The differential processing of proenkephalin A in mouse and human breast tumour cell lines

B K Brar and **P J Lowry**

The School of Animal and Microbial Sciences, The University of Reading, Reading, Berkshire RG6 6AJ, UK

(Requests for offprints should be addressed to P J Lowry)

(B K Brar is now at Department of Molecular Pathology, Middlesex Hospital Medical School, The Windeyer Building, 46 Cleveland Street, London WIP 6DB, UK)

Abstract

We have carried out an investigation into the processing of the enkephalin-like immunoreactivity reported in breast tissue using two human breast tumour cell lines and a mouse tumour cell line. A 46 kDa form of proenkephalin (PE) has been observed in the cell lysates of two human breast tumour cell lines (MCF-7, ZR-75–1) and the mouse androgen-responsive Shionogi breast carcinoma cell line (SC115). PE processing in the cell lysates of these cells was assessed by a specific met-enkephalin RIA. The basal levels of processed PE in the MCF-7, ZR-75–1 and SC115 cell lysates were 30, 30 and 76% respectively. The processing enzymