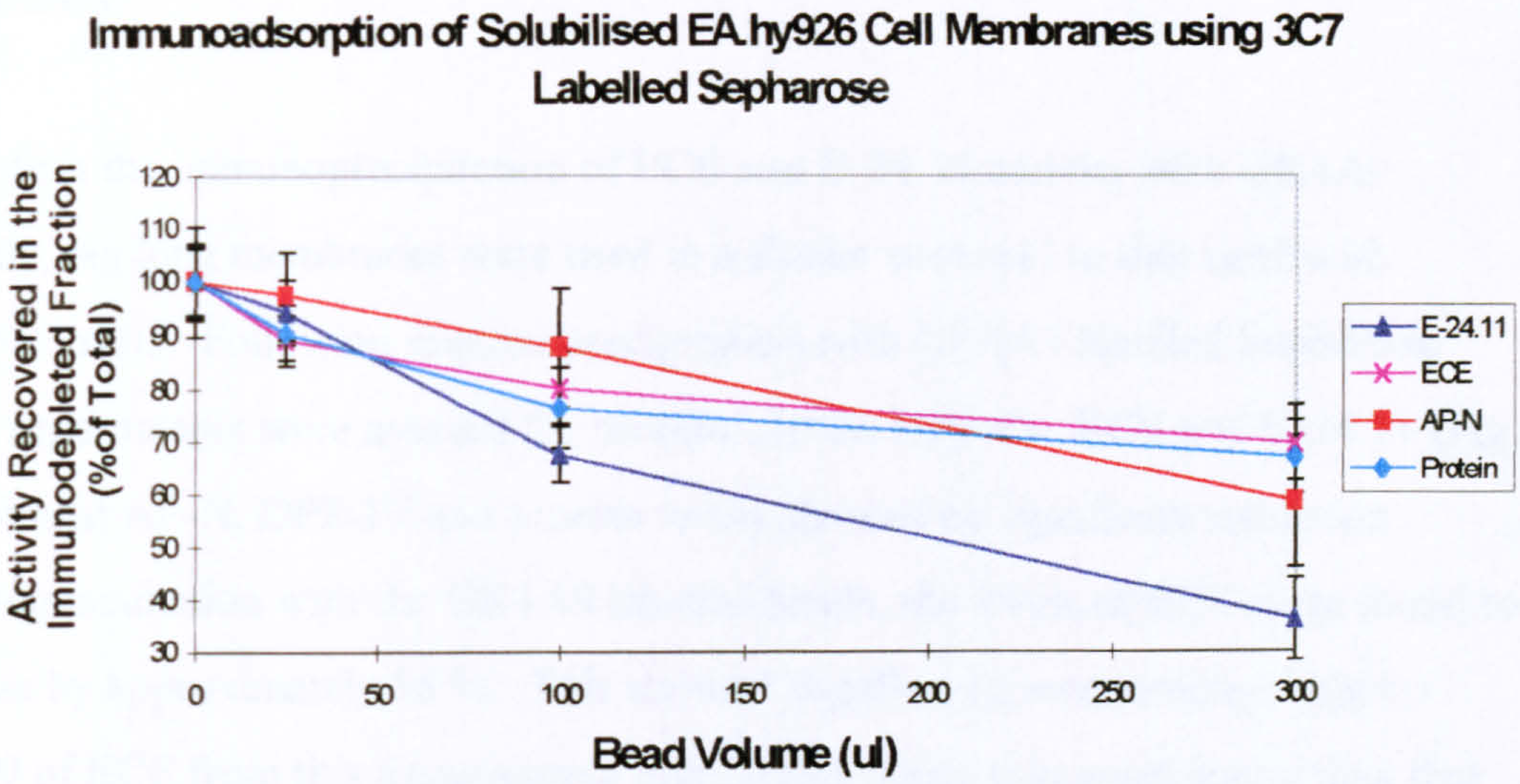
3 Eluted fraction 1

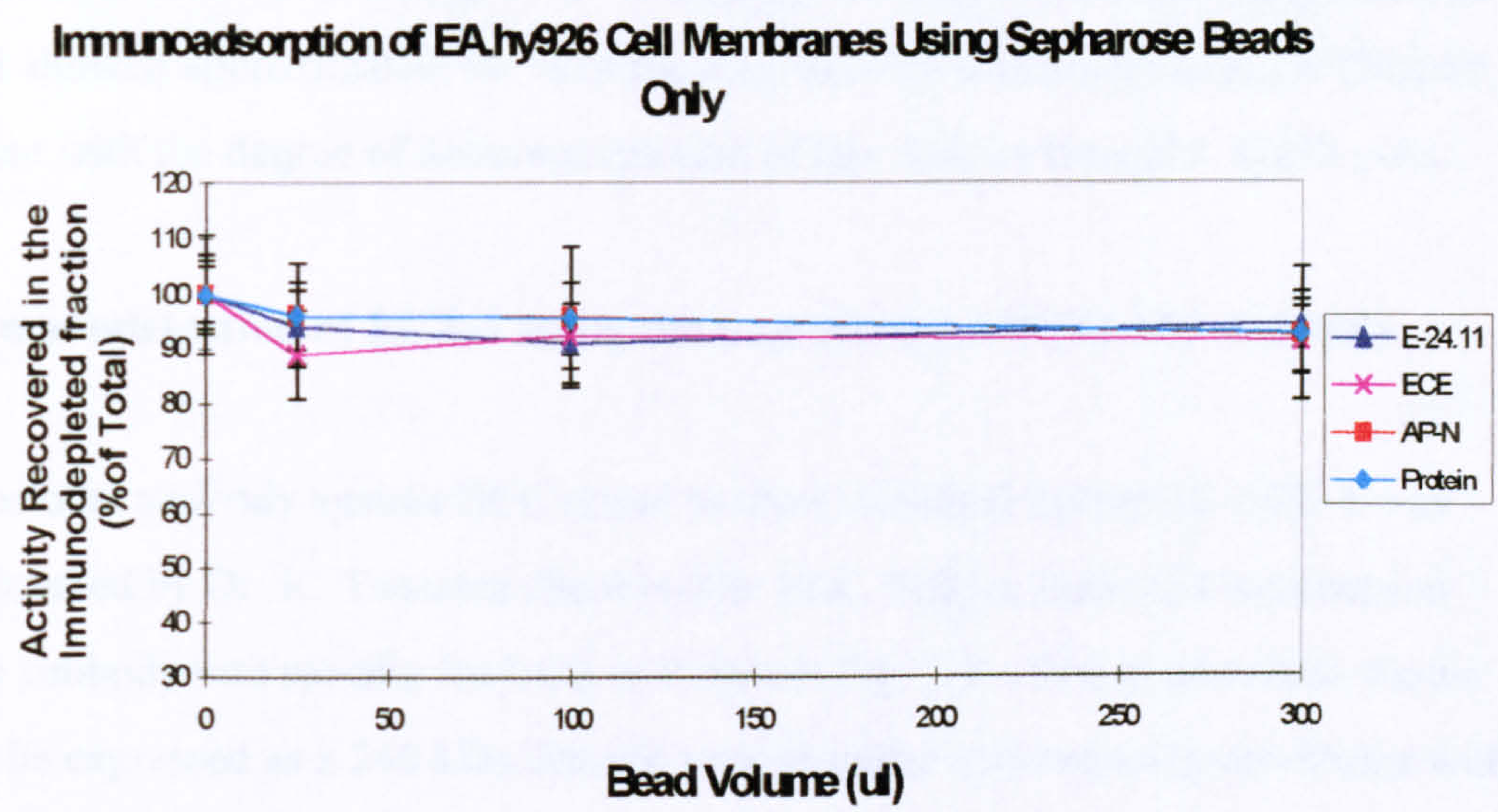
4 Eluted fraction 2

Triton X-100 solubilised membranes (1.4 mg/ml protein) were prepared from CHO cells transfected with ECE cDNA and incubated with GK4A9 labelled Sepharose beads (300 μl) for 24 h at 4 °C. Beads were recovered by centrifugation (10,000 g, 10 min) and the supernatant retained as the immunodepleted fraction. Bound proteins were eluted using glycine (pH 2.3). Volumes applied to the column (lane 1), the immunodepleted fraction (lane 2) and the eluted fractions (lanes 3 and 4) were consistent in volume (100 μl).

Samples (12 μl / well) were examined for ECE protein content by separating proteins on a 10 % SDS-polyacrylamide gel. ECE was detected using the monoclonal antibody to ECE, AEC32-236.

Fig. 3.6 Immunoadsorption of solubilised EA.hy926 cell membranes using 3C7 labelled and unlabelled Sepharose beads





EA.hy926 cell membranes (1 mg/ml) were solubilised using Triton X-100 and incubated with various volumes of unlabelled and 3C7 labelled Sepharose beads for 24 h at 4°C. Beads were removed by centrifugation (10,000 g, 10 min) and the supernatants assayed for E-24.11 (using the spectrophotometric protocol), ECE, AP-N and protein. Results are shown as the mean ± S.D. for 4 experiments.

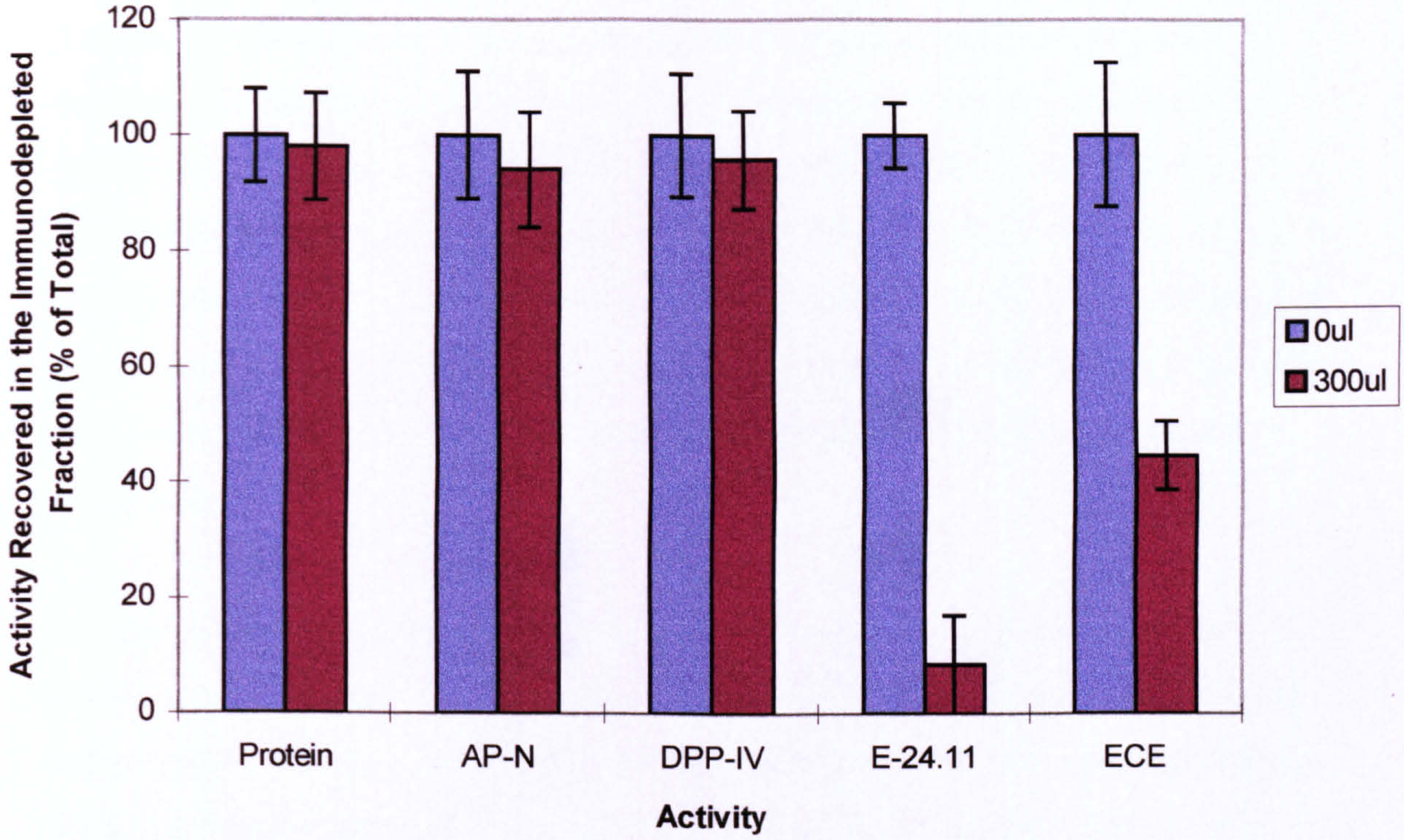
3.5.5 Immunoprecipitation of peptidase activities by GK4A9 from pig lung membranes

To confirm the immunoprecipitation of ECE and E-24.11 activity with GK4A9 antibody, pig lung membranes were used in a similar protocol to that used with EA.hy926 cells. Following immunoprecipitation with GK4A9 labelled Sepharose beads, supernatants were assayed for protein, AP-N, DPP-IV, ECE and E-24.11 (Fig. 3.7). Whilst AP-N, DPP-IV and protein levels showed no significant reduction following incubation with the GK4A9 labelled beads, the levels of ECE were found to decrease by approximately 55 %. This showed significant immunorecognition by GK4A9 of ECE from this tissue source though this figure was much lower than that reported for EA.hy926 cells (Fig. 3.3). In contrast the degree of immunodepletion of E-24.11 showed approximately 90 % of the total activity was precipitated by GK4A9, consistent with the degree of immunodepletion of this activity from EA.hy926 cells.

3.6 Immunodetection of ECE-1 using the monoclonal AEC32-236 antibody

A monoclonal antibody against ECE raised to the C-terminal domain of ECE-1 was kindly donated by Dr. K. Tanzawa (Sankyo Co. Ltd., Tokyo, Japan). Confirmation that this antibody was specific for ECE is shown in Fig. 3.8. This immunoblot shows ECE to be expressed as a 240 kDa dimeric protein under non-reducing conditions with a monomeric weight of 120 kDa as shown by incubation with β-mercaptoethanol.

Fig. 3.7 Immunodepletion of solubilised pig lung membranes by GK4A9

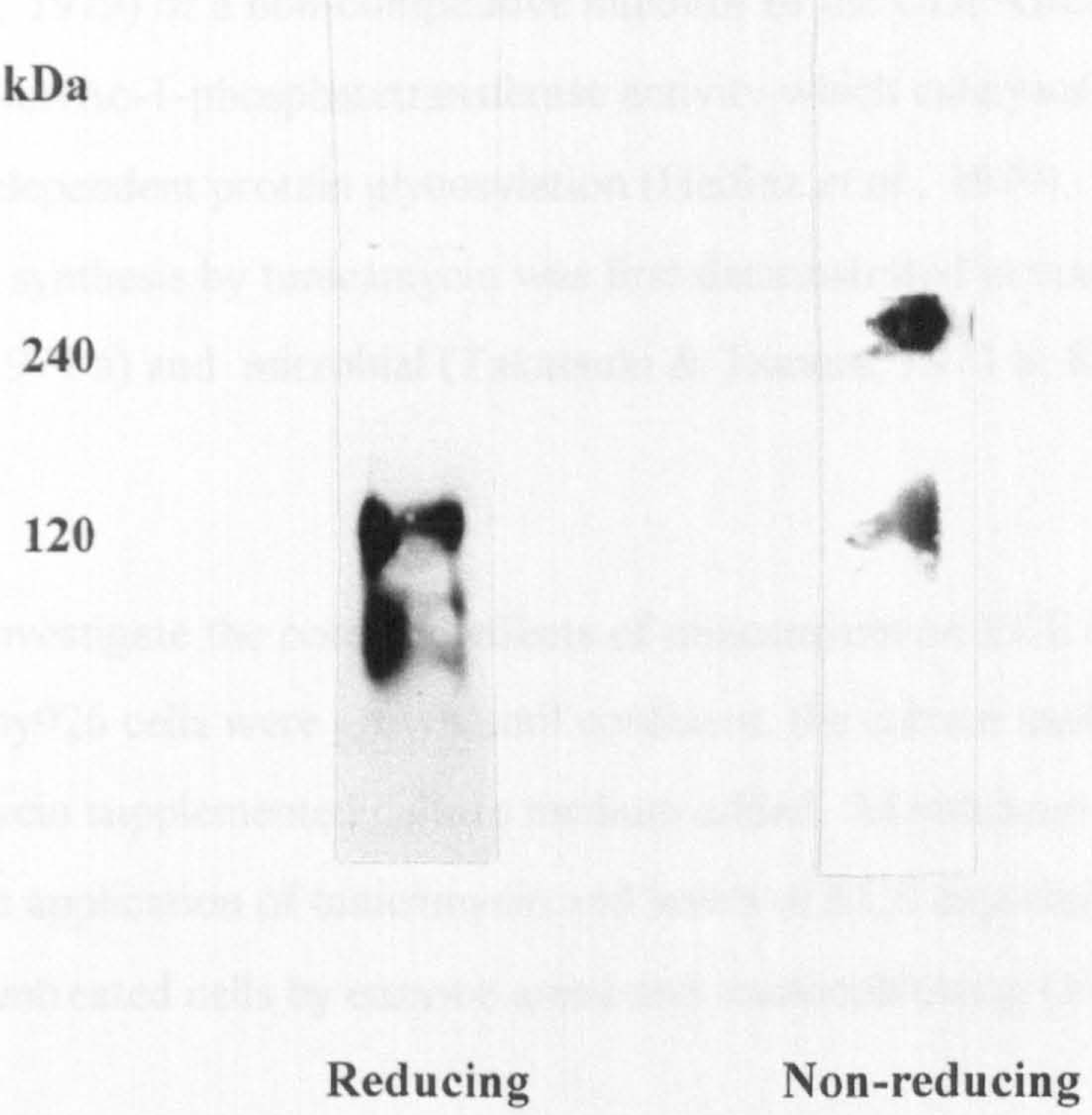


Membranes prepared from pig lung membranes (5 mg/ml) were incubated with GK4A9 labelled Sepharose beads (300 µl) at 4°C for 24 h. Beads were recovered by centrifugation (10,000 g, 10 min) and the supernatant retained as the immunodepleted fraction. Membrane peptidase activities were assayed before and after incubation with the beads and activities in the immunodepleted fraction calculated as a percentage of the total activity prior to incubation with the beads.

E-24.11 was assayed using the spectrophotometric protocol described in section 2.3.1. ACE and DPP-IV were assayed by the protocol described in section 2.3.2. Protein was assayed by the BCA method (section 2.2.1) and ECE assayed using the RIA protocol described in sections 2.3.6 and 2.3.7.

Results are mean ± S.D. for 4 separate experiments

Fig. 3.8 Western blot of EA.hy926 cell membrane preparations under reducing and non-reducing conditions using the monoclonal antibody AEC32-236



EA.hy926 cell membrane proteins (12 μg protein) were separated on a 10 % SDS-polyacrylamide gel under reducing (lane 1) and non-reducing (lane 2) conditions and then immunoblotted using the monoclonal antibody AEC32-236.

3.7 Effect of tunicamycin on ECE expression

Tunicamycin is an antibiotic which inhibits lipid carrier-dependent protein glycosylation (Duksin & Mahoney *et al.*, 1982). The inhibitory properties of this molecule are thought to be due to either its activity as a multi-analogue, competitive inhibitor (Keller *et al.*, 1979) or a non-competitive inhibitor of the UDP-GlcNAc:dolichyl phosphate-GlcNAc-1-phosphatetransferase activity which catalyses the first step in lipid carrier-dependent protein glycosylation (Heifetz *et al.*, 1979). The interference of glycoprotein synthesis by tunicamycin was first demonstrated in mammalian (Takatsuki & Tamura, 1971 a) and microbial (Takatsuki & Tamura, 1971 b; Kuo & Lampen, 1974) cells.

In order to investigate the potential effects of tunicamycin on ECE synthesis and activity EA.hy926 cells were grown until confluent, the culture medium was removed and tunicamycin supplemented culture medium added. Membranes were prepared 48 h following the application of tunicamycin and levels of ECE expression compared in treated and untreated cells by enzyme assay and immunoblotting (Fig. 3.9).

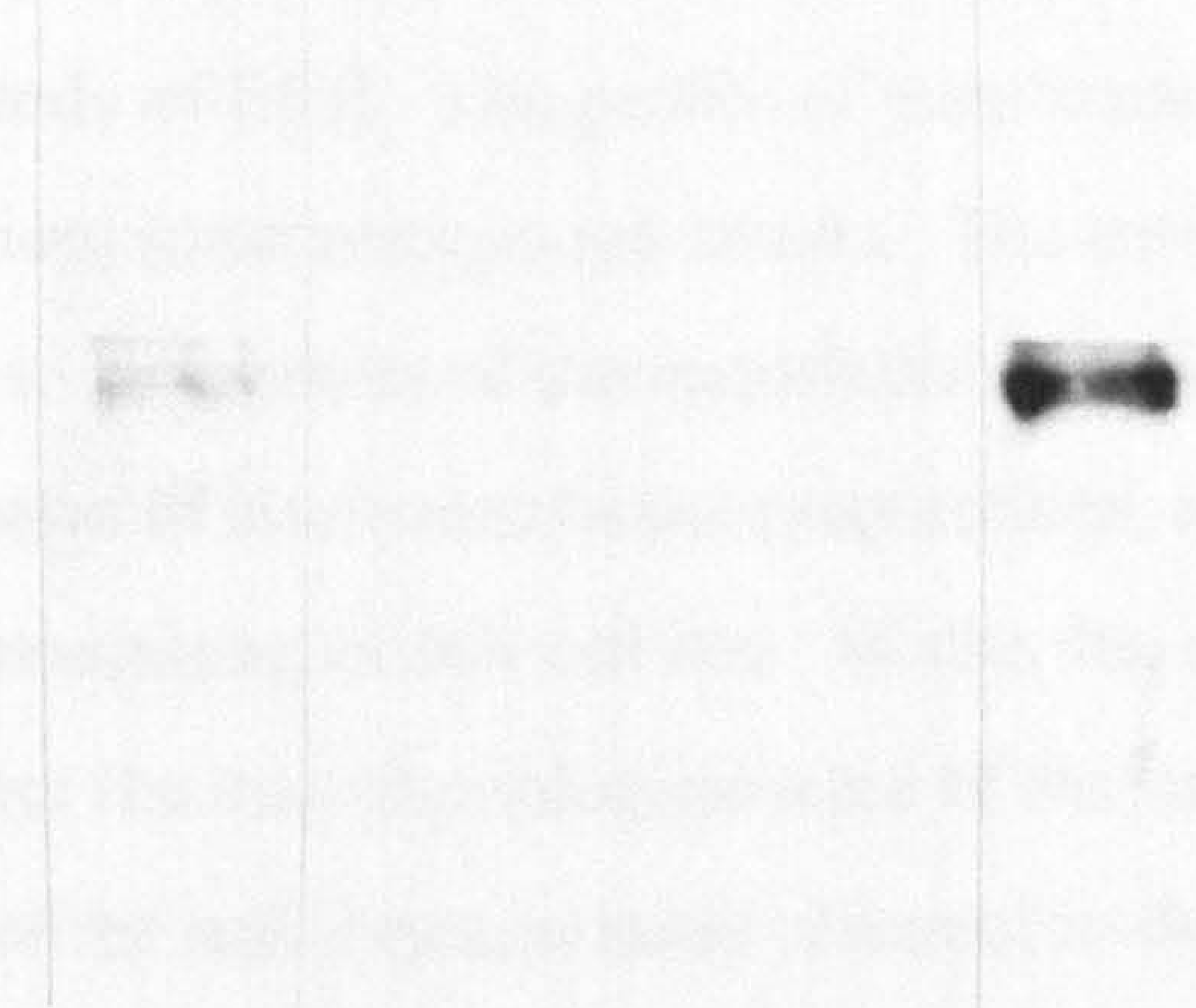
The effect of glycosylation on ECE synthesis and activity can be seen from these data. Comparison of the specific activities of tunicamycin treated and untreated membranes showed levels of ECE to diminish by approximately three-fold following treatment of cells with tunicamycin. These data were consistent with the levels of ECE detected by immunoblotting which showed tunicamycin treated cells to express much lower levels of the immunoreactive protein.

Fig. 3.9 Investigation of the effect of glycosylation on ECE expression and activity

	+ Tunicamycin	- Tunicamycin
ECE activity (pmol/min/mg)	0.49 ± 0.2	1.62 ± 0.33

kDa

120



+ Tunicamycin **- Tunicamycin**

Flask cultured EA.hy926 cells were grown until confluent before the culture media was removed and replaced with tunicamycin supplemented media (final concentration 10 μM). Membrane samples were either assayed for ECE activity or membrane proteins (12 μg / lane) were separated on a 10 % SDS-polyacrylamide gel and then immunoblotted with the monoclonal ECE antibody AEC32-236.

3.8 Discussion

3.8.1 The biochemical characteristics of the ECE protein

In the absence of cloning and structural information regarding ECE at the start of this thesis, it was decided to characterise the protein in terms of its biochemical properties. At the time ECE had yet to be cloned or sequenced, E-24.11 was regarded as a possible, though unlikely candidate for the physiological ECE and there was no sensitive, reproducible assay for ECE activity. The initial work therefore was intended to characterize the EA.hy926 cell line and determine the suitability of this cell line for use as a model system for the study of ECE. The profile of membrane peptidases expressed by this cell line did show some unexpected results. The enzyme activities, ACE and DPP-IV, considered to be markers of the endothelial cell phenotype were found not to be expressed in assays of crude membrane preparations, a result confirmed by subsequent immunostaining of this cell line. Whilst this indicated that these cultured cells did not reflect the true physiological state of the vascular endothelium, the development of the radioimmunoassay protocol to detect ECE activity in these cells showed expression levels suitable for study of this protein. An additional consideration given the apparent ability of E-24.11 to hydrolyse big ET-1 was the relatively low levels of E-24.11 activity expressed by this cell line. In this case, the assayed levels of both enzymes allowed the accurate and specific determination of ECE activity in membranes prepared from these cells.

An important aspect of the biochemistry of membrane peptidases is the mechanism by which they are anchored to the cell membrane. Identification of this was accomplished using several solubilisation techniques and comparing these data with those obtained for E-24.11, a known type II integral membrane protein. The relative resistance of ECE to solubilisation by bacterial phosphatidylinositol-specific PLC gave an initial indication that the protein was not anchored by a G-PI moiety. Detergent solubilisation profiling supported this evidence since both ECE and E-24.11 were effectively solubilised by Triton X-100, a non-ionic detergent of relatively low CMC which was unable to solubilise a known G-PI anchored protein. The similarity of the solubilisation profiles for ECE and E-24.11 suggested, but did not prove, that these proteins also shared a common anchoring mechanism. The data from temperature-induced phase separation studies, designed to identify amphipathic proteins were consistent with this theory. Both ECE and E-24.11 were found to localize in the detergent-rich phase while the hydrophilic protein, LDH, which was included as a positive control was localized in the detergent-depleted phase. All of these studies suggested ECE to be, like E-24.11, an integral transmembrane protein. This was confirmed when ECE was subsequently cloned and sequenced (Xu *et al.,* 1994; Shimada *et al.,* 1994; Schmidt *et al.,* 1994; Ikura *et al.,* 1994; Yorimitsu *et al.,* 1995).

3.8.2 Glycosylation of ECE protein

The importance of glycosylation on ECE synthesis and activity was investigated using the antibiotic tunicamycin. Membranes of eukaryotic cells are usually between 2 % and 10 % carbohydrate, a significant proportion of which is in the form of glycoprotein. Glycosylation of proteins is thought to play a role in both intercellular recognition and protein orientation within the membrane. Tunicamycin is a useful inhibitor of protein glycosylation and its effect on the synthesis and activity of ECE, a known glycoprotein with 10 N-linked glycosylation sites was demonstrated by both assay and immunoblotting. Determination of the specific ECE activity in treated and untreated membranes showed treatment of EA.hy926 cells with 10 μM tunicamycin diminished ECE activity by approximately 3-fold.

Immunoblotting of membranes with the monoclonal ECE antibody AEC32-236 was carried out to determine whether this was due to decreased synthesis of ECE or if tunicamycin was inhibiting ECE breakdown of big ET-1. The decrease in the size of the immunoreactive band in treated cell membranes was consistent with decreased synthesis of the mature ECE-1 protein rather than the inhibition of ECE activity and suggested glycosylation to be an important step in the correct synthesis of the mature active protein.

3.8.3 Co-precipitation of E-24.11 & ECE using monoclonal antibodies

There was mounting evidence to suggest there were many common biochemical characteristics between ECE and E-24.11. Both proteins were now thought to be integral membrane metalloproteases with a pH optimum of around neutral sharing some inhibition similarities. Further characterisation of ECE revealed the possibility that these proteins might have originated from the same protein family and therefore might share a significant degree of sequence and structural homology. Since antibodies recognise epitopes of proteins consisting usually of 6-10 amino acids it was thought that an antibody raised to one of the proteins may also recognise the corresponding sequence if this was conserved between the two.

Three antibodies to E-24.11 were available; a polyclonal antibody RP161 and two monoclonal antibodies GK4A9 and 3C7. Initial work involving the characterisation of E-24.11 levels expressed by the EA.hy926 cell line involved immunoblotting for which RP161 was used (Fig. 3.1). This antibody provided evidence for expression of E-24.11 by this cell line revealing a protein band at 90 kDa. However, no bands of molecular weight greater than this were revealed indicating this antibody did not cross-react with ECE (120 kDa). Further immunoblots of crude EA.hy926 membrane preparations using both of the monoclonal antibodies to E-24.11 showed these were not suitable for use in western blotting since neither detected E-24.11 at 90 kDa.

In order to determine if GK4A9 or 3C7 showed any immunoreactivity toward ECE a different approach of immunoprecipitation of the activities from solubilised membrane preparations was used. Both antibodies were immobilised on Sepharose and incubated with Triton X-100 solubilised membrane preparations from EA.hy926 cells. The 3C7 antibody was found to be poor at removing E-24.11 from membrane preparations and showed no indication of specific immunorecognition of ECE whatsoever. CNBr activated Sepharose lacking any bound antibody ligand showed only limited, non-specific binding of all proteins assayed and total protein content. In contrast to each of these, Sepharose labelled with the GK4A9 ligand appeared to show strong recognition of both E-24.11 and ECE whilst control assays of total protein and

AP-N confirmed this recognition to be specific. This work proved to be reproducible and the inhibitory potency of thiorphan and phosphoramidon (approximately 90 %) was confirmed since any loss of inhibition by these would have suggested big ET-1 breakdown by E-24.11 rather than ECE. The immunodepletion of membrane preparations was compared between pig lung membrane and EA.hy926 cells. A greater proportion of total ECE activity was removed from EA.hy926 membranes, suggesting the GK4A9 antibody to have a greater affinity for ECE expressed by this cell line compared with the form found in pig lung.

From these data, the possibility arose that activity being attributed to ECE was due, in some part to E-24.11 activity. In order to demonstrate this was not the case, CHO cells which had been transfected with ECE cDNA and found to express high levels of ECE but did not express E-24.11 were used in immunoprecipitation studies with GK4A9. Detergent solubilised membranes prepared from these cells were found to have almost (90 %) less ECE activity following incubation with the GK4A9 antibody. These data were supported by the observation that levels of ECE protein in the samples, detected by immunoblotting with AEC32-236, were significantly reduced following immunoprecipitation. In addition, fractions eluted from the GK4A9 column showed detectable levels of ECE protein confirming this antibody to immunoprecipitate ECE activity.

The co-precipitation of E-24.11 and ECE by an antibody raised to E-24.11 suggested the existence of a common epitope between the two proteins and was consistent with the hypothesis that both ECE and E-24.11 originated from the same protein family. This has also been confirmed by genetic analysis (Valdenaire *et al.*, 1995).

The immunoprecipitation of E-24.11 and ECE from membrane samples may also provide a rapid and convenient method of purification. CHO cells transfected with ECE cDNA had been found to have a specific activity for ECE of approximately 1000-fold greater than the levels found in EA.hy926 cells and immunoprecipitation of ECE from these cells was confirmed by the detection of ECE protein in the eluted fractions.

Since CHO cells do not express E-24.11 activity, problems of contamination with this related protein are precluded allowing rapid purification of ECE by this method.

CHAPTER FOUR

THE IDENTIFICATION OF ECE IN NEURONAL AND GLIAL CELL LINES

4.1 AIMS

To quantify levels of ECE expression in neuronal and glial cell types using radioimmunoassay and immunoblotting protocols and to compare levels of E-24.11.

To examine the effect of metalloprotease inhibitors on ECE levels.

4.2 Introduction

4.2.1 Tissue specific expression of ECE

The endothelin (ET) peptide family was originally identified in porcine endothelial cells (Yanagisawa *et al.,* 1988) where the peptides were predicted to be synthesised from larger, inactive precursors, the preproendothelins. The three ET isoforms were found to be expressed in non-vascular tissues (Inoue *et al.,*1989), suggesting ET and its production was not confined to vascular cells. Since then much research has focused on identification of sites of ET production since ET appears to be involved in many disease models afflicting a variety of tissues. It has been proposed that ET production in the central nervous system (CNS) plays a major role in the pathophysiology of stroke, neuronal damage and may act as a signalling neuropeptide involved in the regulation of cerebral blood flow and astrocytic function (Barone *et al.,* 1995). In addition, ETs are known to act as powerful morphogens regulating tissues derived from the neural crest (Baynash *et al.,* 1994; Kurihara *et al.,* 1994; Puffenberger *et al.,* 1994). Whilst the identification of the ET-1 and ET-3 peptides in glial and neuronal cells has long been established (MacCumber *et al.,* 1990; Ehrenreich *et al.,* 1991; Fuxe *et al.,* 1991; Giaid *et al.,* 1991), the expression of ECE by these cell types has remained controversial. Most previous studies have focused on the nature and localisation of endothelial ECE although enzyme assays have detected ET production from big ET by brain homogenates (Warner *et al.,* 1992 a,b) and cultured astrocytes (Deschepper *et al.,* 1995).

Since there was still controversy over the nature of the ECE activity in brain, the aim of this part of the work was to determine the levels of ECE-like activity in a variety of brain cell types and its possible identity with ECE-1.

4.2.2 Regulation of ECE by control of protein degradation

The mechanisms through which the levels of ECE protein expressed by the cell are controlled are not yet fully understood and modulating these may prove to be an effective method of regulating ET production to control disease. One level at which protein levels can be controlled is the rate of protein turnover. In order to identify possible regulatory proteases responsible for the breakdown of ECE, the inhibitors PR and thiorphan, were studied for their effects on membrane peptidase expression in a number of cell lines.

4.2.2.1 Regulation of ECE in response to phosphoramidon (PR)

Previous reports have shown PR to exert a number of effects on the synthesis of ET. Early reports suggested exogenously applied PR was able to suppress the secretion of ET-1 and the generation of ET-1 from exogenously applied big ET-1 (Ikegawa *et al.*, 1990 a) in cultured endothelial cells. In addition, Sawamura *et al.*, (1991) demonstrated PR inhibited the intracellular conversion of big ET-1 to ET-1 in endothelial cells whilst McMahon *et al.*, (1991) reported PR inhibition of big ET-1 *in vitro*. The inhibition of the conversion of exogenously big ET-1 by PR has now been reported in bovine endothelial cells (Takada *et al.*, 1991) and rat lung (Takaoka *et al.*, 1991) in a manner consistent with the inhibition of big ET-1 hydrolysis by ECE. More recently, Corder & Vane (1995) reported that the pressor effect of big ET-1 was due to local conversion to ET-1 and found PR reduced the pressor response to big ET-1 by 93 % whilst not altering its rate of clearance from circulation.

Studies *in vivo* have also demonstrated that administration of PR inhibits both the conversion of big ET-1 and the big ET-1 pressor response, though not the pressor response obtained with ET-1 (Matsumura *et al.*, 1990 c; Shinyama *et al.*, 1991; McMahon *et al.*, 1991; Bird *et al.*, 1992). More recent reports have suggested PR treatment of cultured endothelial cells may have direct effects on the levels of ECE expressed by the cells. Schmidt *et al.*, (1994) reported the up-regulation of PR-sensitive big ET-1 converting activity of up to 10-fold in foetal bovine heart endothelial cells and a 3-5- fold increase in other cell lines (unpublished data). This "up-regulation" of ECE in response to μM concentrations of PR was used to aid the purification of ECE by this group though the mechanism through which expression of ECE was enhanced was not addressed. As part of this work, the effect of PR treatment was studied in several cell lines to determine whether the effect was specific to endothelial cells and if ECE was up-regulated in response to PR treatment of cells.

4.2.2.2 Regulation of ECE in response to thiorphan

Since PR is an inhibitor of certain metalloproteases and appeared to show up-regulation or accumulation of ECE in certain cell types (Schmidt *et al.*, 1994) it was considered possible that the mechanism through which PR caused this was by inhibiting an enzyme usually involved in the degradation of the ECE protein. To further investigate this hypothesis, the effect of a second inhibitor of E-24.11, thiorphan, was examined to determine whether administration of this compound resulted in a comparable accumulation of ECE and therefore provide further evidence for metalloprotease involvement in the physiological regulation of ECE.

4.3 Results

4.3.1 Expression of ECE and E-24.11 in endothelial, glial, neuroblastoma cells and in a pheochromocytoma cell line

To compare the activities of ECE and E-24.11 in membrane preparations of the endothelial cell line, EA.hy926, the human neuroblastoma cell line SH-SY5Y, the human glial cell line Bu17, the rat glial cell line C6 and the cloned human astrocyte line D384 were assayed for ECE by radioimmunoassay and E-24.11 by HPLC These data are shown in Table 4.1.

Phosphoramidon-sensitive ECE activity was found to be highest in the EA.hy926 cells, similar to that found in Bu17 cells and threefold greater than that found in SH-SY5Y cells. Low levels of ECE activity were found in the astrocytoma clone D384 and the rat pheochromocytoma PC12 cells. ECE was not detected in the rat glial cells, C6 (Table 4.1).

In contrast, activity levels of E-24.11 were 4.5 fold higher in the Bu17 than the EA.hy926 cells which showed similar levels of expression to D384 and PC12 cells whilst this peptidase was not detected in C6 or SH-SY5Y cells (Table 4.1).

4.3.2 Immunoblotting of selected cell types to confirm ECE expression

Immunoblotting for ECE confirmed that the protein was expressed most abundantly in the EA.hy926, Bu17 and D384 cell lines compared with the SH-SY5Y line. Limited ECE protein was detected in the PC12 cell line but no protein was detected in the C6 rat glial cells (Fig. 4.1).

Table 4.1 Summary of ECE and E-24.11 specific activities in a variety of cell types

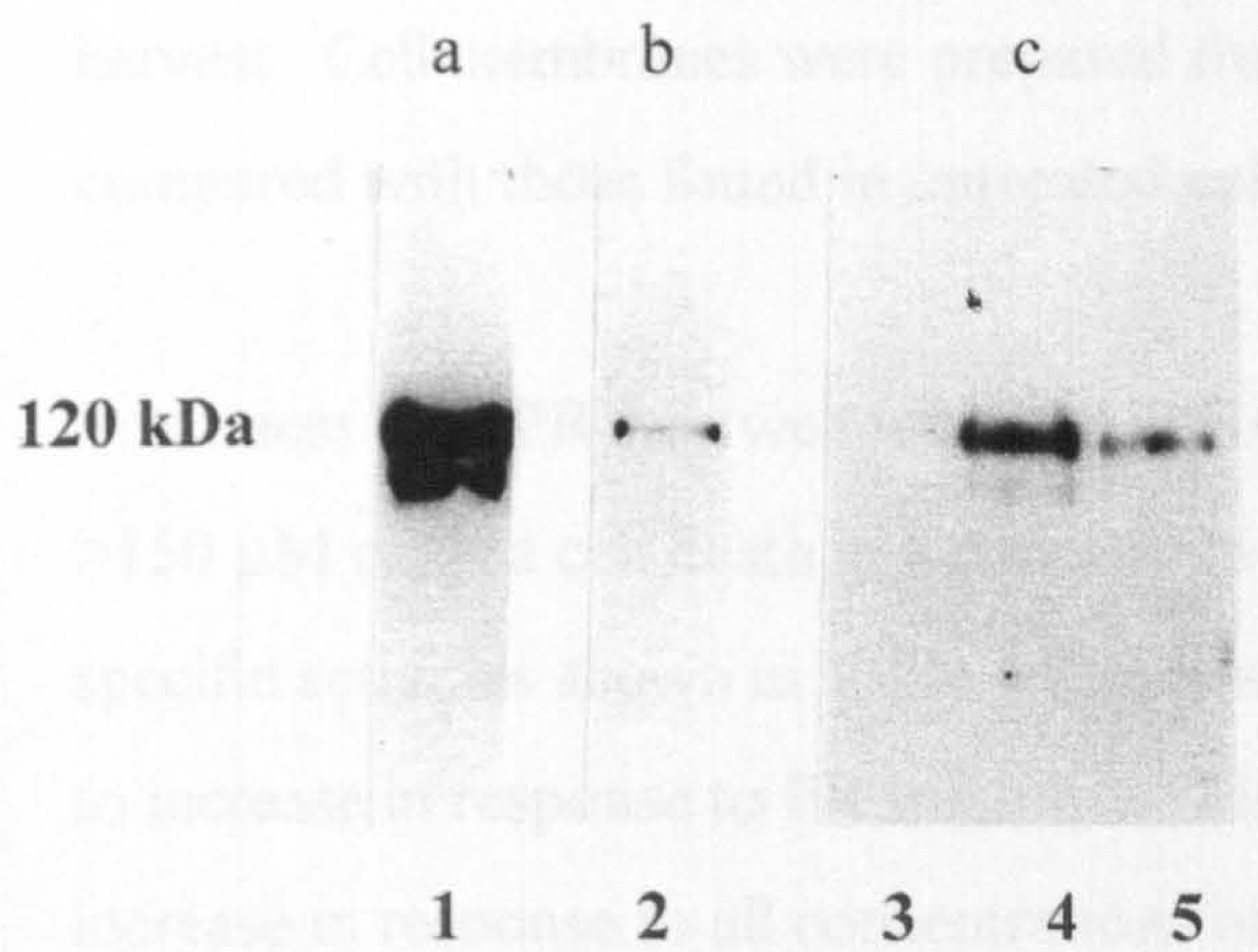
Cell Line	Cell Type	PR-Sensitive ECE (pmol/min/mg)	E-24.11 (nmol/min/mg)
EA.hy926	Human Endothelial	1.5 ± 0.1	4.9 ± 0.2
Bu17	Human Glioma	1.3 ± 0.1	23.3 ± 0.9
C6	Rat Glioma	< 0.1	N.D.
SH-SY5Y	Human Neuroblastoma	0.6 ± 0.1	N.D.
PC12	Rat Pheochromocytoma	N.D.	4.2 ± 0.7
D384	Astrocytoma Clone	0.2 ± 0.1	5.6 ± 1.1

N.D. Not detectable

Membrane preparations of each cell line were prepared and assayed for E-24.11 and ECE activity. Assays for E-24.11 were performed using the HPLC protocol described in section 2.3.1. ECE activity was assayed as described in sections 2.3.6 and 2.3.7.

All specific activities are the results of the mean ± S.E.M. for 5 separate experiments.

Fig. 4.1 Immunoblot analysis of ECE expression in selected cell types using the monoclonal antibody AEC32-236



Membrane preparations of cultured endothelial (**a**), neuroblastoma (**b**) and glioma (**c**) lines. Membrane proteins (16 μg/ lane) from EA.hy926 (lane 1), SH-SY5Y (lane 2), C6 cells (lane 3), Bu17 cells (lane 4), D384 cells (lane 5) were separated on a 7.5 % SDS-polyacrylamide gel under reducing conditions and immunoblotted using the monoclonal antibody AEC32-236 to ECE.

4.4 The effects of PR on membrane peptidase expression

4.4.1 The effects of PR on levels of membrane peptidase expression in EA.hy926 cells.

In order to investigate possible regulatory mechanisms involved in the control of ECE expression, cells were grown in the presence of PR for periods up to 48 h prior to cell harvest. Cell membranes were prepared from treated cells and levels of activity compared with those found in untreated cells by enzyme assay. (Table 4.2).

Treatment with PR was well tolerated in EA.hy926 cells though concentrations of >150 μM caused cell death in a concentration-dependent manner. The comparison of specific activities shown in Table 4.2 shows that neither E-24.11 nor AP-N were found to increase in response to PR treatment. However, in contrast, ECE was found to increase in response to all concentrations of PR examined with a maximal increase of 2.3-fold relative to the untreated cells following treatment with 30 μM PR.

4.4.2 Immunoblotting of membranes prepared from PR treated SH-SY5Y and Bu17 cells

In order to determine whether the accumulation of ECE in response to PR was specific for endothelial cells or if this was a mechanism present in other cells, two cell lines expressing different levels of both E-24.11 and ECE were examined for their response to PR treatment. SH-SY5Y had previously been shown to express low levels of ECE activity whilst Bu17 cells expressed levels of ECE comparable to EA.hy926 cells (Table 4.1). Cells were cultured in 24-well plates and treated with PR concentrations up to 100 μM. Cells were then solubilised with SDS sample buffer prior to separation by SDS-PAGE. Immunoblotting with the AEC32-236 monoclonal antibody to ECE revealed changes in the levels of ECE protein expressed by the cell (Fig. 4.2).

Table 4.2 Dose-response of membrane peptidase expression in EA.hy926 cells following PR treatment

	PR Concentration (μM)				
	0	10	30	50	100
AP-N nmol/min/mg	19.6 ± 1.2	21.7 ± 1.0	16.6 ± 2.4	19.3 ± 3.4	18.3 ± 2.0
E-24.11 nmol/min/mg	5.8 ± 0.9	5.5 ± 1.2	6.1 ± 1.4	5.5 ± 1.6	5.8 ± 1.2
ECE pmol/min/mg	1.4 ± 0.3	1.5 ± 0.2	3.1 ± 0.3	2.3 ± 0.2	1.9 ± 0.3

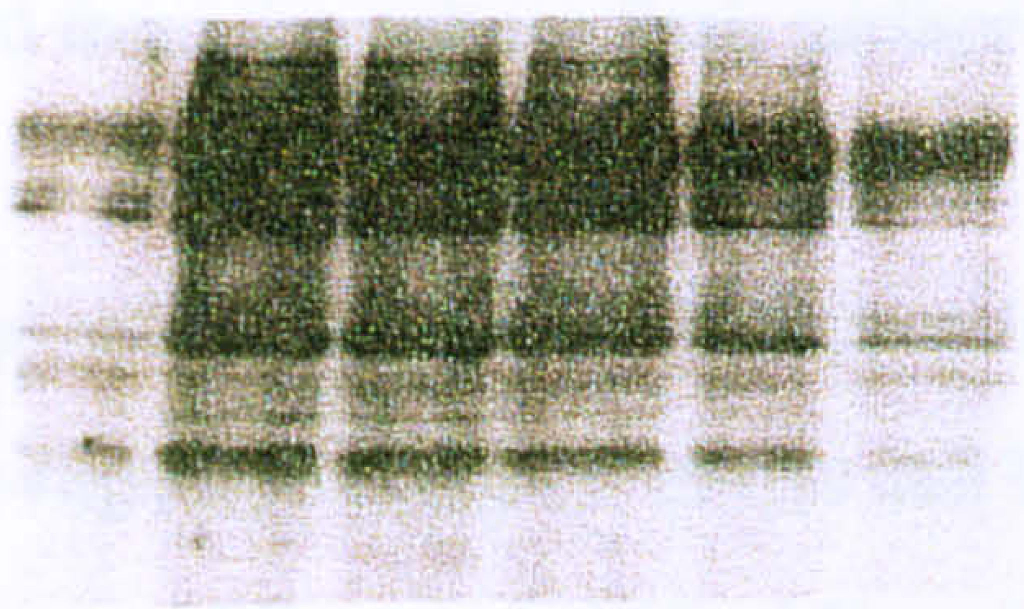
Flask cultured EA.hy926 cells were treated with PR supplemented media (0 - 100 μM final concentration) and cell membranes prepared 48h following media application. Cell membranes were assayed for AP-N, E-24.11 (spectrophotometric protocol) and ECE. The results are the mean ± S.E.M. for 4 experiments.

Fig. 4.2 Dose-response of ECE expression in SH-SY5Y and Bu17 cells following PR treatment

Immunoblotting of SH-SY5Y cells

0 10 30 50 100 150 PR Concentration (μM)

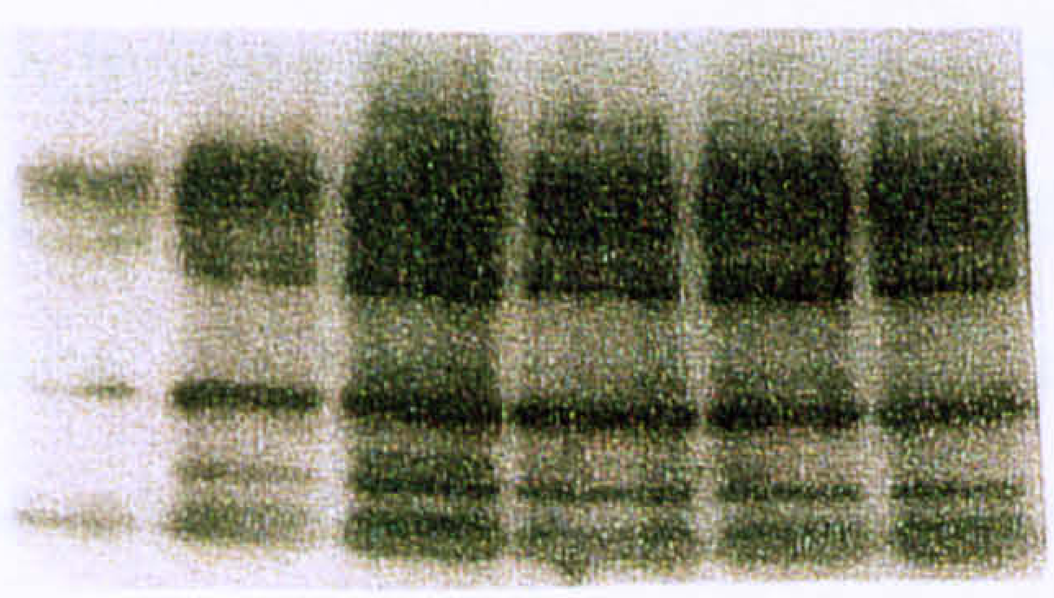
120 kDa



Immunoblotting of Bu17 cells

0 10 30 50 100 150 PR Concentration (μM)

120 kDa



SH-SY5Y and Bu17 cells were cultured in 24-well plates and treated with PR-supplemented media. Cells were harvested by solubilisation with SDS-sample buffer and proteins separated under reducing conditions on a 10 % SDS-polyacrylamide gel and immunoblotted, using the monoclonal antibody AEC32-236 to ECE.

The band intensities seen following immunoblotting with the AEC32-236 antibody show a marked increase in the levels of ECE protein following treatment of cells with PR. Administration of 10 μM PR was sufficient to induce accumulation of ECE protein in both cell lines. The maximal increase in ECE protein in SH-SY5Y cells was in response to 10 μM PR though the effect was reduced at concentrations of >50 μM PR. At these higher concentrations it would appear that PR is toxic to this cell line since cells detached from the culture flask surface.

In Bu17 cells, the greatest increase in ECE protein was seen in response to 20 μM PR treatment. The up-regulation of ECE in this cell line was maintained even at the highest concentration of PR tested (150 μM) suggesting this cell line is more resistant to the toxic effects of PR.

4.5 The effects of thiorphan on membrane peptidase expression in EA.hy926 cells

Pre-treatment of EA.hy926 cells with thiorphan up to 50 μM concentration appeared to have no effect on the ability of cells to grow and replicate and resulted in no visible morphological change. The compound was well tolerated at the highest of concentrations used although higher concentrations proved to be toxic to the cells. Assay data of treated cell membranes is shown in Table 4.3.

These data showed treatment of cells with thiorphan did not cause any significant change in AP-N and E-24.11 since levels of these activities remained constant across the concentration range used. This was in contrast to the results obtained for ECE which showed an increase in specific activity in response to all concentrations of thiorphan used with a maximal increase in response to treatment with 50 μM thiorphan.

Table 4.3 Assays of thiorphan treated EA.hy926 cells

Activity	Final Concentration of Thiorphan (μM)			
	0	10	30	50
AP-N (nmol/min/mg)	24.1 ± 4.1	28.4 ± 4.7	26.5 ± 5.3	32.4 ± 3.8
E-24.11 (nmol/min/mg)	2.6 ± 0.6	2.8 ± 0.2	2.4 ± 0.4	2.4 ± 0.7
ECE (pmol/min/mg)	1.3 ± 0.2	3.0 ± 0.4	2.7 ± 0.3	3.6 ± 0.3

E-24.11 activity was determined using the spectrophotometric protocol.

Specific activities for each activity were determined as the mean ± S.E.M. for 4 separate experiments.

4.6 Discussion

4.6.1 The identification of ECE in brain cell types

ECE expression in brain was found to be both cell and species specific. Human glial cells, Bu17 were found to express levels of ECE comparable with those found in the human endothelial EA.hy926 cell line and approximately 5-fold more E-24.11 activity compared with EA.hy926. In comparison, the rat glioma cell line C6 was found to express neither of these activities. Cultured brain astrocytes D384 were found to express relatively low levels of ECE activity, consistent with the findings of Deschepper *et al.*, (1995). Virtually no ECE activity was detectable in the human neuroblastoma SH-SY5Y or the rat pheochromocytoma PC12 (with characteristics of the adrenal medulla) cell lines. This finding was to prove useful in the studies of ECE regulation since accumulation of ECE could be more readily quantified against a low background level of the enzyme.

These findings have some implications for the pharmacological prevention of ischaemic neuronal damage. Recent research in this field has concerned ET production and the role of ECE with the aim of identifying specific and potent inhibitors of ECE to lower ET synthesis following ischaemia. Aside from problems such as permeating the blood-brain barrier and administration of such inhibitors to their target site additional problems may arise by the tissue and cell variations in ECE expression. In addition, inhibitors used in this way should ideally be highly specific for ECE since E-24.11 activity, thought to play a crucial role in terminating synaptic signalling (Turner *et al.*, 1985), is also expressed by several brain cell types. Inhibition of E-24.11 by inhibitors designed for ECE may have some side-effects.

4.6.2 The regulation of ECE expression

The apparent involvement of ET in so many disease models has promoted intense research activity into its production and much of this research has focused on the control of ET effects by modulating ET activity through inhibitors of ECE and antagonists of ET receptors. In comparison, relatively little is known regarding the pathways by which ECE production is controlled at a cellular level. Whilst EA.hy926 was used initially as a model system, the effect of these peptidase inhibitors on other brain cell lines was investigated.

4.6.2.1 The effect of PR and thiorphan on ECE expression

In this investigation the levels of ECE found in EA.hy926 were found to increase specifically in response to both PR and thiorphan. Following administration of each of these compounds the levels of both E-24.11 and AP-N remained constant, suggestive of a distinct processing pathway involved in ECE turnover. These data suggest a number of possible mechanisms by which levels of ECE may be regulated. Inhibition of ECE activity by PR would lead to an intracellular accumulation of the ET precursor, big ET which in turn could act via a feedback mechanism to increase rates of transcription and translation of ECE. Subsequent work by Barnes *et al.*, (1996) revealed treatment of TRLEC-03 cells did not lead to an increase in ECE-1 α mRNA (the ECE isoform exclusively expressed by TRLEC-03 cells; Shimada *et al.*, 1995) and would therefore suggest the most likely mechanism involves the inhibition of an activity normally involved in the specific degradation of ECE by the cell. Other possible explanations appear unlikely. The up-regulatory effect seen in the case of thiorphan would rule out an autocatalytic effect since ECE is considered to be insensitive to thiorphan (for review see Turner & Murphy, 1996). The possibility that PR treatment alters the post-translational modification of the protein is precluded since this would change the *M*r of the protein. Changes in the *M*r of the immunoreactive band detected by AEC32-236 does not occur indicating PR does not alter ECE expression in this manner. If the effect were at the level of intracellular transport, PR would be expected to alter the expression of AP-N which undergoes the same transport pathway.

The results presented here are indicative of a novel metalloprotease activity involved in the normal degradative processing of ECE which would appear to be different from E-

24.11. The possibility that E-24.11 might be involved in degradation of ECE appears to have been refuted by Barnes *et al.,* (1996) who demonstrated that E-24.11 was unable to cleave ECE and is consistent with the previous finding that E-24.11 has strict specificity for oligopeptides (Kenny *et al.,* 1987).

Recent research has focused on the design of specific inhibitors for ECE in order to regulate the effects of ET-1 at the level of big ET-1 hydrolysis. These data have considerable implications for such research. The use of inhibitors as therapeutic agents would seem to present problems since these may have systemic effects possibly causing an accumulation of ECE when the therapeutic aim is to reduce levels of ECE. Whether this accumulated ECE is functionally active in the cell in ET-1 production remains to be established.

CHAPTER FIVE

INVESTIGATION OF THE POSSIBLE ROLE OF TYROSINE PHOSPHORYLATION IN THE REGULATION OF ECE

5.1 AIMS

To examine whether tyrosine phosphorylation may play a role in the regulation of ECE expression.

To establish dose and time responses to vanadate, an inhibitor of protein tyrosine phosphatases, in cell culture systems.

To use immunoblotting and enzyme assay to quantify changes in membrane peptidase expression in several cell types.

5.2 Introduction

5.2.1 Cellular effects of protein tyrosine phosphorylation

The expression and activity of membrane peptidases is the result of the relative rates of breakdown and synthesis of the protein. The signal transduction pathways which control protein expression are complex and a number of pathways may influence expression levels of any given protein. Many of the cellular responses induced by hormones, growth factors and cytokines are mediated by the action of intracellular kinases, some of which act by phosphorylating specific proteins on tyrosine residues.

The ability of the cell to control phosphorylation levels is crucial to the regulation of the action of certain proteins and breakdown in cellular control of phosphorylation can have damaging physiological consequences on the cell. The phosphorylation state of cellular proteins can indirectly influence the rate of synthesis of a protein or may be crucial to the activity of the protein itself.

The promotion of phosphorylation of intracellular proteins by vanadate treatment has been shown to result in dramatic physiological changes such as effects on the differentiation of a variety of cell types (Gordon, 1991). In addition, cultured cell lines including rat pheochromocytoma PC12 cells have been stimulated to express neurites considered to be a marker for the neuronal phenotype whilst human neuroblastoma SH-SY5Y cells have extended neurites following culture with vanadate supplemented medium (Rogers *et al.*, 1994).

Previous work has demonstrated that cultured endothelial cells (HUVEC) have been shown to suppress ET-1 release following treatment with μM concentrations of vanadate (Shimizu *et al.*, 1994), though the effects on ECE were not reported. In order to determine whether phosphorylation is indeed a key event in the regulation of ECE expression, studies were carried out using the protein tyrosine phosphatase inhibitor vanadate to study the effects on expression levels in membrane preparations prepared from a number of cell lines.

5.2.2 Effects of vanadate on tyrosine phosphorylation

Sodium orthovanadate (Na_3VO_4) has been used in this study as a tool for the study of the role of protein tyrosine phosphorylation in the regulation of ECE. Vanadate acts by mimicking the transition state of phosphate (Gordon, 1991) and is a well established inhibitor of protein tyrosine phosphatases and has been used in this context (Simons 1979; Nechay *et al.*, 1986; Gordon, 1991). However, the cellular effects exhibited by vanadate could be related to its ability to alter the activity of certain other enzymes including ion channel ATPase (Cantley *et al.*, 1977) and protein kinases (Brown & Gordon, 1984) including the insulin receptor kinase (Tamura *et al.*, 1984).

5.2.3 The investigation of tyrosine phosphorylation effects in ECE regulation

In order to investigate the possible role of tyrosine phosphatase inhibition on the levels of ECE expression, the EA.hy926 cell line was examined for changes in membrane peptidase expression following treatment with vanadate. This study was widened to determine whether any effects were specific for endothelial cells or whether a common regulatory mechanism operated in other cell types. In addition, the rat pheochromocytoma PC12 cell line, which had previously been shown not to express ECE (Table 3.1) but had been shown to undergo morphological changes and differentiation in response to vanadate treatment, was examined to determine if differentiation to the neuronal phenotype resulted in induction of ECE expression.

5.3 Results

5.3.1 The response of EA.hy926 cells to vanadate treatment

5.3.1.1 The time-dependent response of EA.hy926 cells to vanadate

Prior to this work, the effects of vanadate on EA.hy926 cells had not been documented and in the absence of any known morphological marker the initial work involved the use of vanadate concentrations in the μM region which were sufficient to induce the cellular changes reported by Rogers *et al.* (1994).

EA.hy926 cells were cultured until confluent and the culture medium replaced by vanadate supplemented medium (40 μM final concentration) or untreated culture medium. Cells were allowed to grow until harvested at intervals up to 48 h and membranes prepared immediately. Membranes were assayed for ECE, AP-N and E-24.11 activity (Table 5.1).

The specific activities of ECE in membranes prepared from cells treated with vanadate showed a marked increase at all time points up to 48 h with a maximal increase of almost 3-fold at 24 h relative to untreated cells at the same time point. Whilst it appeared that treatment of cells with vanadate caused a time-dependent increase in ECE activity, activity over the same period in untreated cells remained essentially constant. (Fig. 5.1).

Immunoblotting of vanadate treated cell membranes showed an increase in levels of ECE protein over the time course period, consistent with the increase in ECE specific activity seen in the same membranes. There was no significant increase in levels of ECE protein over the time course in untreated cells.

5.3.1.2 Dose-response of membrane peptidase expression in EA.hy926 cells to vanadate treatment

Having determined the time period following vanadate application at which up-regulation of ECE was maximal, the most potent concentration of the compound was determined. By using a fixed period of 24 h between application of vanadate-supplemented medium and cell harvest, cells were treated with a range of vanadate

Table 5.1 Time-related effect of vanadate on peptidase expression in EA.hy926 cells

Specific activities of membrane peptidases (untreated EA.hy926 cells)

	0 h	2 h	8 h	24 h	48 h
AP-N	18.8 ± 3.6	13.9 ± 4.0	18.4 ± 4.9	22.4 ± 5.8	21.1 ± 3.8
E-24.11	4.7 ± 0.9	4.3 ± 0.9	4.0 ± 0.9	4.6 ± 1.3	5.1 ± 1.1
ECE	1.3 ± 0.3	1.3 ± 0.1	1.2 ± 0.2	1.2 ± 0.4	1.1 ± 0.2

Specific activities of membrane peptidases in EA.hy926 cells treated with 40 μM vanadate

	0 h	2 h	8 h	24 h	48 h
AP-N	18.8 ± 3.6	16.0 ± 3.2	15.9 ± 4.1	22.7 ± 6.2	18.9 ± 2.1
E-24.11	4.7 ± 1.3	3.6 ± 1.2	3.8 ± 1.5	4.2 ± 1.0	4.5 ± 0.8
ECE	1.3 ± 0.3	1.5 ± 0.2	2.4 ± 0.3	3.5 ± 0.4	3.1 ± 0.3

All activities measured in nmol/min/mg except ECE which is measured in pmol/min/mg

Flask cultured EA.hy926 cells were grown until confluent and the medium replaced with either fresh medium (untreated EA.hy926) or culture medium supplemented with 40 μM vanadate (final concentration). Cell were harvested at a range of times following application and membranes prepared immediately. Specific activities were determined for ECE by radioimmunoassay, E-24.11 (spectrophotometric assay) and AP-N.

Results are given as the mean ± S.E.M. for 4 experiments.

Fig. 5.1 Time-related effect of vanadate on ECE expression in EA.hy926 cells revealed by immunoblotting

Immunoblotting of vanadate treated EA.hy926 cells

0 2 4 8 24 48 Time (h) Following Application of 40 μM Vanadate

120 kDa

Immunoblotting of untreated EA.hy926 cells

0 2 4 8 24 48 Time (h) Following Application of Fresh Culture Medium

120 kDa

EA.hy926 cells, cultured in 24-well plates were treated with vanadate-supplemented culture medium (final concentration 40 μM) and membranes prepared at different time intervals following application of the treated medium by solubilisation with SDS-sample buffer. β-mercaptoethanol (10 % final concentration) was added and proteins separated on a 10 % SDS-polyacrylamide gel and immunoblotted with the ECE monoclonal antibody AEC32-236.

concentrations and membranes were assayed for peptidase activities (Table 5.2 and Fig. 5.2).

Since the samples used in this particular investigation were the same for both enzymic assays and immunoblotting, and immunorecognition was quantified by phosphorimaging, direct comparisons between the two analytical methods was possible. Immunoblotting for ECE (Fig. 5.2) showed the maximal increase in ECE expression was in response to treatment with 30 μM vanadate and this was confirmed by phosphorimaging which suggested this increase to be of the order of 2.7-fold relative to untreated cells.

Determination of ECE activity by radioimmunoassay (Table 5.2) showed a maximal increase of 2.3-fold compared with untreated EA.hy926 cells in response to a concentration of 30 μM vanadate, consistent with phosphorimaging data.

Increase in ECE expression was seen across the concentration range used and treatment with vanadate up to 50 μM did not appear to cause any significant change in the growth abilities of the cell and did not cause any visible morphological change when examined under the microscope. At concentrations above this, significant cell death resulted as seen by the stripping of cells from the culture vessel surface over large surface areas, though no morphological change resulted in this cell line at lower concentrations.

5.3.2 Dose-response of membrane peptidase expression in Bu17 cells to vanadate treatment

Having established and quantified the degree to which ECE expression is increased in EA.hy926 cells in response to vanadate treatment, the human glial cell line, Bu17, which had previously been shown to express levels of ECE activity comparable to those found in EA.hy926 (Table 4.1) was examined by assay and immunoblotting to determine if this cell line showed a similar response to vanadate.

Table 5.2 Dose-response of membrane peptidase expression in EA.hy926 cells to vanadate treatment

	Vanadate (μM)			
	0	10	30	50
AP-N	23.6 ± 3.4	23.8 ± 2.9	24.1 ± 2.8	27.3 ± 2.1
E-24.11	5.5 ± 1.1	6.3 ± 1.0	6.4 ± 0.5	6.7 ± 0.8
ECE	1.4 ± 0.2	2.1 ± 0.1	3.2 ± 0.2	2.9 ± 0.2

All activities measured in nmol/min/mg except ECE which is measured in pmol/min/mg

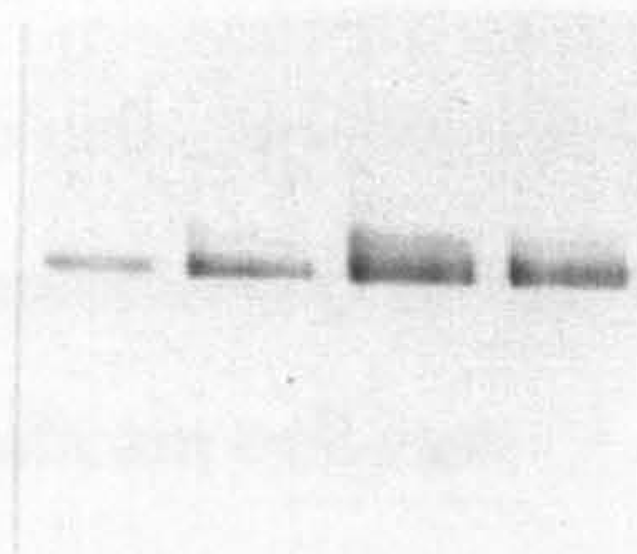
Using the same samples, assays for each untreated and vanadate treated sample were performed to determine the specific activities of ECE, E-24.11 (spectrophotometric protocol) and AP-N.

Assay results are given as the mean ± S.E.M. for 4 separate experiments.

Fig. 5.2 Dose-response of ECE expression in EA.hy926 cells to vanadate treatment revealed by immunoblotting

0 10 30 50 Vanadate Concentration (μM)

120 kDa



Band Intensity 1 1.8 2.7 2.4

Flask cultured EA.hy926 cells were grown until confluent then treated with medium supplemented with vanadate (0 - 100 μM final concentration). Cells were cultured for a further 24 h, harvested and membranes prepared from these. Membrane proteins were separated on a 10 % SDS-polyacrylamide gel and immunoblotted with the ECE monoclonal antibody AEC32-236. The western blot was further analysed by comparing band areas by phosphorimaging. The areas given for each band were determined as a ratio relative to the band obtained for the immunoblotting of untreated membranes.

The effect of vanadate treatment on Bu17 cells was consistent with that observed for EA.hy926 cells. Radioimmunoassay data showed the specific activity of ECE to be 2.2-fold higher in cells treated with 30 μM vanadate compared with untreated cells. (Table 5.3).

The immunoblot in Fig. 5.3 shows an increase in ECE expression in response to all concentrations used though the maximal effect was seen with cells treated with 10 μM vanadate.

In common with EA.hy926 cells, no morphological changes were detected in cells cultured with vanadate supplemented medium though concentrations of >50 μM vanadate again caused significant cell loss.

5.3.3 Dose-response of membrane peptidase expression in PC12 cells to vanadate treatment

Previous studies on this cell line (Rogers *et al.*, 1994) had demonstrated vanadate to induce the expression of neurites, a prominent morphological marker of the neuronal phenotype. In this study, neurite extension was seen when cells were treated with vanadate concentrations of >10 μM. Concentrations of vanadate above 75 μM were found to be toxic to this cell line.

Vanadate treated PC12 cells were examined to determine the effect of vanadate on ECE expression in this cell line. Previous assays had demonstrated that ECE could not be detected in this cell line (Table 4.1). Assays of membranes from untreated and vanadate treated cells were assayed for ECE activity (Table 5.4).

Assays of vanadate treated cells demonstrated an apparent increase in ECE activity compared with untreated cells. Untreated cells were found to have negligible ECE activity whilst cells grown in the presence of vanadate showed significant levels of activity. The maximal effect was seen in response to treatment with 30 μM vanadate.

Immunoblot analysis of membrane preparations from vanadate treated and untreated cells showed a similar response in terms of the effect on ECE protein levels (Fig. 5.4).

Table 5.3 Dose-response of E-24.11 and ECE activity in Bu17 cells following vanadate treatment

	Vanadate (μM)			
	0	10	30	50
E-24.11 (nmol/min/mg)	15.4 ± 2.7	13.7 ± 2.0	13.7 ± 3.4	18.1 ± 3.0
ECE (pmol/min/mg)	1.3 ± 0.1	2.0 ± 0.3	2.8 ± 0.3	1.9 ± 0.3

Flask cultures of Bu17 cells were allowed to grow until confluent and the culture medium then replaced with vanadate supplemented medium (10 - 50 μM). Cells were cultured for a further 24 h before membranes were prepared from these. E-24.11 (HPLC protocol), and ECE activities were determined for each sample.

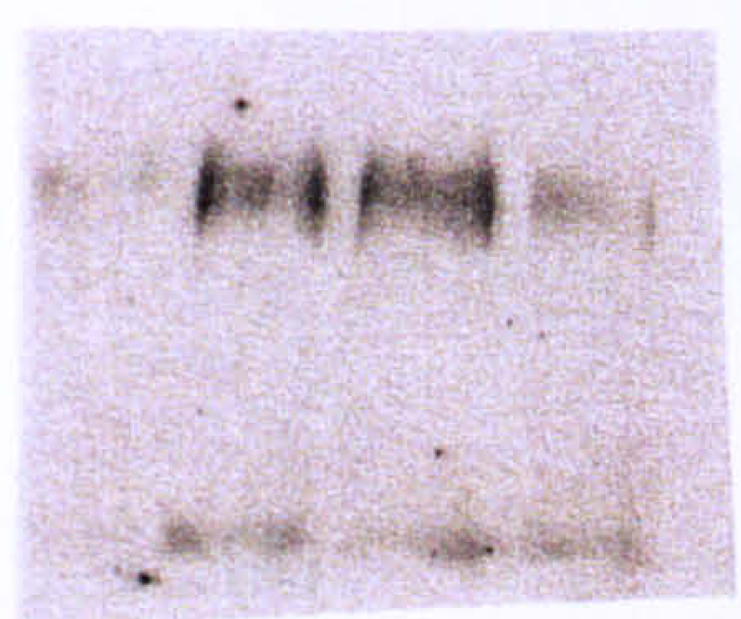
Results are given as mean ± S.E.M. for 4 experiments.

Fig. 5.3 Dose-response of ECE expression in Bu17 cells following treatment with vanadate detected by immunoblotting

Vanadate (uM)

0 10 30 50

120 kDa



Bu17 cells were cultured in 24-well plates and treated with varying concentrations of vanadate. After 24 h cells were solubilised with SDS-sample buffer and β-mercaptoethanol added to a final concentration of 10 %. Proteins were then separated on a 10 % SDS-polyacrylamide gel and immunoblotted with the monoclonal antibody AEC32-236.

Table 5.4 Dose-response of E-24.11 and ECE in PC12 cells following vanadate treatment

	Vanadate (μM)			
	0	10	30	50
E-24.11 (nmol/min/mg)	4.2 ± 0.9	3.6 ± 0.4	3.9 ± 0.4	4.1 ± 0.9
ECE (pmol/min/mg)	N.D.	1.0 ± 0.2	2.8 ± 0.2	1.9 ± 0.3

N.D. Not detectable

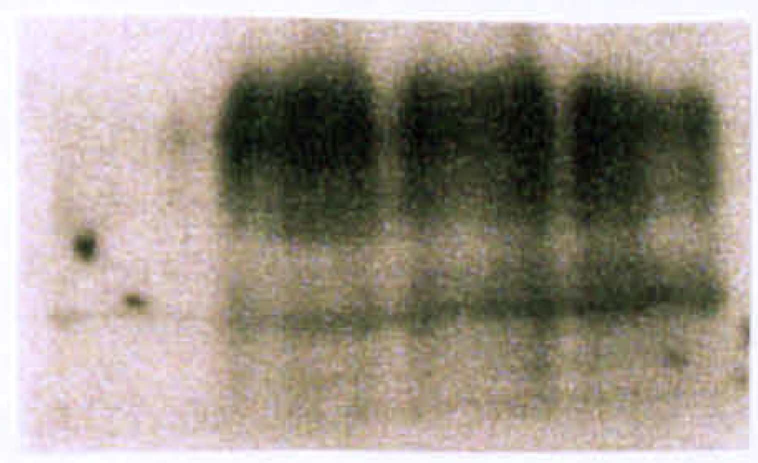
Suspension cultures of PC12 cells were grown and cell numbers adjusted to 10^6 cells per flask. The existing cell culture medium was then replaced with vanadate supplemented medium and cells allowed to grow for a further 48 h. The cells were then sedimented by centrifugation and membranes prepared from each flask.

All membrane preparations were assayed for E-24.11 (HPLC method) and ECE activity. Results are the mean ± S.D. for 4 different membrane preparations.

Fig. 5.4 Dose-response of ECE expression in PC12 cells to vanadate treatment detected by immunoblotting

0 10 30 50 Vanadate Concentration (μM)

120 kDa



Flask cultured PC12 cells were treated with media supplemented with a range of vanadate concentrations (0-50 μM). Following treatment with supplemented media (24 h) cells were harvested by centrifugation (1000 g, 10 min) and membranes prepared. Membrane proteins (14 μg protein/lane) were separated on a 10 % SDS-polyacrylamide gel under reducing conditions then immunoblotted using the ECE monoclonal antibody AEC32-236.

The immunoblot of PC12 cells in Fig. 5.4 appears to show a dramatic increase in levels of ECE protein expressed by this cell line in response to all concentrations of vanadate used. This effect was seen to be maximal in response to 30 μM, consistent with the assays of specific activities.

5.3.4 Protein tyrosine phosphatase inhibition using phenylarsine oxide

Phenylarsine oxide has been described as a potent inhibitor of protein tyrosine phosphatases (Carey *et al.*, 1995). It has been used in previous studies to maintain the phosphorylated state of proteins on tyrosine residues (Garcia-Morales *et al.*, 1990; Bernier *et al.*, 1987; Santini and Beaven, 1993) and in common with vanadate, treatment of cells with phenylarsine oxide induces several additional intracellular effects in addition to tyrosine phosphatase inhibition.

Studies using a number of cell lines, including EA.hy926, Bu17 and SH-SY5Y cells, carried out in conjunction with the study of the effects of vanadate on ECE expression showed that phenylarsine oxide prepared as described in section 2.4.6 was apparently toxic at levels of >30 μM, whilst at lower concentrations the potential up-regulatory effects on ECE were not potentiated by phenylarsine oxide. Whilst it would appear likely that the toxic effect on the cells was due primarily to the phenylarsine oxide content of the media supplement, control cultures of cells supplemented with the solvent used to solubilise phenylarsine oxide (HCl acidified DMSO) showed significant toxicity to the cells (approximately 50% cell death). This method was recommended by the manufacturer and other methods proved ineffective at solubilising phenylarsine oxide at the concentrations needed to induce phosphotyrosine phosphatase inhibition.

5.4 Discussion

In this study, the effects of protein tyrosine phosphatase activity on the levels of membrane peptidases expressed by a number of cell lines has been determined. Previous studies had shown μM concentrations of vanadate were sufficient to induce differentiation in SH-SY5Y cells and cause PC12 cells to exhibit a neuronal phenotype. In addition, vanadate had previously been shown to suppress ET-1 release from HUVEC cells (Shimizu *et al.,* 1994). The effects of vanadate on levels and activity of ECE protein expressed by the human endothelial cell line, EA.hy926, PC12 cells and the human glioma cell line were studied by immunoblot analysis and enzyme assay.

Many of these studies had demonstrated that cells were responsive to treatment with μM concentrations of vanadate and on this evidence initial experiments were used to determine the optimum vanadate concentrations to influence ECE expression. Vanadate effects were studied in EA.hy926 cells to determine whether application of this compound had any detectable effects on levels of ECE expressed by this cell line. Time-course studies of treated and untreated cells revealed EA.hy926 cells treated with 40 μM vanadate prior to cell harvest had levels of ECE specific activity approximately 3-fold higher after 24 h compared with untreated cells allowed to grow for a similar time period (Table 5.1). This apparent vanadate induced up-regulation of ECE activity was consistent with an increase in levels of ECE protein expressed by this cell line as demonstrated by immunoblotting (Fig. 5.1).

Having established 24 h as the optimal period between vanadate administration and cell harvest, this period was used in all subsequent experiments. The dose-response of membrane peptidase expression in EA.hy926 cells demonstrated a maximal increase following treatment with 30 μM vanadate, demonstrating an approximate 2.3 fold increase in levels of ECE activity relative to untreated cells (Table 5.2). This effect was confirmed by immunoblotting and phosphorimaging analysis (Fig. 5.2) which quantified the band intensity of the immunoblot. Using this method band intensity was demonstrated to be 2.7 fold higher in cells treated with 30 μM vanadate compared with untreated cells.

The effects of vanadate were further studied on other cell lines to determine if the up-regulation of ECE in response to vanadate treatment was specific to EA.hy926 cells or whether the effect was common to other cell lines. A human glioma cell line, Bu17, which had previously been shown to express levels of ECE activity comparable with those found in EA.hy926 cells was studied to determine whether ECE was up-regulated to a similar extent. Treatment of this cell line with 30 μM vanadate resulted in a similar degree of ECE up-regulation (2.2-fold) as determined by enzyme assay (Table 5.3).

A third cell line, the rat pheochromocytoma PC12 line which exhibited a chromaffin cell phenotype and had previously been demonstrated to express negligible levels of ECE activity (Table 4.1) was investigated to determine if vanadate could "switch on" ECE expression in this cell line. Following treatment of PC12 cells with vanadate, this cell line was found to express significant levels of ECE activity, demonstrated by enzyme assay (Table 5.4) and immunoblotting (Fig. 5.4). ECE activity and levels of protein were seen in response to 10 μM up to 50 μM vanadate. Neurite extension was seen in this cell line at concentrations of >10 μM vanadate and concentrations in excess of 75 μM vanadate were found to be toxic to this cell line.

These data appear to show inhibition of tyrosine phosphatase to be a crucial event in the control of ECE levels in cells though the mechanism through which this occurs is not common to the related membrane peptidase, E-24.11 since this activity showed no comparable increase following vanadate treatment of EA.hy926 cells (Table 5.2).

Whilst the response to vanadate treatment suggests phosphorylation of cellular proteins to be a key event in the regulation of ECE protein expression, the site at which the up-regulatory effect is caused cannot be concluded from these data. Vanadate has been shown to stimulate protein tyrosine phosphorylation by inhibiting protein tyrosine phosphatases or by activating certain protein tyrosine kinases. Previous work by Shimizu *et al.*, (1994) demonstrated that treatment of HUVEC cells with μM concentrations of vanadate resulted in a suppression of ET-1 release from this cell line. This effect was reproducible on further exposure of the cells to vanadate suggesting these effects could be attributable neither to cell growth, nor to cell damage but was due to the influence of vanadate on a specific biochemical pathway through the phosphorylation of intermediates.

The effects on ECE activity following vanadate treatment of the cell lines studied in this work would appear to demonstrate there is a specific up-regulation of ECE expression as demonstrated by immunoblotting resulting in higher specific activities in treated cells. This represents higher activity due to the level of ECE protein produced by the cell being increased relative to total cellular protein. These data in parallel with the findings of Shimizu *et al.*, (1994) suggest that the response of the cell to suppression of ET-1 release is to stimulate greater production of ECE. The hypothesis of a direct feedback mechanism in response to suppression of ET-1 release is supported by the fact that similar concentrations of vanadate were needed to suppress ET-1 release from HUVEC and to up-regulate levels of ECE in EA.hy926 cells.

Whilst this mechanism may operate through protein tyrosine phosphorylation, other cellular effects known to be induced by vanadate cannot be discounted. Previous work has demonstrated IL-1, IL-2 and insulin to be implicated in the regulation of ET-1 production in endothelial cells whilst IL-2 and insulin have both been shown to operate through tyrosine kinase activity (Saltzman *et al.*, 1988; Merida & Gaulton, 1990; Petruzzelli, 1984). Insulin has been shown to increase ET-1 production (Hu *et al.*, 1993) whilst Taniguchi *et al.*, (1992) demonstrated suppression of ET-1 release in response to IL-2 in cultured endothelial cells.

The data presented in this work therefore suggest that protein tyrosine phosphorylation may be a key event in a direct feedback mechanism between ECE and ET-1 levels produced by the cell. This regulation may be mediated by cytokines acting on endothelial cells.

CHAPTER SIX

THE IDENTIFICATION OF ECE-1 ISOFORMS USING ANTI-PEPTIDE ANTIBODIES

6.1 AIMS

To design immunogenic peptide sequences corresponding to each of the reported ECE-1 isoforms.

To invoke an immune response to these peptides in rabbits and purify specific antibodies from the antisera.

To screen cell lines and tissues for the expression of each isoform using western blotting.

6.2 Introduction

The alternative to raising antibodies to a whole protein is to synthesise short peptide sequences corresponding to unique regions of the protein sequence. Previous use of this strategy has been used for the biochemical and structural characterisation of many other proteins (e.g. Kirkness and Turner, 1988; Stephenson and Duggan, 1991; Molnar *et al.*, 1993).

Following the molecular cloning of ECE by several groups in 1993 the generation of antibodies to ECE by using the purified protein could now be by-passed by this more convenient strategy, particularly since the purification of ECE was not a trivial task. Since the yield of ECE from tissues or cell lines was relatively low and the procedure for purification time consuming, the alternative of using short peptide sequences deduced from the published cDNA sequence offered several advantages. Following the discovery of the existence of 2 ECE-1 isoforms, α and β (Shimada *et al.*, 1994, 1995; Schmidt *et al.*, 1994; Xu *et al.*, 1994; Ikura *et al.*, 1994) (Fig. 6.1) (these isoforms were termed b and a respectively by Valdenaire *et al.*, 1995), it also became possible to raise antibodies to distinguish between the individual isoforms by targeting the distinct peptide sequences at the N-termini.

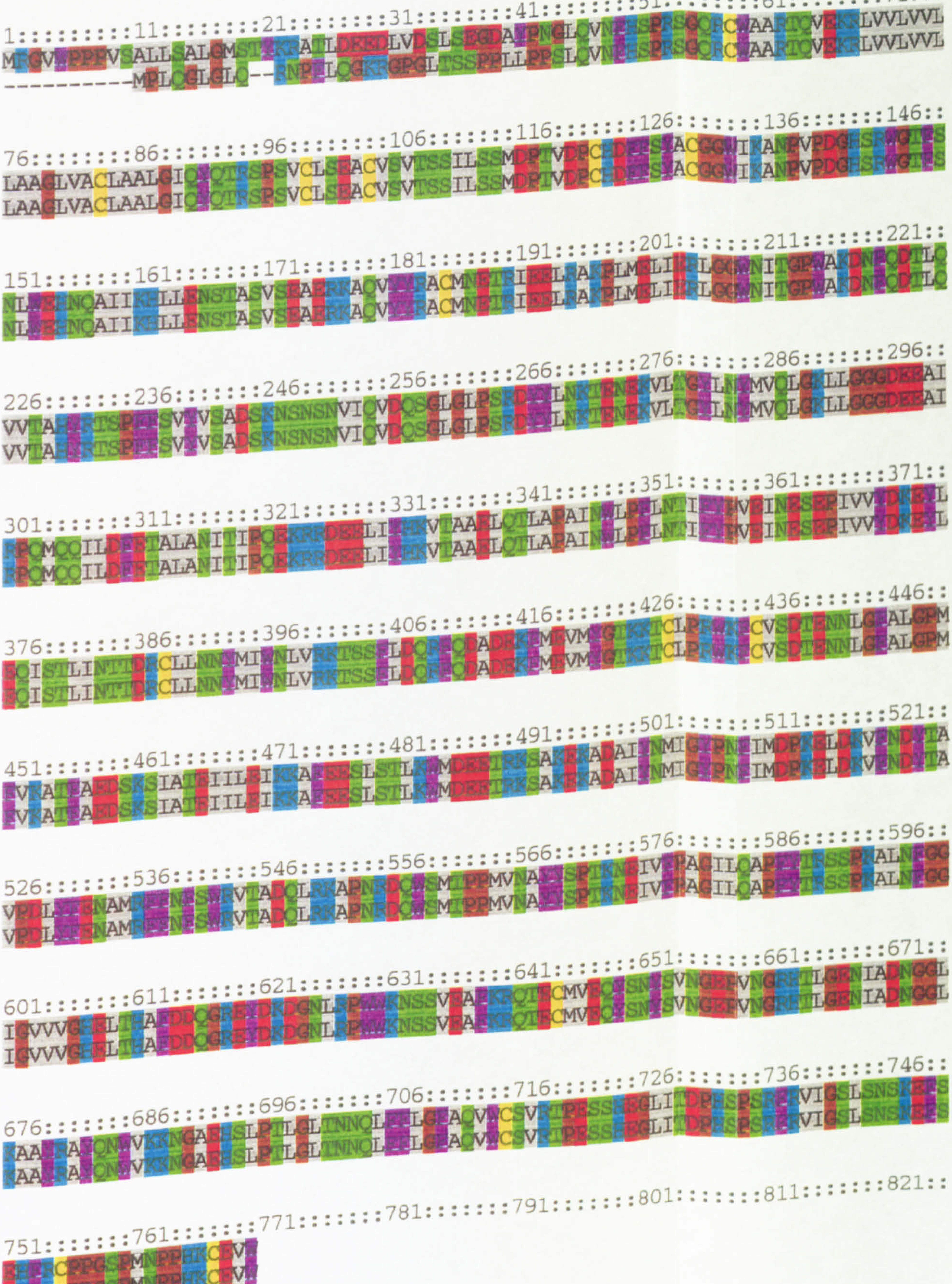
The anti-peptide antibody strategy is not without some disadvantages. For instance, the antibodies raised may not recognise the sequence when it is presented as part of the native protein and for this reason anti-peptide antibodies are rarely suited to immunoprecipitation and occasionally the peptide itself is not sufficiently immunogenic and the specific antibody titre of the serum remains low. The production of antibodies in this way was carried out with the intention of studying tissue specific expression of the ECE-1 isoforms and relied on the identification of sequences unique to each isoform which were also predicted to be immunogenic as defined by several criteria outlined in section 6.4.

Fig. 6.1 Sequence alignment of ECE-1 α and β isoforms (page 119)

Sequences of the ECE-1 α (Schmidt *et al.*, 1994) and β (Shimada *et al.*, 1995) isoforms were aligned to give the best fit between the two sequences. Amino acids were colour coded according to characteristics as follows:

Red, acidic residues (E, D)
Blue, basic residues (H, K, R)
Green, polar residues (N, Q, S, T)
Grey, non-polar residues (A, I, L, M, V)
Purple, aromatic residues (F, W, Y)
Brown, structure-disrupting residues (G, P)
Yellow, cysteine residues (C)

HUMECE1A	**Human ECE-1 α sequence**
HUMECE1B	**Human ECE-1 β sequence**



6.3 Selection of peptides

6.3.1 Selection of peptides for synthesis

Certain criteria have been established to identify regions of a protein which would be most likely to stimulate antibody production in the host animal (Stephenson and Duggan, 1991) and while such identification is no guarantee of immunogenicity, this method does maximise the chances of generating a specific immune response against the peptide. The peptide should correspond to a region of the protein most likely to be exposed in the protein. In addition, highly mobile regions of a protein are likely to be recognised by anti-peptide antibodies (Niman *et al.*, 1983) and so the N and C termini, which are generally less conformationally constrained than other regions, are likely candidates. This has been used in the strategies successfully adopted by Stephenson and Duggan (1991) and Wenthold *et al.*, (1992). In the present instance, the α and β isoforms of ECE-1 differ only in terms of their N-terminal sequence and this offered an ideal opportunity for the raising of specific antibodies to each isoform by this method.

The likelihood of a given peptide sequence being exposed in the native protein is linked to the degree of hydrophilicity and whether the sequence forms or is part of a turn/loop structure. Previous work has shown that the ideal sequence length is at least 6 residues, up to a maximum of 20. Generally peptides of 12-15 amino acids are used for antibody production. Whilst shorter peptides may not be sufficiently immunogenic, longer sequences may also have the advantage of being able to mimic the conformation of the native protein and may also reduce the chances of antibodies cross-reacting with other proteins. In addition another important consideration when selecting peptides for use is that regions of sequence which are likely to be glycosylated should be avoided to ensure structural consistency between the peptide and native protein.

Assessment of the antigenicity of amino acid residues has been accomplished by analysing the frequency with which they occur in known epitopes of proteins. The order established, in decreasing antigenicity is histidine, lysine, alanine, leucine, aspartate and arginine (Scheidtman, 1990). Examining the frequency of these residues within the selected peptide can be used to give an indication of the potential immunogenicity of the peptide. The immunogenicity is also increased by presentation of the peptide to the immune system by coupling the peptide to a carrier protein, in this case Keyhole Limpet Haemocyanin (KLH).

As mentioned above, the termini of proteins are likely to be exposed and are therefore targets frequently selected for peptide synthesis. Whilst this increases the likelihood of raising an antigenic response it is no guarantee of success and it is still advisable to check these regions for hydrophilicity. Potential peptides adhering to the criteria described were checked with the OWL database for similarity to other proteins and were synthesised with a cysteine at the C-terminus to allow linkage to the carrier protein.

In the case of the N-terminal cytosolic sequences of the isoforms, there was a high degree of similarity towards the C-terminus but significant differences were seen in the N-terminal regions (Fig. 6.1). In order to raise antibodies to each isoform it was necessary to identify likely immunogenic regions which were unique to each isoform.

The rationale for peptide selection involved analysis of the human ECE 1 α and β isoforms sequenced by Shimada *et al.* (1994) and identification of the sequences most likely to prove immunogenic using a number of algorithms described below.

6.4 Computer analysis of potential peptide sequences

Computer programs were used to identify sequence differences between the ECE-1 α and β isoforms (Fig. 6.1) and analyse certain biochemical characteristics of these regions to determine their suitability for use in the production of antibodies.

6.4.1 Prediction of hydrophilicity/hydrophobicity

A prediction of the hydropathy of the protein sequence was created using a program design first used by Kyte & Doolittle (1982). The relative degree of hydrophobicity of any amino acid side chain within the peptide sequence is presented as a graph about a zero x-axis, with hydrophobic regions shown above the x-axis and hydrophilic regions below. The hydrophobic transmembrane sequences can thus be potentially identified although these need to be confirmed by additional biochemical criteria.

6.4.2 Surface probability

Surface probability is calculated according to the formula of Emini *et al.*, (1985) and describes the likelihood of any given residue being exposed at the surface of the protein. This algorithm is therefore strongly influenced by the hydrophilic nature of the window of amino acids being examined. Regions above the base line are predicted to be at the surface.

6.4.3 Flexibility

Flexibility is calculated according to the formula of Karplus & Schulz (1985), and is an analysis of the structural constraints on any given residue and is therefore allied to the probability of any given residue participating in secondary structures. The measurement of flexibility is given on a scale of 0.8 to 1.2 where the higher the rating, the greater the flexibility of the sequence at that point.

6.4.4 Prediction of secondary structure

Prediction of secondary structure of proteins is based on the character of amino acids in the polypeptide chain and their interaction with adjacent amino acids. Structural analysis of many proteins has identified trends of amino acids occurring in certain secondary structures. Examples of this include β-pleated sheet structures which have a high isoleucine content whereas α helices contain more glutamate, alanine and leucine.

Two algorithms were used to predict the occurrence of secondary structures; the Chou and Fasman method (Chou & Fasman, 1978) and the Robson Garnier method (Garnier *et al.*, 1978).

6.4.4.1 Chou & Fasman method

This method is used to predict helices, sheets and turns. It resolves overlapping regions of alpha helices and beta sheets by using the probability procedure introduced by Nishikawa (1983). This same procedure is also able to locate turns which are not in conflict with other secondary structures. The original Chou Fasman parameters are modified as follows: The conditions that p(bound) be greater than 1.0 and that p(alpha) be greater than p(beta) are not used and sheet structures must be of 5 residues or more.

6.4.4.2 Robson Garnier method

This method predicts secondary structure according to the method described in Garnier *et al.* (1978). The minimum length of an alpha helix is six residues and beta sheet is four. Regions where accurate predictions are not possible are assigned the conformational state of the next best probability.

A strong prediction for a secondary structure is represented by a large area above the baseline shown at the extreme left of the sequence (Figs. 6.2 and 6.3). The accuracy of secondary structure prediction is questionable (Creighton, 1993) therefore a selection of peptide sequences should be subjected to other parameters to determine their immunogenic potential.

Fig. 6.2 Analysis of ECE-1 α peptide sequence features

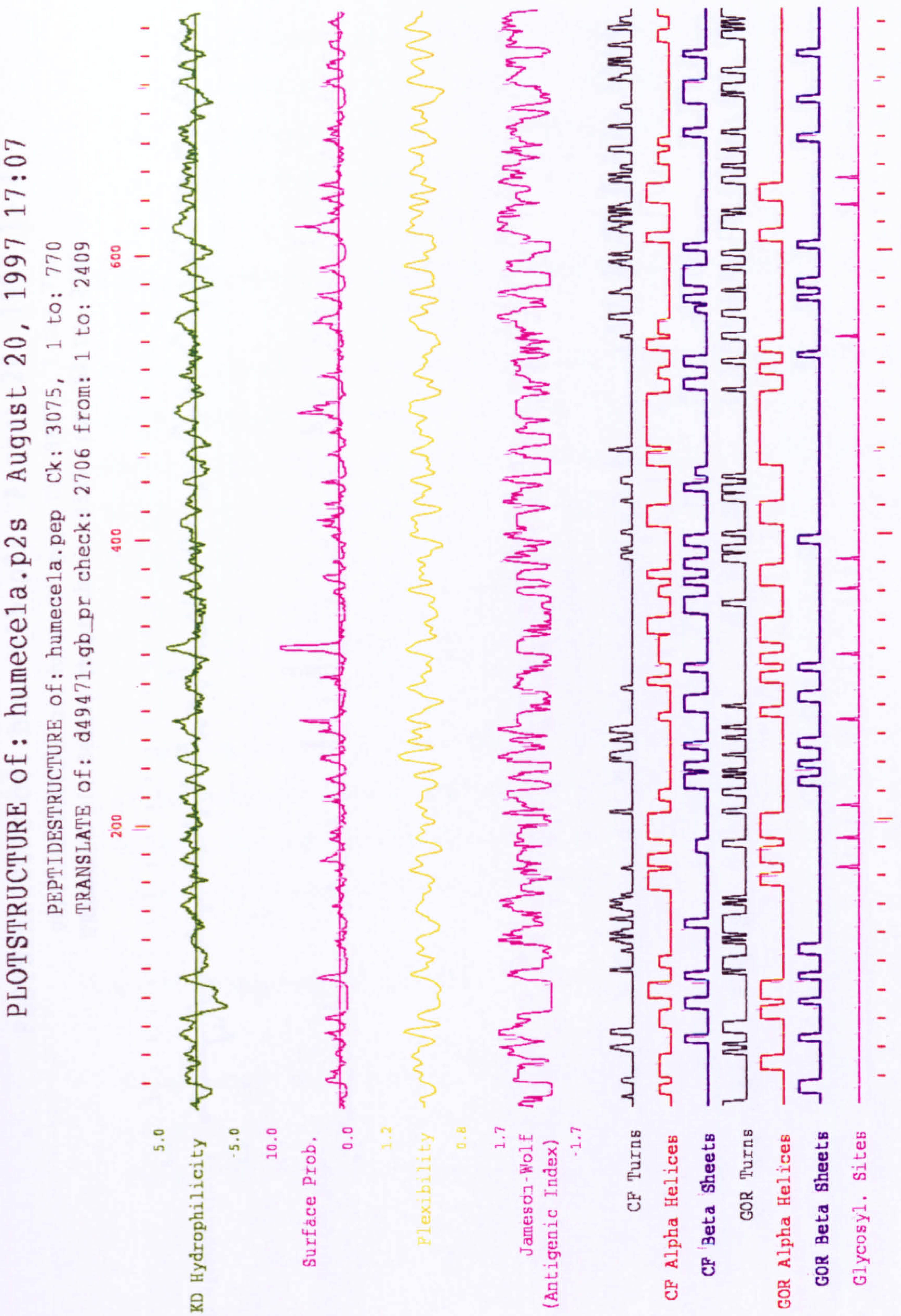
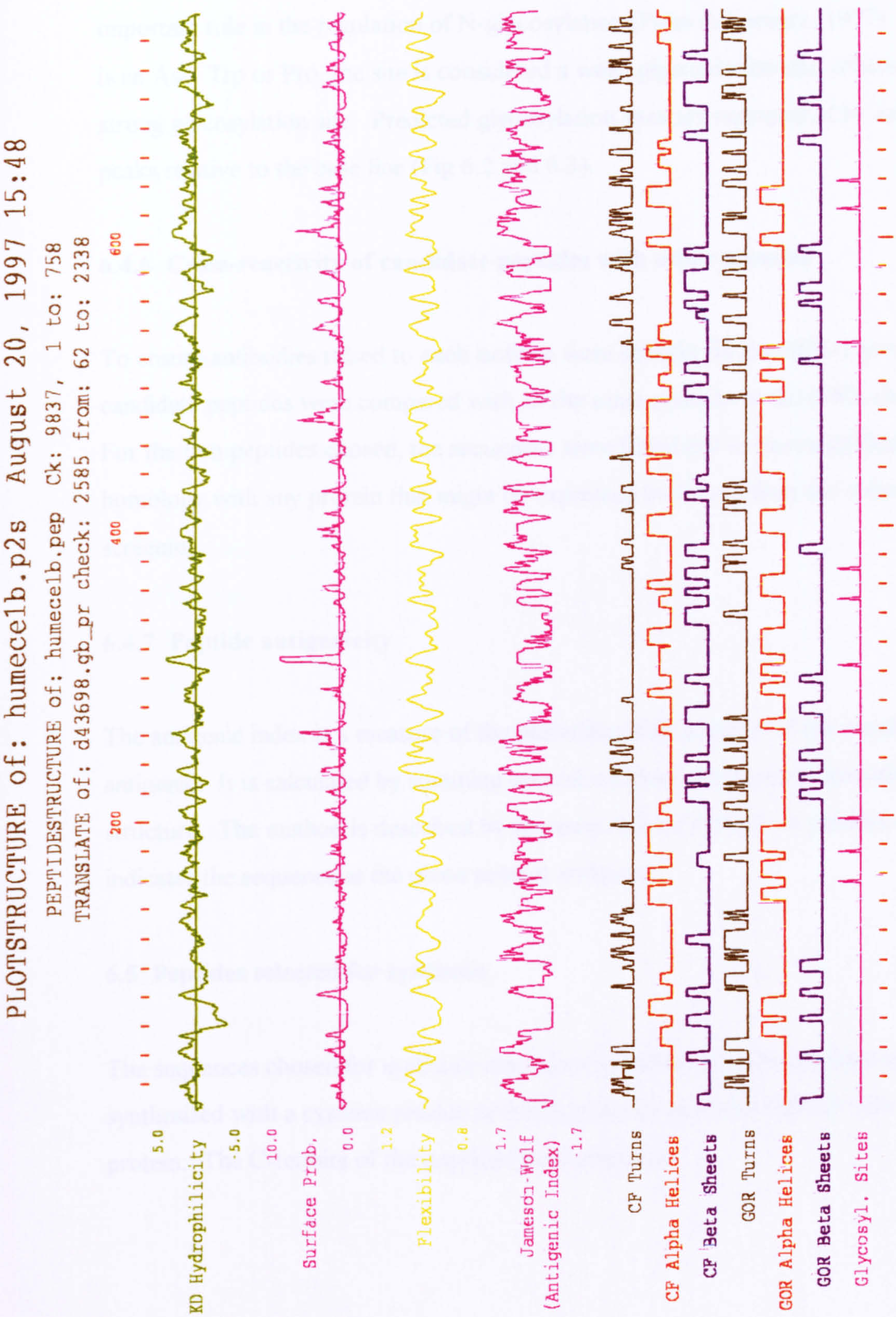


Fig. 6.3 Analysis of ECE-1 β peptide sequence features



6.4.5 Glycosylation sites

N-linked glycosylation sites are specific to the consensus motif Asn-X-Ser/Thr (Marshall, 1972) although the occurrence of this tripeptide sequence is not sufficient to conclude that an asparagine residue is glycosylated since protein folding plays an important role in the regulation of N-glycosylation (Pless & Lennarz, 1977). Where X is an Asp, Trp or Pro, the site is considered a weak glycosylation site, otherwise it is a strong glycosylation site. Predicted glycosylation sites are represented by narrow peaks relative to the base line (Fig 6.2 and 6.3).

6.4.6 Cross-reactivity of candidate peptides with other proteins

To ensure antibodies raised to each isoform were specific for the ECE-1 protein, candidate peptides were compared with all the other proteins in the OWL database. For the two peptides chosen, the sequences were found not to have significant homology with any protein that might be expressed in the cell lines and tissues used for screening.

6.4.7 Peptide antigenicity

The antigenic index is a measure of the probability that a region of the sequence is antigenic. It is calculated by summing several weighted measures of secondary structure. The method is described by Jameson & Wolf (1988). A positive rating indicates the sequence at the given point is antigenic.

6.5 Peptides selected for synthesis

The sequences chosen for synthesis are shown in Table 6.1. The peptides were synthesised with a cysteine residue at the N-terminus to enable linkage with the carrier protein. The C-termini of the peptides were amidated.

Table 6.1 Peptides selected for synthesis

	Peptides Selected	
	$CTYKRATLDEED\text{-}NH_2$	$CQRNPFLQGKRG\text{-}NH_2$
Source protein	ECE-1 α	ECE-1 β
Amino Acid Position	3-13	9-19
Reference	Schmidt *et al.*, 1994	Shimada *et al.*, 1995
Mr of peptide (Da)	1443.6	1403.6
Purity Determined by Manufacturer (%)	87	90

Both peptides were selected on the basis of several algorithms designed to identify antigenic regions of proteins. The peptides selected were also scanned by using the OWL database and found to have no significant similarities with other proteins likely to be expressed in the target cells and tissues.

Following receipt, peptides were stored under vacuum until required. Purity was checked by the manufacturer by using HPLC and further analysis by mass spectroscopy was performed by Dr. J. Keen, University of Leeds. Both peptides were found to be sufficiently pure for coupling to the carrier protein.

6.5.1 Peptide purity

Following synthesis of the peptides the purity was checked using mass spectrometry performed by Dr. J. Keen, University of Leeds. The degree of purity is shown in Table 6.1. Both peptides were found to be sufficiently pure to require no further treatment prior to coupling to the carrier protein.

6.5.2 Peptide coupling to the carrier protein

Peptides are linked to a carrier protein in order to increase their immunogenicity and to prevent their rapid degradation in the rabbit. In this study, each peptide was synthesised with a cysteine residue at the N-terminus to enable coupling with the carrier protein, keyhole limpet haemocyanin (KLH). KLH has been used successfully although it does have the disadvantages of precipitating due to its high molecular weight and generating a high immune response itself.

Both peptides were coupled to KLH using a linker molecule, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) which attaches to amino groups on the KLH protein while the free maleimide group on the linker reacts with the free thiol of the peptide, which is reduced prior to joining of the peptide (Fig. 6.4).

Peptide coupling is described in section 2.7.3. Binding of the protein to the KLH-MBS complex was monitored using Ellman's reagent. The peptide-carrier protein conjugate was stored in aliquots at -20°C.

6.5.3 Injection protocol

Peptide conjugates were injected into New Zealand white rabbits (2 rabbits per peptide). Pre-immune serum was collected prior to any injections being given. Antisera were collected following each boost injection (every 2-3 weeks) and stored at -20°C for long term storage or 4°C for short term storage. Since two rabbits were injected per peptide, there were a total of 4 samples of antisera. The nomenclature regarding these is as follows:

ECE-1 α peptide antisera designated ECE1a 1 and ECE1a 2
ECE-1 β peptide antisera designated ECE1b 1 and ECE1b 2

6.6 Analysis of antisera following final injection

6.6.1 Assessment of immunoreactivity of antisera

Immunoreactivity against the immobilised peptide was tested using an enzyme linked immunoassay (E.L.I.S.A.) as described in section 2.7.5. Serial dilutions of antisera were incubated on plastic plates, the wells of which had been coated with each peptide. Immunoreactivity was determined by measuring colour development at 405 nm. Controls were performed by using pre-immune serum and, in each case, cross-reactivity with the other peptide was assessed (Figs. 6.5 & 6.6). The results of the E.L.I.S.A showed only antiserum ECE1a 1 was sufficiently immunogenic and specific for the peptide against which it was raised. A further booster injection (the fifth in total) of each of the rabbits immunised to the ECE-1 β peptide failed to stimulate a powerful and specific response and the only antiserum to undergo further treatment was therefore ECE1a 1.

Fig. 6.4 Structure of the peptide conjugate linkage

KLH - MBS - peptide

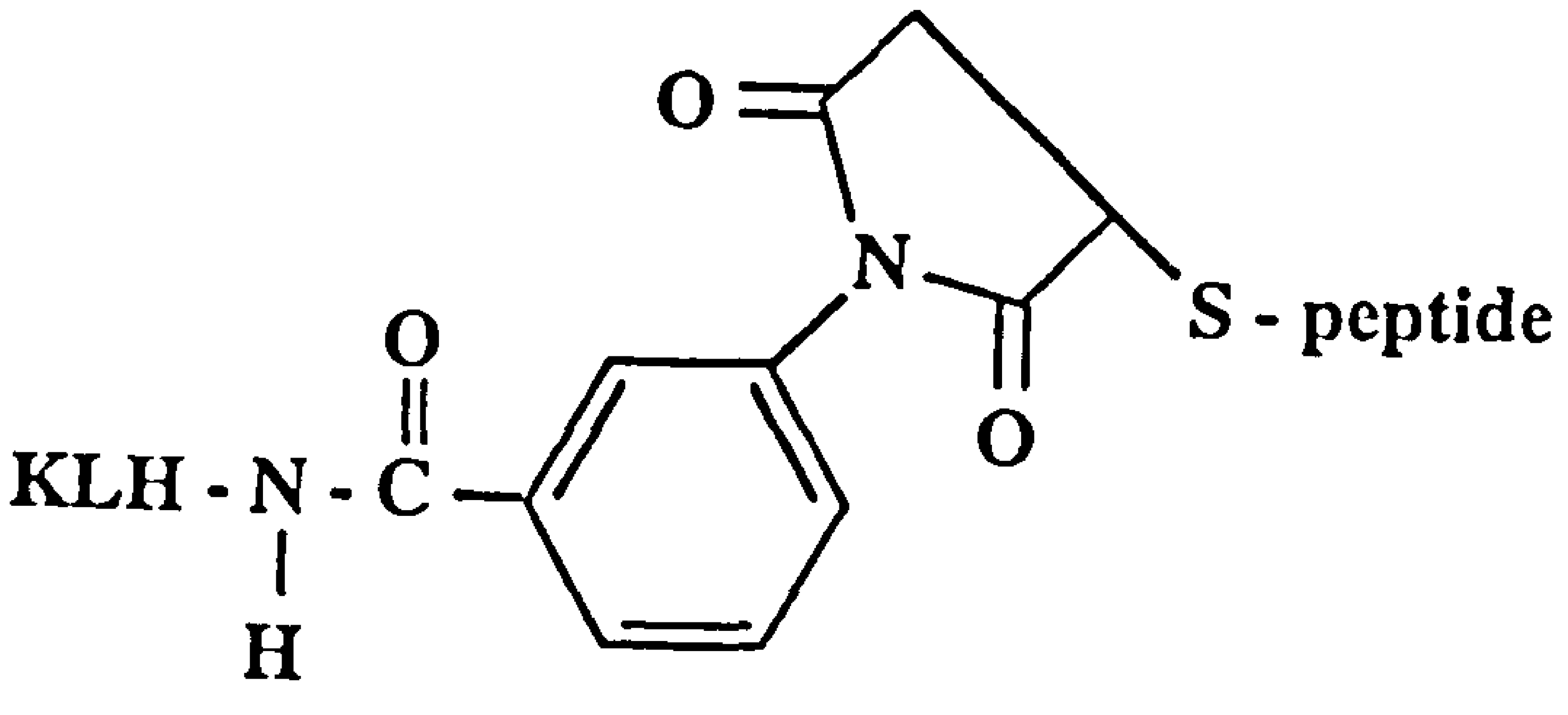
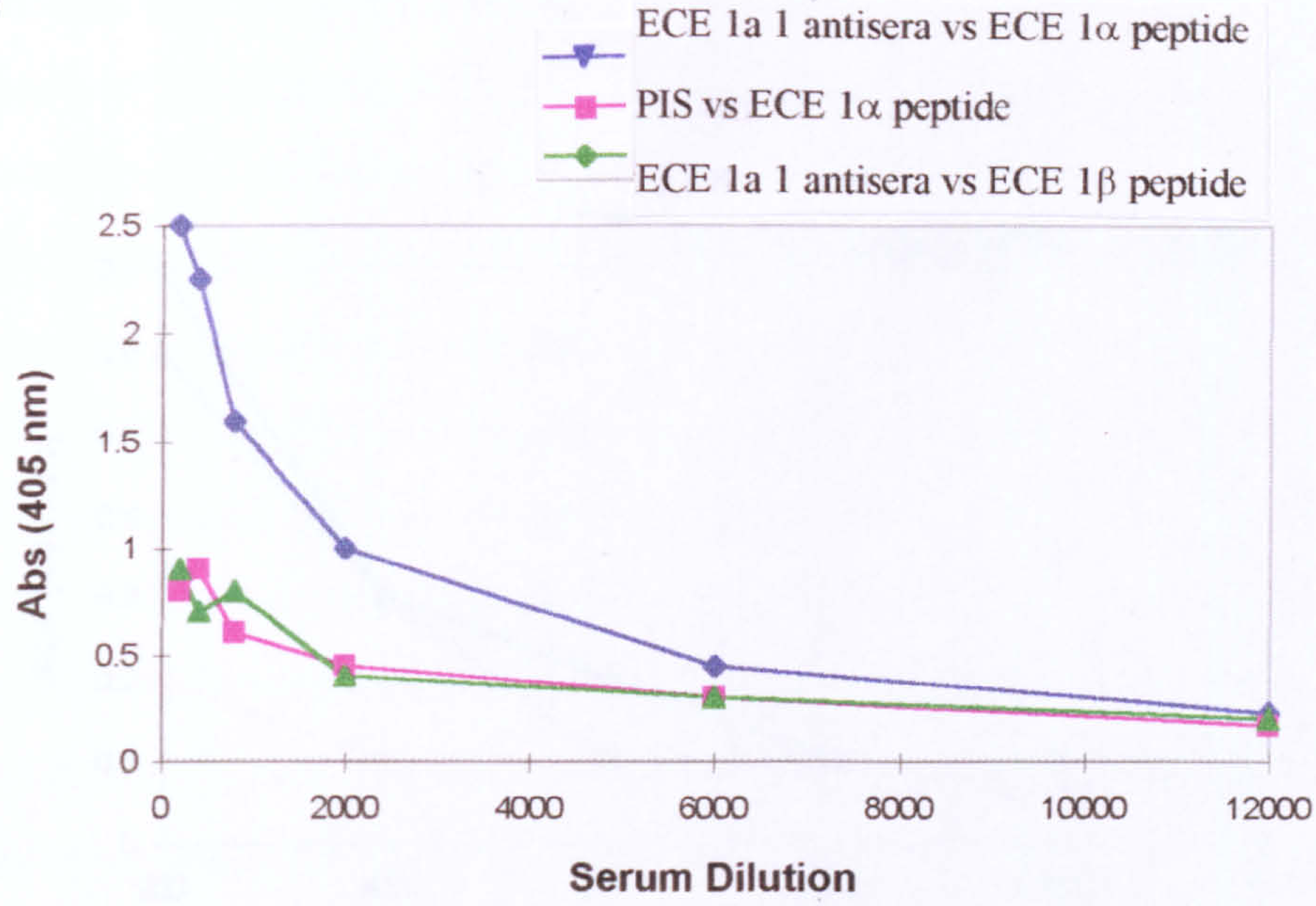


Fig. 6.5 E.L.I.S.A. analysis of immunoreactivity of ECE1a 1 and ECE1a 2 antisera

i)



ii)

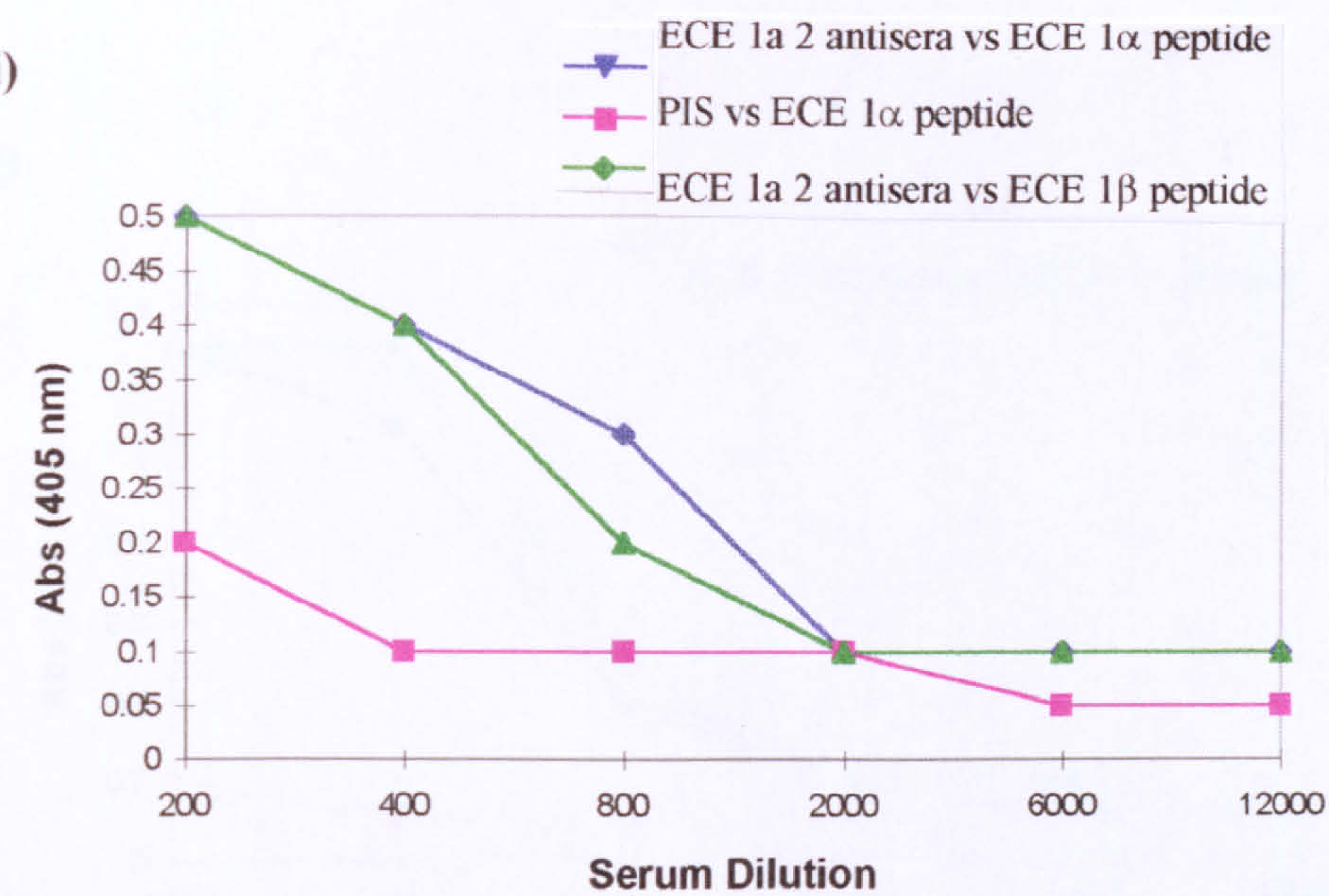
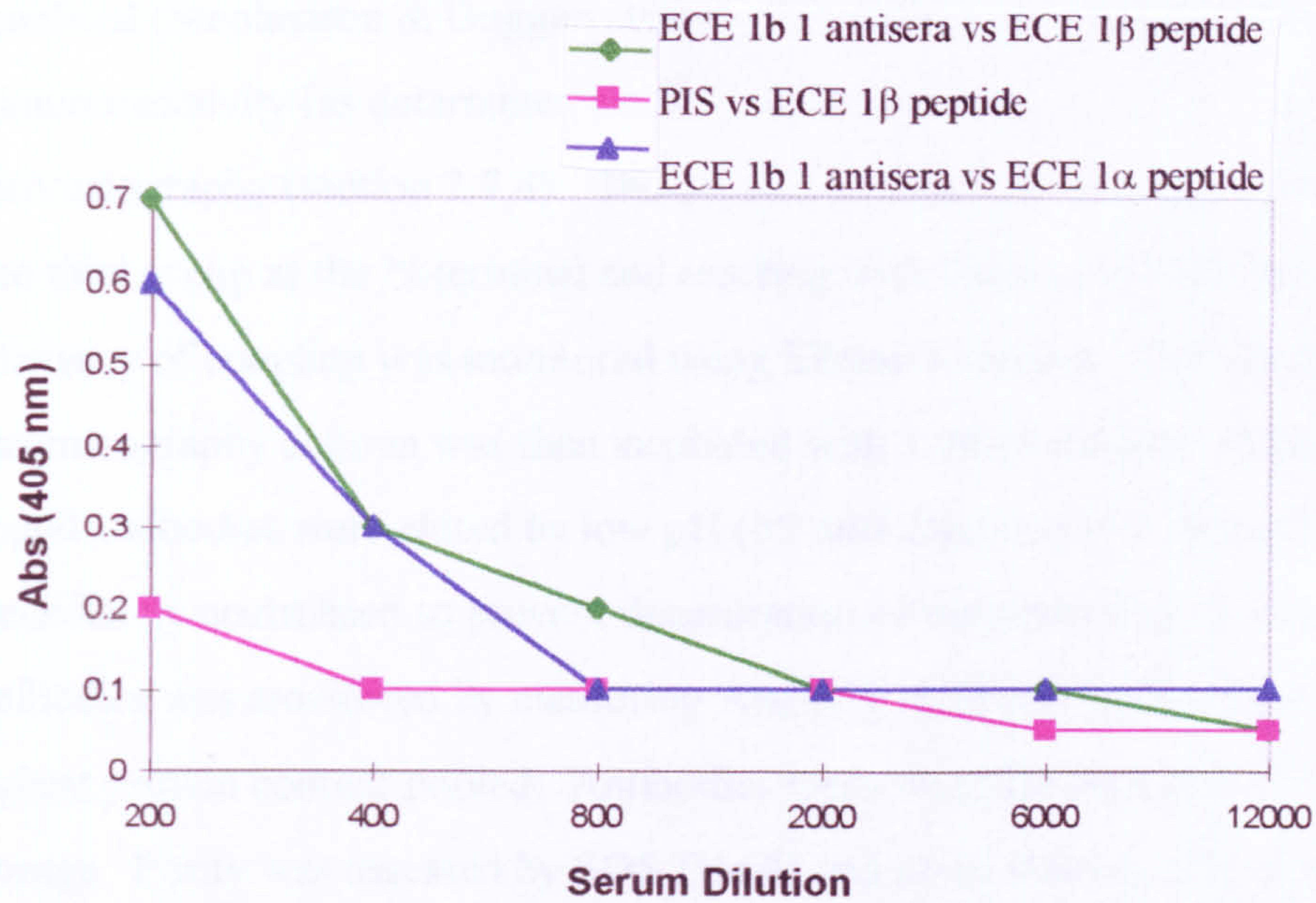


Plate wells were coated with 20 ng of peptide 1 and incubated with dilutions of antisera ECE1a 1 (i) and ECE1a 2 (ii) and pre-immune serum (PIS). Plate wells coated with the peptide corresponding to the ECE-1 α protein (20 ng/well) were incubated to check if this cross-reacted with either of the ECE-1 antisera.

Fig. 6.6 E.L.I.S.A. analysis of immunoreactivity of ECE1b 1 and ECE1b 2 antisera

i)



ii)

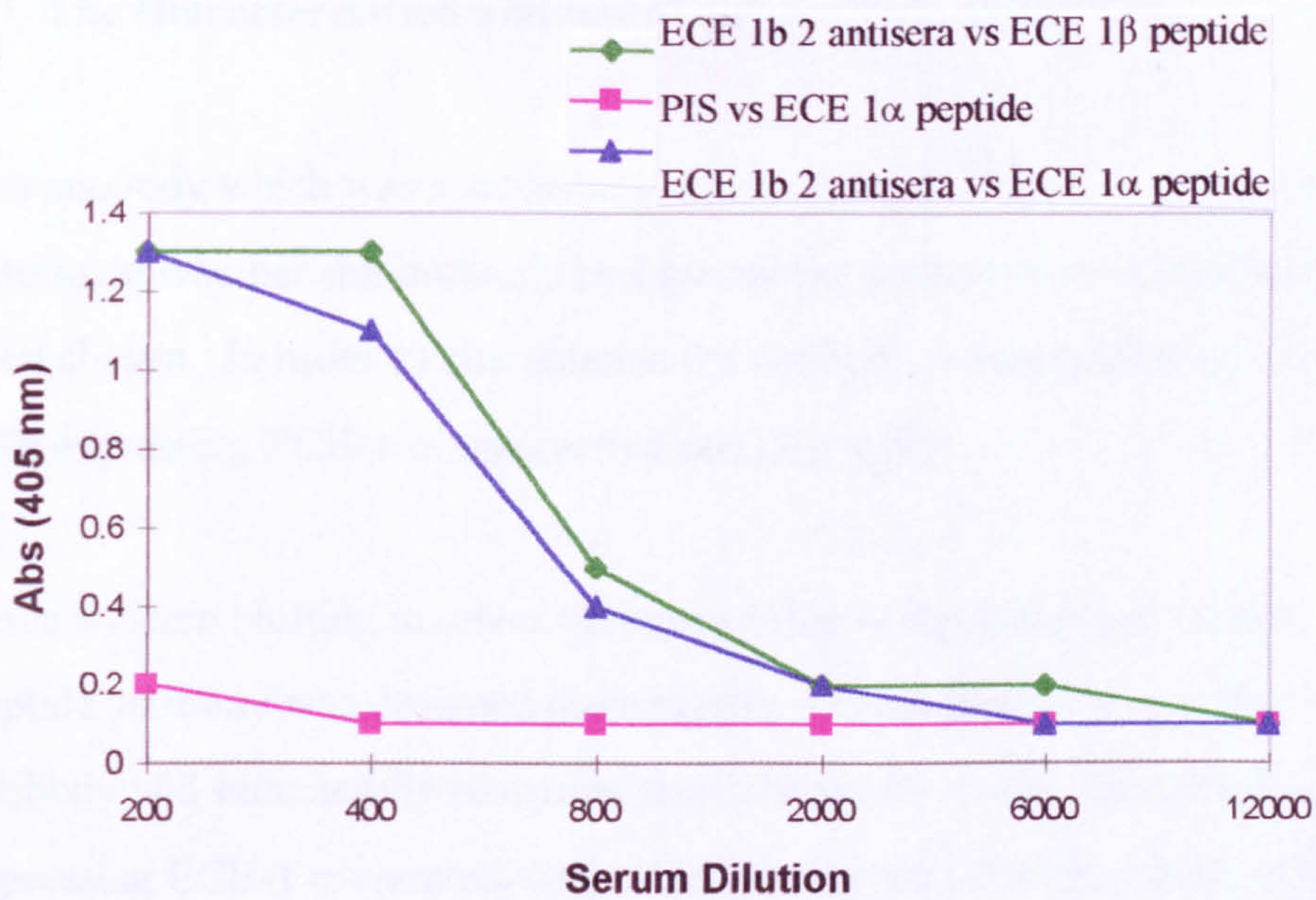


Plate wells were coated with 20 ng of peptide 1 and incubated with dilutions of antisera ECE1b 1 (i) and ECE1b 2 (ii) and pre-immune serum (PIS). Plate wells coated with the peptide corresponding to the ECE-1 β protein (20 ng/well) were included to check if this cross-reacted with either of the ECE-1 antisera.

6.6.2 Affinity purification of anti-peptide antibodies

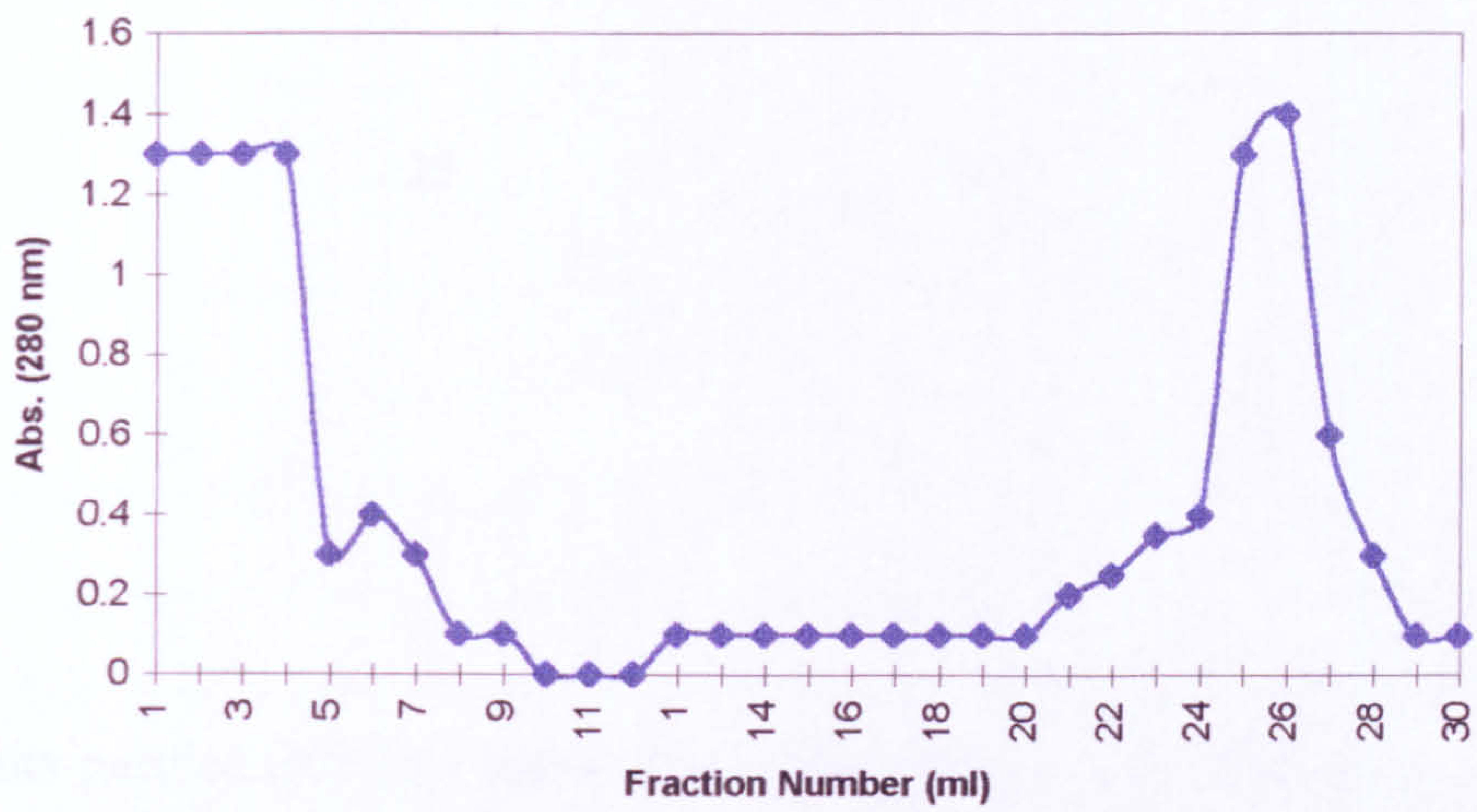
Previous studies have shown that affinity purification of anti-peptide antibodies is beneficial (Stephenson & Duggan, 1991). The antisera with the highest immunoreactivity (as determined by E.L.I.S.A. above) were purified by peptide affinity chromatography (section 2.7.4). The peptide was immobilised first by reducing the free thiol group at the N-terminal and reacting with thiopropyl Sepharose-6B. The efficiency of coupling was monitored using Ellman's reagent. The affinity chromatography column was then incubated with 5 ml of antisera. After washing, bound antibodies were eluted by low pH (50 mM glycine, pH 2.3) and the eluant immediately neutralised to prevent denaturation of the antibodies. Elution of the antibodies was monitored by measuring A_{280} (Fig. 6.7) and the fractions with the highest protein content pooled. Antibodies were stored in PBS at -70°C for long term storage. Purity was assessed by SDS-PAGE and silver staining (Fig. 6.8).

6.7 The characterisation and use of anti-peptide antibodies

The antibody which was successfully raised to ECE-1 α was characterised to determine whether this antibody recognised the protein from which the peptide had been chosen. In order to characterise the antibody, western blotting of transfected cells expressing ECE-1 α was carried out (Fig. 6.9).

Since western blotting involves the recognition of the denatured protein and the anti-peptide antibody was designed to recognise a linear epitope it is probable that the antibody will successfully recognise the denatured protein. Samples suspected of expressing ECE-1 α were applied to denaturing SDS-PAGE and the separated proteins electrophoretically transferred to a PVDF membrane. Binding of antibodies to polypeptides was detected using the ECL enhancement system and the relative molecular mass (*M*r) of the polypeptides recognised was then calculated by comparison with molecular weight standards.

Fig. 6.7 Elution profile of ECE1a 1 antibody from ECE-1 α peptide affinity column



The ECE1a 1 antiserum was affinity purified using the method described in section 2.7.4. Once the initial peak in absorbance had decreased to <0.05, representing the removal of non-specific proteins bound to the column, antibodies were eluted using 0.2 M glycine-HCl, pH 2.4 and fractions of 1 ml were collected and immediately neutralised using 1 M Tris-HCl buffer (pH 8.0). Absorbance at 280 nm was monitored and peak fractions with the greatest absorbance were combined and dialysed against PBS.

Fig. 6.8 SDS-PAGE of affinity purified ECE1a 1 antibody

55

25

1

Affinity purified ECE1a 1 antibody was loaded on a 10 % SDS-polyacrylamide gel (lane 1, 5 μg) under reducing conditions. Following separation, proteins were detected by silver staining and molecular weights calculated by comparison to commercially available standards.

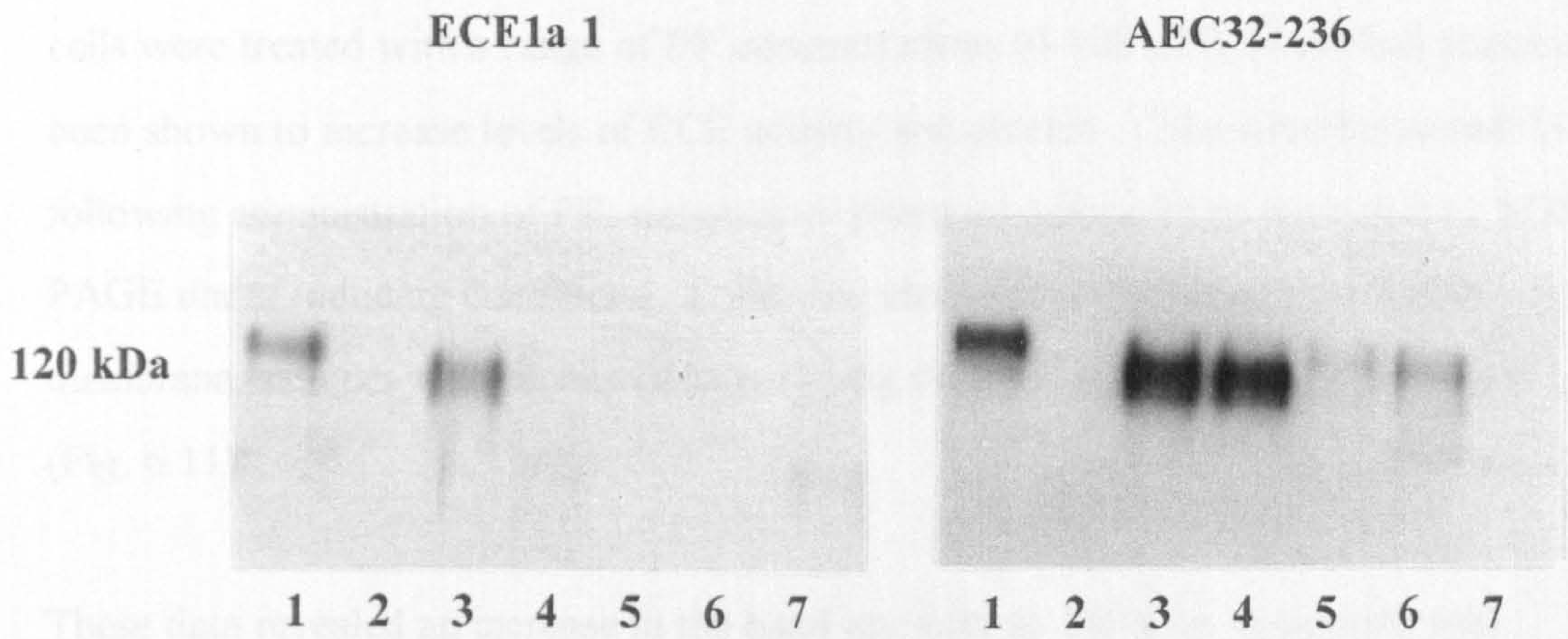
6.7.1 The specificity of immunorecognition by the ECE1a 1 antibody

The purity of the antibody was seen by protein separation on a polyacrylamide gel and subsequent silver staining (Fig. 6.8). This resolved the heavy and light chains of the antibody and demonstrated the affinity purification had been successful. In order to determine if the antibody recognised the ECE-1 α protein and was specific in this recognition, CHO and COS cells expressing ECE-1 α and COS cells expressing ECE-1 β were applied to SDS-PAGE and immunoblotted with the ECE1a 1 antibody (Fig. 6.9'). The western blotting procedure used goat anti-(rabbit IgG) IgG as the second antibody. The results show strong immunorecognition of the ECE band at 120 kDa in transfected cells expressing ECE-1 α and no band detected in the cells expressing ECE-1 β or in COS and CHO cells which had not been transfected. This indicated the antibody was specific for the ECE-1 α isoform. Blotting of the same cells with the AEC32-236 antibody showed ECE recognition at 120 kDa in all transfected cell lines expressing either of the ECE-1 isoforms.

6.7.2 Immunoblotting of cultured cells and pig lung membranes using the ECE1a 1 antibody

To further examine expression of the α form of the ECE-1 protein, membranes were prepared from a range of cultured cell lines and tissues and immunoblotted with the ECE1a 1 antibody (Fig. 6.10). Under reducing conditions, TRLEC-03 cells showed immunorecognition at 120 kDa consistent with the findings of Shimada *et al.*, (1995). ECE-1 α was also detected in membranes prepared from EA.hy926 cells, smooth muscle cells and pig lung membranes though the clarity of recognition was diminished by the high degree of non-specific binding.

Fig. 6.9 Immunorecognition of ECE-1 α expressed by transfected and non-transfected cell lines using ECE1a 1 and AEC32-236 antibodies



1 CHO cells transfected with ECE-1 α

2 CHO cells not transfected

3 COS cells transfected with ECE-1 α

4 COS cells transfected with ECE-1 β

5 COS cells not transfected

6 EA.hy926 cell membranes

7 COS cells transfected with E-24.11

All lanes except (6) were loaded with 6 μg protein. Lane (6) was loaded with 10 μg of protein. Proteins were separated on a 10 % SDS-polyacrylamide gel under reducing conditions. Following electrophoretic transfer to PVDF membrane, proteins were detected with either the ECE1a 1 or AEC32-236 antibody. Immunorecognition was detected using the ECL detection system.

6.7.3 Immunoblotting of PR treated EA.hy926 cell membranes using the ECE1a 1 antibody

In order to enhance immunorecognition of ECE-1 α in cultured cell lines, EA.hy926 cells were treated with a range of PR concentrations (0-100 μM) which had previously been shown to increase levels of ECE activity and protein. Cells were harvested 24 h following administration of PR, membranes prepared and proteins separated by SDS-PAGE under reducing conditions. Following electrophoretic transfer to PVDF membrane, samples were immunoblotted using the ECE1a 1 antibody to ECE-1 α (Fig. 6.11).

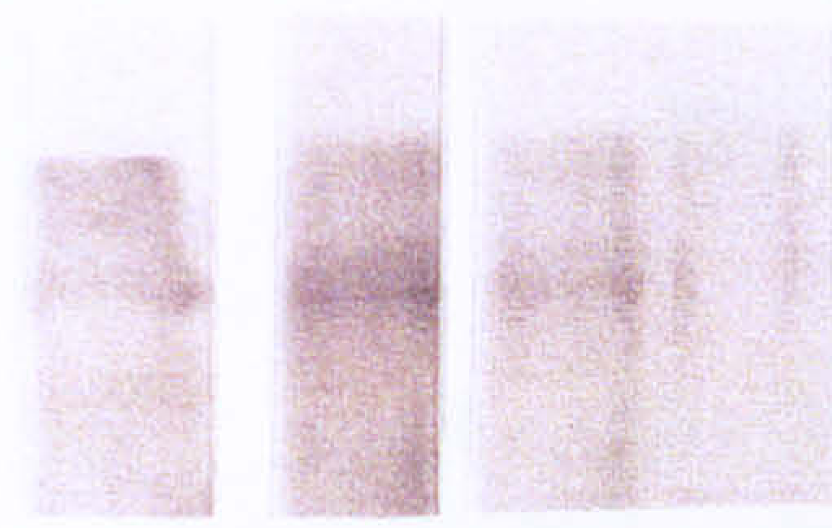
These data revealed an increase in the band intensity at 120 kDa, consistent with previous findings (section 4.2) which showed ECE to be up-regulated in this cell line in response to PR treatment. In common with this previous work, the effect was maximal following treatment with 30 μM PR.

6.7.4 Immunoblotting of PR treated Bu17 cell membranes using the ECE1a 1 antibody

The up-regulatory effect of PR on ECE expression had previously been reproduced in Bu17 cells (section 4.2). In order to determine if this enhanced ECE-1 α protein levels, membranes prepared from Bu17 cells treated with 0-100 μM PR, 24 h prior to cell harvest, were separated by SDS-PAGE under reducing conditions. Proteins were electophoretically transferred to PVDF membrane and ECE-1 α expression detected using ECE1a 1 antibody (Fig. 6.12).

In common with the immunorecognition profile seen in EA.hy926 cells in response to PR, Bu17 cells were seen to express detectable levels of ECE-1 α following treatment with PR. A PR concentration of 30 μM induced the greatest increase in ECE-1 α.

Fig. 6.10 Immunoblotting of membranes prepared from cells and tissues with the ECE1a 1 antibody to ECE-1 α



1 Pig lung membranes

2 TRLEC-03 cell membranes

3 EA.hy926 cell membranes

4 Porcine smooth muscle cell membranes

All lanes were loaded with 14 μg of protein. proteins were separated on a 10 % SDS-polyacrylamide gel. Following separation, proteins were electrophoretically transferred to a PVDF membrane. Samples were immunoblotted using the ECE1a 1 polyclonal antibody to the ECE-1 α isoform. Immunorecognition was detected using the ECL development system.

Fig. 6.11 Immunoblotting of PR treated EA.hy926 cell membranes using the ECE1a 1 polyclonal antibody to ECE-1 α

PR (μM)

0 10 30 50 100

kDa 120

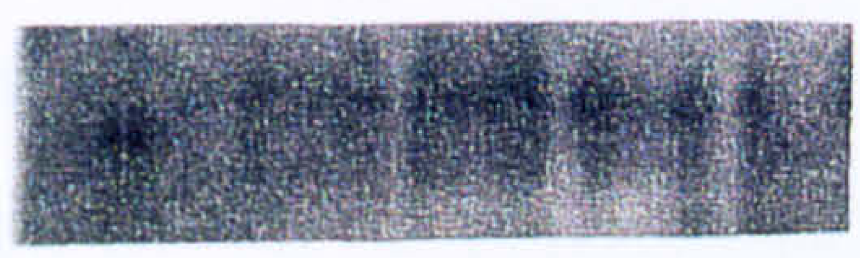
Flask cultured EA.hy926 cells were treated with 0-100 μM PR 24 h prior to cell harvest. Membranes were prepared and proteins separated by SDS-PAGE on a 10 % SDS-polyacrylamide gel (10 μg/well). Following transfer to PVDF membrane, proteins were detected using the ECE1a 1 polyclonal antibody to ECE-1 α.

Fig. 6.12 Immunoblotting of PR treated Bu17 cells using the ECE1a 1 polyclonal antibody to ECE-1 α

PR (μM)

0 10 30 50 100

kDa 120



Flask cultured Bu17 cells were treated with 0-100 μM PR 24 h prior to cell harvest. Membranes were prepared and proteins separated by SDS-PAGE on a 10 % polyacrylamide gel (10 μg/well). Following transfer to PVDF membrane, proteins were detected using the ECE1 a1 polyclonal antibody to ECE-1 α.

6.8 Discussion

The objective of this part of the work was to raise antibodies to each of the ECE-1 isoforms, α and β which were cloned and expressed by Schmidt *et al,* (1994) and Shimada *et al.* (1995). Protein sequences were analysed using several computer based algorithms to determine the immunogenicity of peptide sequences within the protein. N-terminal sequence differences between the α and β isoforms allowed immunogenic peptides unique to each isoform to be designed. These peptides were synthesised and coupled to a carrier protein (KLH) for presentation to the immune system of a rabbit. A course of 4 injections of the protein were administered (5 in the case of the ECE-1 β peptide). Test bleeds were taken throughout the injection protocol and analysed by E.L.I.S.A. to determine the strength and specificity of the immune recognition. Whilst one rabbit elicited a strong immune reaction to the ECE-1 α (designated ECE-1a 1) there was no significant immune response by either the second rabbit injected with the ECE-1 α peptide or the rabbits immunised with the ECE-1 β peptide.

The antiserum which did show specific immunorecognition of the ECE-1 α peptide was affinity purified by immobilising the peptide to an inert resin and bound antibodies eluted and neutralised. Antibody samples isolated in this way were examined for purity by SDS-PAGE which revealed two bands corresponding to the light and heavy chains of the antibody protein which demonstrated the purity of the antibody.

The purified antibody, which had been raised to a peptide sequence of 11 amino acids was analysed to determine if it recognised the entire protein sequence expressed by CHO and COS cells transfected with ECE-1 α. These cells, which had been shown to express high levels of the ECE-1 α protein, were immunoblotted using the ECE-1a 1 antibody. Bands were detected at approximately 120 kDa under reducing conditions in cells transfected with the ECE-1 α cDNA indicating the antibody did recognise the ECE-1 α protein. The specificity of this recognition was further supported by the fact that non-transfected cells and cells transfected with ECE-1 β did not show any immunoreactivity. Whilst this demonstrated that specific antibodies to ECE-1 α had been successfully raised, the recognition of the entire protein had only been demonstrated in transfected cell lines known to express high levels of ECE.

Previous work by Shimada *et al.*, (1995) had demonstrated ECE-1 α mRNA to be more abundant in a range of cells and tissues including brain, kidney, heart and lung. In addition, ECE-1 α mRNA was found to be exclusively expressed by TRLEC-03 cells. Western blotting using the ECE1a 1 antibody revealed a polypeptide of approximately 120 kDa in this cell line corresponding to ECE1α 1, but the detection was weak with a relatively high degree of background staining (Fig. 6.10). Western blotting of other cell lines detected some ECE-1 α expression in EA.hy926 cells and in pig lung membranes.

CHAPTER SEVEN

DISCUSSION

At the commencement of the work in this thesis, prior to the molecular cloning of ECE by several groups in 1994 little was known concerning the biochemical characteristics and topology of the ECE protein. With no antibodies available to the protein, many of the cells and tissues which expressed ECE were unidentified. The cloning and expression of ECE revolutionised the study of this protein with considerable information now accumulated regarding ECE and its role in the production of the potent vasoconstrictive peptide, ET and the expression of the two isoforms of ECE-1, α and β.

In order to study ECE activity, a human endothelial cell line, EA.hy926, was used. This was a fusion between HUVECs and a continuous human epithelial cell line (A549), originally derived from a human lung cell carcinoma (Edgell *et al.*, 1983). EA.hy926 cells had been shown to exhibit sustained expression of many differentiated functions of the vascular endothelium, though were found not to express ECE or DPP-IV, considered to be markers of the endothelial phenotype. The EA.hy926 cell line had been demonstrated to express prepro ET-1 mRNA and to secrete both ET-1 and big ET-1 into the culture medium in a PR-sensitive manner and was considered a good model for the system of ET-1 production by the physiologically relevant ECE activity.

ECE was thought to share several different biochemical characteristics with E-24.11 including a neutral pH optimum, sensitivity to metal chelators and sensitivity to PR. The observation that both ECE and E-24.11 were expressed at detectable levels in membranes prepared from EA.hy926 cells facilitated the study of the mechanism by which ECE was anchored to the plasma membrane.

Since E-24.11 was known to be a type II integral membrane protein, characterisation of the ECE membrane anchor was attempted by comparing the behaviour of the two activities in temperature-induced phase separation studies, detergent solubilisation profiling and their ability to resist solubilisation by bacterial PI-PLC. Generally, detergents which are anchored by a G-PI moiety are resistant to solubilisation by detergents of relatively low CMC, such as Triton X-100, though these are often solubilised by detergents of high CMC such as octyl glucoside. The ability of both Triton X-100 and Nonidet P-40 to effectively solubilise both ECE and E-24.11 suggested neither of these proteins was G-PI anchored. This was later confirmed by studies which demonstrated both ECE and E-24.11 to be resistant to bacterial PI-PLC solubilisation. These data were compared with the solubilisation of a known G-PI anchored protein, 5'-Nucleotidase, which was found to be effectively solubilised by this activity. Temperature-induced phase separation using Triton X-114 of EA.hy926 cell membranes allowed the comparison of E-24.11 and ECE with a known cytosolic protein, LDH. Both E-24.11 and ECE were found to localise predominantly in the detergent-rich phase whilst the majority of LDH activity was found to localise in the detergent-depleted or aqueous phase. This observation suggested both ECE and E-24.11 to be integral membrane proteins which, when considered with other data, indicated ECE was not G-PI anchored. ECE was later confirmed to be a type II integral membrane protein with a large extracellular C-terminal catalytic domain.

The identification of an ECE activity distinct from E-24.11 was confirmed by the selective inhibition of E-24.11 by thiorphan though this did not preclude the possibility of these proteins shared significant structural and sequence homology. This hypothesis was investigated by the screening of mono and polyclonal antibodies to E-24.11 for possible cross-reactivity to the ECE protein. Whilst the polyclonal antibody, RP161 and the monoclonal antibody, 3C7 did not show any detectable immunoreactivity to the ECE protein, a monoclonal antibody, GK4A9 immobilised to an inert resin was found to immunoprecipitate ECE from Triton X-100 solubilised EA.hy926 membranes. Following immunoprecipitation by GK4A9, both ECE and E-24.11 activities were reduced by >80 % in the solubilised membrane sample though there was no comparable decrease in AP-N or protein content, suggesting this immunorecognition to be specific for E-24.11 and ECE. To further examine the immunorecognition of ECE by GK4A9, the experiment was repeated using CHO cells transfected with ECE. This cell line had been shown to express approximately 500-1000-fold more ECE activity per mg of protein compared with EA.hy926 cells and did not express E-24.11 ensuring any activity attributed to ECE was not due to substrate hydrolysis by E-24.11. Incubation of GK4A9bound toSepharose with Triton X-100 solubilised transfected CHO cell membranes resulted in removal of >85 % of the total ECE activity. This observation was supported by the results of immunoblotting which confirmed removal of the ECE protein from the membrane sample. These data supported the hypothesis that this antibody raised to E-24.11 also recognised the ECE protein and suggested that ECE and E-24.11 shared a common epitope. This was consistent with hypothesis that these proteins originated from the same protein family, later confirmed by genetic analysis (Valdenaire *et al.,* 1995). In addition, ECE protein was detected by immunoblotting with AEC32-236 in fractions eluted from the GK4A9 column, suggesting that ECE might be rapidly affinity purified by GK4A9 from cell membranes. This process would be greatly aided by the use of transfected cell lines overexpressing large amounts of ECE whilst avoiding contamination with E-24.11 by using cells which do not express this protein. Using large scale preparations, this observation may be used to rapidly purify large quantities of ECE.

Studies by Schmidt *et al.,* (1994) reported that treatment of FBHE cells with PR resulted in an accumulation of ECE, an observation which was exploited to aid purification of ECE from this cell line. In this study, the effect was reproduced in an endothelial cell line (EA.hy926), a human neuroblastoma (SH-SY5Y) and the human glial cell line (Bu17). Consistent with the findings of Schmidt *et al.,* (1994), the increase in ECE protein and activity was found to be in response to treatment of cells with PR in the μM range. The increase in ECE in FBHE cells reported by Schmidt *et al.* (1994) was found to be 10-fold though the increase seen in other cell lines was found to be between 3 and 5-fold. The increase in ECE in the cell lines used in this study was found to be approximately 3-fold following treatment with 30 μM PR as estimated by enzymic assay and immunoblotting.

Further investigation into the regulation of ECE using thiorphan, an inhibitor of E-24.11, resulted in an apparent increase in the levels of ECE in response to treatment of cells with μM concentrations of thiorphan. The ability of both these peptidase inhibitors to increase ECE levels detectable in cell membrane preparations ruled out the possibility of autocatalytic regulation of ECE since ECE is generally considered to be insensitive to thiorphan.

The possibility that inhibition of ECE was resulting in an intracellular accumulation of the ET processing intermediate, big ET-1 which in turn regulated ECE transcription or translation could be discounted by the observation that the administration of thiorphan would not diminish the rate of big ET-1 conversion, and would not be expected to increase ECE levels by this mechanism. In addition, TRLEC-03 cells, which were found not to express ECE-1 β (Shimada *et al.*, 1995) showed there was no increase in ECE-1 α mRNA following PR treatment suggesting the mechanism did not involve control of ECE-1 transcription.

The most likely explanation consistent with these data is that treatment of cells with either PR or thiorphan inhibits the turnover of ECE resulting in an intracellular increase in the levels of this protein. Since E-24.11 is the only known mammalian enzyme which has been found to be sensitive to both PR and thiorphan, this was considered a candidate activity for ECE degradation. However, Barnes *et al.*, (1996) demonstrated that E-24.11, which has been shown to have a strict specificity for oligopeptides (Kenny *et al.*, 1987), was unable to degrade ECE protein and these findings, together with the data presented in this work suggests the existence of a novel metalloprotease activity, sensitive to both PR and thiorphan which is involved in the turnover of ECE and possibly other, as yet unidentified proteins. These data suggest this activity may be a protein of the E-24.11, ECE and Kell family of proteins.

Further studies concerning the regulation of ECE expression involved the study of the role of protein tyrosine phosphorylation using the inhibitor of the protein tyrosine phosphatase inhibitor, vanadate. Previous studies had demonstrated that ET-1 release from HUVECs was suppressed following treatment of cells with vanadate. In this study, the effect of vanadate was studied in several cell lines and found to increase levels of ECE using enzymic assay and immunoblotting. The levels of increase were found to be approximately 2-3-fold in EA.hy926 and Bu17 cell lines. Previous work by Rogers *et al.*, (1994) had demonstrated that rat pheochromocytoma PC12 cells responded to differentiation stimuli, including vanadate, by developing a phenotype resembling that expressed by neurons involving neurite extension to form a network. Using this observation as a morphological marker for this cell line, the effects of vanadate on ECE expression were studied. Interestingly, vanadate treatment appeared to induce ECE expression which showed negligible levels of ECE activity in untreated cells but was found to express significant levels of ECE following treatment of cells with vanadate suggesting differentiation to a neuronal phenotype induced ECE expression.

The effect of vanadate on ECE expression observed in these cell lines , in parallel with the findings of Shimizu *et al.,* (1994) suggest a possible feedback mechanism between the release of ET-1 and ECE production. Though the pathway through which this occurs cannot be conclusively proved from these data, the increase of ECE in response to vanadate treatment would suggest protein tyrosine phosphorylation is likely to be involved and this may prove to be a key event in the regulation of ECE expression levels by the cell. Cytokine regulation of ET production and ECE expression is a likely candidate.

The publication of ECE-1 cDNA sequence data, and the identification of two distinct isoforms of ECE-1, α and β allowed the generation of specific antibodies to each of these. Using a number of computer algorithms, including predictions of secondary structure, hydrophilicity and glycosylation sites, immunogenic regions of amino acids within the protein sequence were identified. Polyclonal antibodies were raised to these sequences and antibodies were affinity purified from antisera. One antibody specific for the ECE-1 α protein was successfully purified and was used to identify cells and tissues expressing this isoform. However, whilst immunoblotting of transfected CHO cells expressing high levels of the ECE-1 α isoform revealed the antibody to be highly specific for the α isoform compared with the β form, the lower levels of ECE expressed in cultured cell lines and tissues, coupled with the lack of a positive control for the β form made accurate quantification of ECE-1 α expression levels difficult. The identification of ECE-1 α in tissues may be accomplished by adapting the immunoblotting protocol to reduce background staining, and altering the antibody incubation conditions to enhance immunorecognition of the protein.

The molecular cloning of ECE led to rapid advances in the understanding of ECE and its physiological role in the generation of the vasoconstrictive peptide, ET in a variety of cells and tissues. There are however a number of questions concerning ECE which have remained unanswered. Data presented in this work have some implications regarding the treatment of pathophysiological conditions by inhibitors of ECE. The co-expression of ECE and E-24.11 in several brain cell types was identified by immunoblotting and enzymic assay (chapter 4) and emphasises the need for the design of specific inhibitors for both E-24.11 and ECE if these are to prove of therapeutic value. The accumulation of ECE in response to treatment of cells with PR or thiorphan raises the possibility that inhibitors of ECE, designed with the intention of inhibiting ECE to reduce levels of circulating ET may cause an increase in the levels of ECE in cells. Candidate inhibitors with the potential for therapeutic value would need to be thoroughly characterised in terms of their effect on overall ECE expression.

The regulatory mechanisms involved in ECE production have not yet been identified but may represent novel targets for the pharmacological intervention of ET production by ECE. The effects of vanadate suggest protein tyrosine phosphorylation may prove to be a key event in the control of ECE expression, though other pathways involving kinase activity may be involved.

The identification of the ECE-1 isoforms is intriguing. No significant difference in substrate specificity has been reported though it appears ECE-1 α is the predominant form in all cells and tissues examined so far (Shimada *et al.*, 1995). To date, the physiological significance of the ECE-1 β isoform remains unclear, though the exclusive expression of the ECE-1 α in transformed cell lines such as TRLEC-03 cells supports the possibility that ECE-1 β may be down-regulated under certain conditions.

To date, the apparent therapeutic potential of regulating production of ET by ECE has not yet been realised. In addition to the disease models in which ET has been implicated (Table 1.1), recent studies indicate ET-1 may play a role in the pathogenesis of diseases other than those afflicting the cardiovascular system. Nelson *et al.*, (1995), identified ET-1 overproduction in the pathogenesis of prostate cancer, and in cases such as this where direct reduction in ET levels is required, the inhibition of ECE-1 activity may prove to be of therapeutic value. To date, there are no specific inhibitors selective for ECE over E-24.11. In addition, ECE has recently been shown to hydrolyse bradykinin at the Pro^7-Phe^8 bond (Hoang & Turner, 1997) suggesting further physiological substrates may exist for ECE. This observation may have implications concerning the design and therapeutic potential of ECE inhibitors.

REFERENCES

Amberger, V.R., Paganetti, P.A., Seulberger, H., Eldering, J.A. & Schwab, M.E. (1994) *Cancer Res.* **54,** 4017-4025.

Ames, B. (1966) *Methods Enzymol.* **8,** 115-118.

Arai, H., Hori, S., Aramori, I., Ohkubo, H. & Nakanishi, S. (1990) *Nature* **348,** 730-732.

Aronson, N.N. & Touster, O. (1974) *Methods Enzymol.* **31,** 90-102.

Auguet, M., Delaflotte, S., Chabrier, P.E. & Braquet, P. (1992) *Eur. J. Pharmacol.* **224,** 101-102.

Bacon, C.R., Carey, N.R. & Davenport, A.P. (1996) *Circulation Res.* **79,** 794-801.

Balmforth, A.J., Ball, S.G., Freshney, R.I., Graham, D.I., McNamee, H.B. & Vaughan, P.F.T. (1986) *J. Neurochem.* **47,** 715-719.

Barnes, K., Matsas, R., Hooper, N.M., Turner, A.J. & Kenny, A.J. (1988) *Neuroscience* **27,** 799-817.

Barnes, K., Bourne, A., Cook, P.A., Turner, A.J. & Kenny, A.J. (1991) *Neuroscience* **44,** 245-261.

Barnes, K., Shimada, K., Takahashi, M., Tanzawa, K. & Turner, A.J. (1996) *J. Cell. Sci.* **109,** 919-928.

Barone, F.C., Willette, R.N., Yue, T.L. & Feuerstein, G. (1995) *Neurol. Res.* **4,** 259-264.

Baynash, A.G., Hosoda, K., Giaid, A., Richardson, J.A., Emoto, N., Hammer, R.E. & Yanagisawa, M. (1994) *Cell* **79,** 1277-1285.

Bdolah, A., Wollberg, Z., Fleminger, G. & Kochva, E. (1989) *FEBS Lett.* **256,** 1-3.

Benigni, A., Zoja, C., Corna, D., Orisio, S., Langaretti, L., Bertani, T. & Remuzzi, G. (1993) *Kidney Int.* **44,** 440-444.

Bernier, M., Laird, D.M. & Lane, M.D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1844-1848

Bessey, O.A., Lowry, O.H. & Brock, M.J. (1946) *J. Biol. Chem.* **184,** 321-329.

Bird, J.E., Waldron, T.E., Little, D.K., Asaad, M.M., Dorso, C.R., DiDonato, G. & Norman, J.A. (1992) *Biochem. Biophys. Res. Commun.* **182,** 224-231.

Booth, A.G. & Kenny, A.J. (1974) *Biochem. J.* **142,** 575-581.

Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604-1607.

Brown, D.J. & Gordon, J.A. (1984) *J. Biol. Chem.* **259,** 9580-9586.

Budzik, G.P., Dillon, T.P., Devine, E.M., Marselle, C.A., Wu-Wong, J.R, & Opgenorth, T.J. (1991) *FASEB J.* **5,** A1017.

Burris, J.F. (1995) *J. Clin. Pharmacol.* **35,** 337-342.

Caldwell, P.R.B., Seegal, B.C. & Hsu, K.C. (1976) *Science* **191,** 1050-1051.

Cantley, R.C., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C. & Guidotti, G. (1977) *J. Biol. Chem.* **252,** 7421-7423.

Cardell, L.O., Uddman, R. & Edvinsson, L. (1992) *Br. J. Pharmacol.* **105,** 376-380.

Carey, J.O., Azevedo, J.L., Morris, P.G., Pories, W.J. & Dohm, G.L. (1995) *Diabetes* **44,** 682-688.

Chou, P.Y. & Fasman, G.D. (1978) *Adv. Enzymol.* **47,** 45-158.

Clozel, M., Gray, G.A., Breu, W., Loffler, B.M. & Osterwalder, R. (1992) *Biochem. Biophys. Res. Commun.* **186,** 867-873.

Clozel, M., Breu, V., Burri, K., Cassal, J., Fischli, W., Gray, G.A., Hirth, G., Loffler, B.M., Muller, M., Neidhart, W. & Ramuz, H. (1993) *Nature* **365,** 759-761.

Connelly, J.C., Skidgel, R.A., Schulz, W.W., Johnson, A.R. & Erdos, E.G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8737-8741.

Corder, R., Harrison, V.J., Khan, N., Anggard, E.E. & Vane, J.R. (1993) *J. Cardiovasc. Pharmacol.* **22,** S73-S78.

Corder, R., Khan, N. & Harrison, V.J. (1995) *Biochem. Biophys. Res. Commun.* **207,** 355-362.

Corder, R. & Vane, J.R. (1995) *Biochem. Pharmacol.* **49,** 375-380.

Creighton, T.E. (1993) In: *Proteins, structures and molecular properties* pp. 255-257, W.H. Freeman & Co.

D'Adamio, L., Shipp, M.A., Masteller, E.L. & Reinherz, E.L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7103-7107.

Danthuluri, N.R. & Brock, T.A. (1990) *J. Pharmacol & Exp. Ther.* **254,** 393-399.

Davitz, M.A., Hereld, D., Shak, S., Krakow, J., Englund, P.T. & Nussenzweig, V. (1987) *Science* **238,** 81-84.

Davitz, M.A., Hom, J. & Schenkman, S. (1989) *J. Biol. Chem.* **264,** 13760-13764.

DeClerck, Y.A. & Imren, S. (1994) *Eur. J. Cancer* **30A,** 2170-2180.

De Mey, J.G. & Vanhoutte, P.M. (1983) *J. Physiol. (Lond.)* **335,** 65-74.

Denault, J.B., Claing, A., D'Orleans-Juste, P., Sawamura, T., Kido, T., Masaki, T. & Leduc, R. (1995) *FEBS Lett.* **362,** 276-280.

Deng, Y., Savage, P., Shetty, S.S., Martin, L.L. & Jeng, A.Y. (1992) *J. Biochem. (Tokyo)* **111,** 346-351.

Deschepper, C.F., Houweling, A.D. & Picard, S. (1995) *Eur. J. Pharmacol.* **275,** 61-66.

Devault, A., Lazure, C., Nault, C., LeMoual, H., Seidah, N.G., Chretien, M., Kahn, P., Powell, J., Mallet, J., Beaumont, A., Roques, B.P., Crine, P. & Boileau, G. (1987) *EMBO J.* **6,** 1317-1322.

Devine, E.M., Marselle, C.A., Pederson, T.M., Wu-Wong, J.R. Budzik, G.P., Dillon, T.P. & Opgenorth, T.J. (1991) *FASEB J.* **5,** A1417

Duksin, D. & Mahoney, W.C. (1982) *J. Biol. Chem.* **257,** 3105-3109.

Edgell, C.-J.S., McDonald, C.C. & Graham, J.B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3734-3737.

Egan, R.W., Jones, M.A. & Lehninger, A.L. (1976) *J. Biol. Chem.* **251,** 4442-4447.

Ehrenreich, H., Kehrl, J.H., Anderson, R.W., Rieckmann, P., Vitkovic, L., Coligan, J.E. & Fauci, A.S. (1991) *Brain Res.* **538,** 54-58.

Ellman, G.L. (1959) *Arch. Biochem. Biophys.* **82,** 70-74.

Elshourbagy, N.A., Adamou, J.E. Gagnon, A.W., Wu, H.-L., Pullen, M. & Nambi, P. (1996) *J. Biol. Chem.* **271,** 25300-25307.

Emini, E.A., Hughes, J.V., Perlow, D.S & Boger, J. (1985) *J. Virol.* **55,** 836-839.

Emori, T., Hirata, Y., Ohta, K., Shichiri, M., Shimokado, K. & Marumo, F. (1989) *Biochem. Biophys. Res. Commun.* **162,** 217-223.

Emoto, N & Yanagisawa, M. (1995) *J. Biol. Chem.* **270,** 15262-15268.

Ferguson, M.A.J., Low, M.G. & Cross, G.A.M. (1985) *J. Biol. Chem.* **260,** 14547-14555

Fox, J.A., Soliz, N.M. & Saltiel, A.R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2663-2667.

Fulcher, I.S. & Kenny, A.J. (1983) *Biochem. J.* **211,** 743-753.

Furchgott, R.F. & Zawadzki, J.V. (1980) *Nature* **288,** 373-376.

Fuxe, K., Tinner, B., Staines, W., Hemsen, A., Hersh, L. & Lundberg, J.M. (1991) *Neurosci. Lett.* **123,** 107-111.

Garcia-Morales, P., Minami, Y., Luong, E., Klausner, R.D. & Samelson, L.E. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9255-9259.

Gardner, J.P., Tokudome, G., Tomonari, H., Maher, E., Hollander, D. & Aviv, A. (1992) *Am. J. Physiol.* **262,** C148-C155.

Garnier, J., Osguthorpe, D.J. & Robson, B. (1978) *J. Mol. Biol.* **120,** 97-120.

Gee, N.S., Matsas, R. & Kenny, A.J. (1983) *Biochem. J.* **214,** 377-386.

Gee, N.S. & Kenny, A.J. (1985) *Biochem. Soc. Trans.* **13,** 101-102.

Gee, N.S., Bowes, M.A., Buck, P. & Kenny, A.J. (1985) *Biochem. J.* **228,** 119-126.

Giaid, A., Gibson, S.J.,Herrero, M.T., Gentleman, S., Legon, S., Yanagisawa, M., Masaki, T., Ibrahim, N.B.N., Roberts, G.W., Rossi, M.L. & Polak, J.M. (1991) *Histochemistry* **95,** 303-314.

Gillespie, M., Owasoyo, J., McMurty, I. & O'Brien, R. (1986) *Exp. Ther.* **236,** 339-343.

Gordon, J.A. (1991) *Meth. Enzymol.* **201,** 477-482.

Goto, K., Kasuya, Y., Matsuki, N., Takuwa, Y., Kurihara, H., Ishikawa, T., Kimura, S., Yanagisawa, M. & Masaki, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3915-3918.

Gray, G.A. & Clozel, M. (1994) *Br. J. Pharmacol.* **112,** U62.

Gray, G.A., & Webb, D.J. (1996) *Pharmacol. Ther.* **72,** 109-148.

Greene, L.A. & Tischler, A. (1976) *Proc. Natl. Acad. Sci.* **73,** 2424-2428.

Grover, G.J., Dzwonczyk, S. & Parham, C.S. (1993) *Cardiovasc. Res.* **27,** 1613-1618.

Gui, G., Xu, D., Emoto, N. & Yanagisawa, M. (1993) *J. Cardiovasc. Pharmacol.* **22,** S53-S56.

Harrison, V.J., Corder, R., Anggard, E.E. & Vane, J.R. (1993) *J. Cardiovasc. Pharmacol.* **22,** S57-S60.

Hartley, B.S. (1960) *Annu. Rev. Biochem.* **29,** 45-72.

Heifetz, A., Keenan, R.W. & Elbein, A.D. (1979) *Biochemistry* **18,** 2186-2192.

Hickey, K.A., Rubanyi, G., Paul,R.I. & Highsmith, R.F. (1985) *Am. J. Physiol.* **248,** C550-C556.

Hioki,Y., Okada, K., Ito, H., Matsuyama, K. & Yano, M. (1991) *Biochem. Biophys. Res. Commun.* **174,** 446-451.

Hoang, M.V. & Turner, A.J. (1997) *Biochem. J.* **327,** 23-26.

Hooper, N.M., Low, M.G. & Turner, A.J. (1987) *Biochem. J.* **244,** 465-469.

Hooper, N.M. & Turner, A.J. (1988 a) *FEBS Lett.* **229,** 340-344.

Hooper, N.M. & Turner, A.J. (1988 b) *Biochem. J.* **250,** 865-869.

Hooper, N.M. (1992) Identification of a glycosyl-phosphatidylinositol anchor on membrane proteins. In: *Lipid modification of proteins, a practical approach* (Eds. Hooper, N.M. & Turner, A.J.), pp. 89-115, Oxford University Press, Oxford, U.K.

Hosoda, K., Hammer, R.E., Richardson, J.A., Baynash, A.G., Cheung, J.C., Giaid, A. & Yanagisawa, M. (1994) *Cell* **79,** 1267-1276.

Howell, S., Murray, H., Turner, A.J. & Kenny, A.J. (1991) *Biochem. J.* **278,** 417-421.

Hu, R.-M., Levin, E.R., Pedram, A. & Frank, H.J.L. (1993) *Diabetes* **42,** 351-358.

Hunziker, W., Spiess, M., Semenza, G. & Lodish, H.F. (1986) *Cell* **46,** 227-234.

Ichai, C., Chevallier, N., Delaere, P., Dournaud, P., Epelbaum, J., Hauw, J.J., Vincent, J.P. & Checler, F. (1994) *J. Neurochem.* **62,** 645-655.

Ignarro, L., Buga, G., Wood, K., Byrns, R. & Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9265-9269.

Ikegawa, R., Matsumura, Y., Tsukahara, Y., Takaoka, M. & Morimoto, S. (1990 a) *Biochem. Biophys. Res. Commun.* **171,** 669-675.

Ikegawa, R., Matsumura, Y., Takaoka, M. & Morimoto, S. (1990 b) *Biochem. Biophys. Res. Commun.* **167,** 860-866.

Ikezawa, H. & Taguchi, R. (1981) *Methods Enzymol.* **71,** 731-741.

Ikura, T., Sawamura, T., Shiraki, T., Hosokawa, H., Kido, T., Hoshikawa, H., Shimada, K., Tanzawa, K., Kobayashi, S., Miwa, S. & Masaki, T. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1417-1422.

Indig, F.E., Ben-Meir, D., Spungin, A. & Blumberg, S. (1989) *FEBS Lett.* **255,** 237-240.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. & Masaki, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2863-2867.

Itoh, S., Sasaki, T., Ide, K., Ishikawa, K., Nishikibe, M. & Yano, M. (1993) *Biochem. Biophys. Res. Commun.* **195,** 969-975.

Jaffe, E.A., Nachman, R.L. & Becker, C.G. (1973) *J. Clin. Invest.* **52,** 2745-2758.

Jaffe, E.A. (1987) *Human Path.* **18,** 234-239.

Jameson, B.A. & Wolf, H. (1988) *Computer Applications in the Biosciences* **4,** 181-186.

Jochum, M., Machleidt, W. & Fritz, H. (1993) *Agents Actions Suppl.* **42,** 51-69.

Jones, C.R., Hiley, C.R., Pelton, J.J. & Mohr, M. (1989) *Neurosci. Lett.* **97,** 276-279.

Kai, H., Kanaide, H. & Nakamura, M. (1989) *Biochem. Biophys. Res. Commun.* **158,** 235-243

Kametani, F., Haga, S., Tanaka, K. & Ishii, T. (1990) *J. Neurol. Sci.* **97,** 43-52.

Karplus, P.A. & Schulz, G.E. (1985) *Naturwissenschaften* **72,** 212-213.

Kashiwabara, T., Inagaki, Y., Ohta, H., Iwamatsu, A., Nomizu, M., Morita, A. & Nishikori, K. (1989) *FEBS Lett.* **247,** 73-76.

Kasuya, Y., Takuwa, Y., Yanagisawa, M., Kimura, S., Goto, K. & Masaki, T. (1989) *Biochem. Biophys. Res. Commun.* **161,** 1049-1055.

Keller, R.K., Boon, D.Y. & Crum, F.C. (1979) *Biochemistry* **18,** 3946-3952.

Kennedy, R.L., Haynes, W.G. & Webb, D.J. (1993) *Clin. Endocrinol. (Oxf.)* **39,** 259-265.

Kenny, A.J. (1977) Proteinases associated with cell membranes. In : *Proteinases in mammalian cells and tissues* (Ed. Barrett, A.J.), pp 393-444, Elsevier/North Holland Biochemical Press, Amsterdam.

Kenny, A.J., Stephenson, S.L. & Turner, A.J. (1987) Cell Surface Peptidases. In : *Mammalian ectoenzymes* (Eds. Kenny, A.J. & Turner, A.J.), pp. 169-210, Elsevier, Amsterdam.

Kenny, A.J. & Hooper, N.M. (1991) Peptidases involved in the metabolism of bioactive peptides. In: *Degradation of bioactive substances: physiology and pathophysiology* (Ed. Henriksen, J.H.), pp. 47-79, CRC Press, Florida.

Kerr, M.A. & Kenny, A.J. (1974 a) *Biochem. J.* **137,** 489-495.

Kerr, M.A. & Kenny, A.J. (1974 b) *Biochem. J.* **137,** 477-488.

Kido, T., Sawamura, T., Hoshikawa, H., D'Orleans-Juste, P., Denault, J.B., Leduc, R., Kimura, J. & Masaki, T. (1997) *Eur. J. Biochem.* **244,** 520-526.

Kimura, S., Kaysuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. & Masaki, T. (1989) *J. Cardiovasc. Pharmacol.* **13,** S5-S7.

Kirkness, E.F. & Turner, A.J. (1988) *Biochem. J.* **256,** 291-294.

Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. & Bdolah, A. (1988) *Science* **242,** 268-270.

Kuo, S.-C. & Lampen, J.O. (1974) *Biochem. Biophys. Res. Commun.* **58,** 287-295.

Kurihara, H., Yoshizumi, M., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T., Hamaoki, M., Kato, H. & Yazaki, Y. (1989) *Biochem. Biophys. Res. Commun.* **159,** 1435-1440.

Kurihara, M., Ochi, A., Kawaguchi, T., Niwa, M., Kataoka, Y. & Mori, K. (1990) *Neurosurgery* **27,** 275-281.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa T., Kumada, M. & Yazaki, Y. (1994) *Nature* **368,** 703-710.

Kyte, J. & Doolittle, R.F. (1982) *J. Mol. Biol.* **157,** 105-132.

Laemmli, U.K. (1970) *Nature* **227,** 680-685.

Laperche, Y., Bulle, F., Aissani, T., Chobert, M., Aggerbeck, M., Hanoue, J. & Gullaen, G. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 937-941.

Lazarini, F., Strosberg, A.D., Couraud, P.O. & Cazaubon, S.M. (1996) *J. Neurochem.* **66,** 459-465.

Lee, M.E., de la Monte, S.M., Ng, S.C., Bloch, K.D. & Quertermous, T. (1990) *J. Clin. Invest.* **86,** 141-147.

Lee, S., Zambas, E.D., Marsh, W.L. & Redman, C.M. (1991) *Proc. Natl. Acad. Sci USA* **88,** 6353-6357.

Lee, S., Zambas, E.D., Green, E.D. & Redman, C.M. (1995) *Blood* **85,** 1364-1370.

Lees, W.A., Kalinka, S., Meech, J., Capper, S.J., Cook, N.D. & Kay, J. (1990) *FEBS Lett.* **273,** 99-102.

Letarte, M., Vera, S., Tran, R., Addis, J.B., Onizuka, R.J., Quackenbush, E.J., Jongeneel, C.V. & McInnes, R.R. (1988) *J. Exp. Med.* **168,** 1247-1253.

Low, M.G. (1989) *Biochem. Biophys. Acta* **988,** 427.

Low, M.G. (1990) Degradation of glycosyl-phosphatidylinositol anchors by specific phospholipases. In: *Cell and molecular biology of membrane proteins* (Ed. Turner, A.J.), pp. 35-63, Ellis Horwood, Chichester.

Luscher, T.F. (1992) *Eur. Heart. J.* **13,** D50-D55.

MacCumber, M.W., Ross, C.A. & Snyder, S.H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2359-2363.

Mahadevan, L.C. & Bell, J.C. (1989) In: *Receptors, a practical approach* (Ed. Hulme, E.), pp. 181-201, IRL Press, Oxford, U.K.

Malfroy, B., Schofield, P.R., Kuang, W.-J., Seeburg, P.H., Mason, A.J. & Henzel, W.J. (1987) *Biochem. Biophys. Res. Commun.* **144,** 59-66.

Malfroy, B., Kuang, W.-J., Seeburg, P.H., Mason, A.J. & Schofield, P.R. (1988) *FEBS Lett.* **229,** 206-210.

Marshall, R.D. (1972) *Annu. Rev. Biochem.* **41,** 673-702.

Masaki, T., Kimura, S., Yanagisawa, M. & Goto, K. (1991) *Circulation* **84,** 1457-1468.

Masaki, T. & Yanagisawa, M. (1992) *Essays Biochem.* **27,** 79-89.

Masuda, Y., Miyazaki, H., Kondah, M., Watanabe, H., Yanagisawa, M., Masaki, T. & Murakami, K. (1989) *FEBS Lett.* **257,** 208-210.

Matsas, R., Fulcher, I.S., Kenny, A.J. & Turner, A.J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3111-3115.

Matsumura, Y., Ikegawa, R., Takaoka, M. & Morimoto, S. (1990 a) *Biochem. Biophys. Res. Commun.* **167,** 203-210.

Matsumura, Y., Ikegawa, R., Tsukahara, Y., Takaoka, M. & Morimoto, S. (1990 b) *FEBS Lett.* **272,** 166-170.

Matsumura, Y., Hisaki, K., Takaoka, M. & Morimoto, S. (1990 c) *Eur. J. Pharmacol.* **185,** 103-106

Matsumura, Y., Ikegawa, R., Tsukahara, Y., Takaoka, M. & Morimoto, S. (1991 a) *Biochem. Biophys. Res. Commun.* **178,** 531-538.

Matsumura, Y., Ikegawa, R., Tsukahara, Y., Takaoka, M. & Morimoto, S. (1991 b) *Biochem. Biophys. Res. Commun.* **178,** 899-905.

McMahon, E.G., Fok, K.F., Moore, W.M., Smith, C.E., Siegel, N.R. & Trapani, A.J. (1989) *Biochem. Biophys. Res. Commun.* **161,** 406-413.

McMahon, E.G., Palomo, M.A., Moore, W.M., McDonald, J.F. & Stern, M.K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 703-707.

Medeiros, M.S., Balmforth, A.J., Vaughan, P.F.T. & Turner, A.J. (1991) *Neuroendocrinology* **54,** 295-302.

Merida, I. & Gaulton, G.N. (1990) *J. Biol. Chem.* **265,** 5690-5694.

Mikulewicz, W., Berdowska, I., Jarmulowicz, J. & Siewinski, M. (1993) *Anti-cancer Drugs* **4,** 341-344.

Molnar, E., Baucle, A., Richmond, S.A., Patel, P.B., Somogyi, P. & McIlhinney, R.A.J. (1993) *Neuroscience* **53,** 307-326.

Moncada, S., Gryglewski, R., Bunting, S. & Vane, J.R. (1976) *Nature* **263,** 663-665.

Murphy, L.J., Corder, R., Mallet, A.I. & Turner, A.J. (1994) *Br. J. Pharmacol.* **113,** 137-142.

Murphy, N.P., Ball, S.G. & Vaughan, P.F.T. (1991) *J. Neurochem.* **56,** 1810-1814.

Nambi, P., Pullen, M. & Feuerstein, G. (1990) *Neuropeptides* **16,** 195-199.

Nechay, B.R., Nanninga, L.B., Nechay, P.S.E., Post, R.L., Grantham, J.J., Macara, I.G., Kubena, L.F., Phillips, T.D. & Nielsen, F.H. (1986) *Fedn. Proc.* **45,** 123-132.

Nelson, J.B., Hedican, S.P., George, D.J., Reddi, A.H., Piantadosi, S., Eisenberger, M.A. & Simons, J.W. (1995) *Nature Med.* **1,** 944-949.

Niman, H.L., Houghton, R.A., Walker, L.E., Reisfeld, R.A., Wilson, I.A., Hogle, J.M. & Lerner, R.A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 4949-4953.

Nishikawa, K. (1983) *Biochim. Biophys. Acta* **748,** 285-299.

Oakley, B.R., Kirsch, D.R. & Morris, N.R. (1980) *Anal. Biochem.* **105,** 361-363.

O'Brien, R., Robbins, R.J. & McMurty, I.F. (1987) *J. Cell Physiol.* **135,** 263-270.

Ohlstein, E.H., Nambi, P., Douglas, S.A., Edwards, R.M., Gellai, M., Lago, A., Leber, J.D., Cousins, R.D., Gao, A., Frazee, J.S., Peishoff, C.E., Bean, J.W., Eggleston, D.S., Elshourbagy, N.A., Kumar, C., Lee, J.A., Brooks, D.P., Weinstock, J., Feuerstein, G., Poste, G., Ruffolo, R.R., Gleason, J.G. & Elliot, J.D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8052-8056.

Ohnaka, K., Takayanagi, R., Yamauchi, T., Okazaki,H., Ohashi, M., Umeda, F. & Nawata, H. (1990) *Biochem. Biophys. Res. Commun.* **168,** 1128-1136.

Ohnaka, K., Takayanagi, R., Nishikawa, M., Haji, M. & Nawata, H. (1993) *J. Biol. Chem.* **268,** 26759-26766.

Okada, K., Miyazaki, Y., Takada, J., Matsuyama, K., Yamaki, T. & Yano, M. (1990) *Biochem. Biophys. Res. Commun.* **171,** 1192-1198.

Palmer, R., Ferrige, A. & Moncada, S. (1987) *Nature* **327,** 524-526.

Pekonen, F., Nyman, T., Ammala, M. & Rutanen, E.-M. (1995) *Br. J. Cancer* **71,** 59-63.

Petruzzelli, L., Herrera, R. & Rosen, O.M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3327-3331.

Pless, D.D. & Lennarz, W.J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 134-138.

Pollock, D.M. & Opgenorth, T.J. (1991) *Am. J. Physiol.* **261,** R257-R263.

Puffenberger, E.G., Hosoda, K., Washington, S.S., Nakao, K., deWit, D., Yanagisawa, M. & Chakravarti, A. (1994) *Cell* **79,** 1257-1266.

Relton, J.M., Gee, N.S., Matsas, R., Turner, A.J. & Kenny, A.J. (1983) *Biochem. J.* **215,** 519-523.

Remme, W.J. (1995) *Eur. Heart J.* **16,** 73-78.

Resink, T.J. Scott-Burden, T. & Buhler, F.R. (1988) *Biochem. Biophys. Res. Commun.* **157,** 1360-1368.

Robinson, P.S., Lees, W.E., Kay, J. & Cook, N.D. (1992) *Biochem. J.* **284,** 407-409.

Rogers, M.V., Buensuceso, C., Montague, F. & Mahadevan, L. (1994) *Neuroscience* **60,** 479-494.

Ross, R., Spengler, B. & Biedler, J. (1985) *J. Cancer Inst.* **71,** 741-747.

Saijonmaa, O., Nyman, T., Hohenthal, U. & Fyhrquist, F. (1991) *Biochem. Biophys. Res. Commun.* **181,** 529-536.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. & Masaki, T. (1990) *Nature* **348,** 732-735.

Sakurai, T., Yanagisawa, M. & Masaki, T. (1992) *Trends. Pharmacol. Sci.* **13,** 103-108.

Saltzman, E.M., Thom, R.R. & Casnellie, J.E. (1988) *J. Biol. Chem.* **263,** 6956-6959.

Samson, W.K., Skala, D., Alexander, B.D. & Huang, F.L.S. (1990) *Biochem. Biophys. Res. Commun.* **169,** 737-743.

Samson, W.K., Skala, K.D., Alexander, B.D. & Huang, F.L. (1991 a) *J. Cardiovasc. Pharmacol.* **17,** S346-S349.

Samson, W.K., Skala, K.D., Huang, F.L., Gluntz, S., Alexander, B. & Gomez-Sanchez, C.E. (1991 b) *Brain Res.* **539,** 347-351.

Samson, W.K., Skala, K.D., Alexander, B.D. & Huang, F.L. (1991 c) *Endocrinology* **128,** 1465-1473.

Santini, F. & Beaven, M.A., (1993) *J. Biol. Chem.* **268,** 22716-22722.

Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M., & Masaki, T. (1989) *Biochem. Biophys. Res. Commun.* **162,** 1287-1294.

Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Kobayashi, M., Mitsui, Y., Yanagisawa, M., Goto, K. & Masaki, T. (1990 a) *Biochem. Biophys. Res. Commun.* **169,** 1138-1144.

Sawamura, T., Kimura, S., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. & Masaki, T. (1990 b) *Biochem. Biophys. Res. Commun.* **168,** 1230-1236.

Sawamura, T., Shinmi, O., Kishi, N., Sugita, Y., Yanagisawa, M., Goto, K., Masaki, T. & Kimura, S. (1990 c) **172,** 883-889.

Scheidtmann, K.H. (1990) In: *Protein Structure, a practical approach* (Ed. Creighton, T.E.), IRL Press, Oxford, U.K.

Schmidt, M., Kroger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. & Hillen, H. (1994) *FEBS Lett* **356,** 238-243.

Schwartz, M.K. & Bodansky, O. (1966) *Methods Enzymol.* **9,** 294-302.

Shields, P.P., Gonzales, T.A., Charles, D., Gilligan, J.P. & Stern, W. (1991) *Biochem. Biophys. Res. Commun.* **177,** 1006-1012.

Shimada, K., Takahashi, M. & Tanzawa, K. (1994) *J. Biol. Chem.* **269,** 18275-18278.

Shimada, K., Takahashi, M., Ikeda, M. & Tanzawa, K. (1995) *FEBS Lett.* **371,** 140-144.

Shimizu, H., Takayama, H., Lee, J.-D., Satake, K., Taniguchi, T., Yamamura, H. & Nakamura, T. (1994) *Thrombosis and Haemostasis* **72,** 973-978.

Shinyama, H., Uchida, T., Kido, H., Hayashi, K., Watanabe, M., Matsumura, Y., Ikegawa, R., Takaoka, M. & Morimoto, S. (1991) *Biochem. Biophys. Res. Commun.* **178,** 24-30.

Shipp, M.A., Vijayaraghavan, J., Schmidt, E.V., Masteller, E.L., D'Adamio, L., Hersh, L.B. & Reinherz, E.L. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 297-301.

Simons, T.J.B. (1979) *Nature* **281,** 337-338.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) *Anal. Biochem.* **150,** 76-85.

Sokolovsky, M., Galron, R., Kloog, Y., Bdolah, A., Indig, F.E., Blumberg, S. & Fleminger, G. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4702-4706.

Sokolovsky, M., Ambar, I. & Galron, R. (1992) *J. Biol. Chem.* **267,** 20551-20554.

Sossin, W.S., Fisher, J.M. & Scheller, R.H. (1989) *Neuron* **2,** 1407-1417.

Stein, P.D., Hunt, J.T., Floyd, D.M., Moreland, S., Dickinson, K.E.J., Mitchell, C., Liu, E.C.-K., Webb, M.L., Murugesan, N., Dickey, J., McMullen, D., Zhang, R., Lee, V.G., Serafino, R., Delaney, C., Schaeffer, T.R. & Kozlowski, M. (1994) *J. Med. Chem.* **37,** 329-331.

Stephenson, F.A. & Duggan, M.J. (1991) Chapter 9 in: *Molecular Neurobiology, a practical approach* (Eds. Chad, J. & Wheal, H.), pp 183-204, IRL Press, Oxford, U.K.

Suda, H., Aoyagi, T., Takeuchi, T. & Umezawa, H. (1973) *J. Antibiot. (Tokyo)* **26,** 621-623.

Sumner, M.J., Cannon, T.R., Mundin, J.W., White, D.G. & Watts, I.S. (1992) *Br. J. Pharmacol.* **107,** 858-860.

Takada, J., Okada, K., Ikenaga, T., Matsuyama, K. & Yano, M. (1991) *Biochem. Biophys. Res. Commun.* **176,** 860-865.

Takahashi, M., Matsushita, Y., Iijima, Y. & Tanzawa, K. (1993) *J. Biol. Chem.* **268,** 21394-21398.

Takahashi, M., Fukuda, K., Shimada, K., Barnes, K., Turner, A.J., Ikeda, M., Koike, H., Yamamoto, Y. & Tanzawa, K. (1995) *Biochem. J.* **311,** 657-665.

Takaoka, M., Miyata, Y., Takenobu, Y., Ikegawa, R., Matsumura, Y. & Morimoto, S. (1990 a) *Biochem. J.* **270,** 541-544.

Takaoka, M., Takenobu, Y., Miyata, Y., Ikegawa, R., Matsumura, Y. & Morimoto, S. (1990 b) *Biochem. Biophys. Res. Commun.* **166,** 436-442.

Takaoka, M., Hukumori, Y., Shiragami, K., Ikegawa, R., Matsumura, Y. & Morimoto, S. (1990 c) *Biochem. Biophys. Res. Commun.* **173,** 1218-1223.

Takaoka, M., Shiragami, K., Fujino, K., Miki, K., Miyake, Y., Yasuda, M., Wang, G.F., Hisaki, K., Matsumura, Y. & Morimoto, S. (1991) *Biochem. Intl.* **25,** 697-704

Takatsuki, A. & Tamura, G. (1971 a) *J. Antibiot.* **24,** 785-794.

Takatsuki, A. & Tamura, G. (1971 b) *J. Antibiot.* **24,** 224-231.

Takuwa, Y., Kasuya, Y., Takuwa, N., Kudo, M., Yanagisawa, M., Goto, K., Masaki, T. & Yamashita, K. (1990) *J. Clin. Invest.* **85,** 653-658.

Takuwa, Y., Masaki, T. & Yamashita, K. (1991) *Contributions To Nephrology* **90,** 99-104.

Tamura, S., Brown, T.A., Whipple, J.H., Fujita-Yamaguchi, Y., Dubler, R.E., Cheng, K. & Larner, J. (1984) *J. Biol. Chem.* **259,** 6650-6658.

Tanford, C. & Reynolds, J.A. (1976) *Biochim. Biophys. Acta* **457,** 113-170.

Taniguchi, K., Morimoto, S., Fukuo, K., Yanagisawa, M., Masaki, T., Suzuki, N. & Ogihara, T. (1992) *Hypertens. Res.* **15,** 171-175.

The HYP Consortium (1995) *Nature Genet.* **11,** 130-136.

Turner, A.J., Matsas, R. & Kenny, A.J. (1985) *Biochem. Pharmacol.* **34,** 1347-1356.

Turner, A.J., Hooper, N.M. & Kenny, A.J. (1989) Neuropeptide-degrading enzymes. In: *Neuropeptides: a methodology* (Eds. Fink, G. & Harmer, A.J.), pp. 189-223, John Wiley & Sons, Chichester.

Turner, A.J. & Murphy, L.J. (1996) *Biochem. Pharmacol.* **51,** 91-102.

Turner, A.J. & Tanzawa, K. (1997) *FASEB J.* **11,** 355-363.

Valdenaire, O., Rohrbacher, E., Mattei, M.-G. (1995) *J. Biol. Chem.* **270,** 29794-29798.

Vane, J.R., Anggard, E.E. & Botting, R.M. (1990) *New England J. Med.* **323,** 27-36.

Vijayaraghavan, J., Scicli, A.G., Carretero, O.A., Slaughter, C., Moomaw, C. & Hersh, L.B. (1990) *J. Biol. Chem.* **265,** 14150-14155.

Wagner-Mann, C.E., Bowman, L. & Sturek, M. (1991) *Am. J. Physiol.* **260,** C763-C770.

Wagner-Mann, C.E. & Sturek, M. (1991) *Am. J. Pharmacol.* **260,** C771-C777.

Walsh, D.A., Mapp, P.I., Wharton, J., Polak, J.M. & Blake, D.R. (1993) *Am. J. Pathol.* **142,** 1610-1621.

Warner, T.D., de Nucci, G. & Vane, J.R. (1989 a) *Eur. J. Pharmacol.* **159,** 325-326.

Warner, T.D., Mitchell, J.A., de Nucci, G. & Vane, J.R. (1989 b) *J. Cardiovasc. Pharmacol.* **13,** S85-S88.

Warner, T.D., Budzik, G.P., Matsumoto, T., Mitchell, J.A., Forstermann, U. & Murad, F. (1992 a) *Br. J. Pharmacol.* **106,** 948-952.

Warner, T.D., Budzik, G.P., Mitchell, J.A., Huang, Z.-J. & Murad, F. (1992 b) *J. Cardiovasc. Pharmacol.* **20,** S19-S21.

Warner, T.D., Allcock, G.H., Corder, R. & Vane, J.R. (1993 a) *Br. J. Pharmacol.* **110,** 777-782.

Warner, T.D., Allcock, G.H., Mickley, E.J., Corder, R. & Vane, J.R. (1993 b) *J. Cardiovasc. Pharmacol.* **22,** S117-S120.

Watanabe, K., Miyazaki, H., Kondoh, M., Masuda, Y., Kimura, S., Yanagisawa, M., Masaki, T. & Murakami, K. (1989) *Biochem. Biophys. Res. Commun.* **161,** 1252-1259.

Watanabe, K., Tamaru, N. & Yoshida, M. (1991) *Cell Biol. Int. Rep.* **15,** 205-210.

Watanabe, K. & Jaffe, E.A. (1993) *Prostaglandins Leukot. Essent. Fatty Acids* **49,** 955-958.

Wenthold, R.J., Yokotani, N., Doi, K. & Wada, K. (1992) *J. Biol. Chem.* **267,** 501-507.

Widdows, K.B., Kirkness, E.F. & Turner, A.J. (1987) *FEBS Lett.* **222,** 125-128.

Wu-Wong, J.R., Budzik, G.P., Devine, E.M. & Opgenorth, T.J. (1990) *Biochem. Biophys. Res. Commun.* **171,** 1291-1296.

Wu-Wong, J.R., Devine, E.M., Budzik, G.P. & Opgenorth, T.J. (1991) *J. Cardiovasc. Pharmacol.* **17,** 520-525.

Wu-Wong, J.R., Chiou, W.J., Magnuson, S.R. & Opgenorth, T.J. (1995) *J. Pharmacol. Exp. Therapeutics* **274,** 499-507.

Wu-Wong, J.R., Chiou, W.J., Magnuson, S.R., Bianchi, B.R. & Lin, C.W. (1996) *Biochim. Biophys. Acta* **1311,** 155-163.

Wypij, D.M., Nichols, J.S., Novak, P.J., Stacy, D.L., Berman, J. & Wiseman, J.S. (1992) *Biochem. Pharmacol.* **43,** 845-853.

Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. & Yanagisawa, M. (1994) *Cell* **78,** 473-485.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988) *Nature* **332,** 411-415.

Yorimitsu, K., Moroi, K., Inagaki, N., Saito, T., Masuda, Y., Masaki, T., Seino, S. & Kimura, S. (1995) *Biochem. Biophys. Res. Commun.* **208,** 721-727.

Yoshizumi, M., Kurihara, H., Morita, T., Yamashita, T., Oh-hashi, Y., Sugiyama, T., Takaku, F., Yanagisawa, M., Masaki, T. & Yazaki, Y. (1990) *Biochem. Biophys. Res. Commun.* **166,** 324-329.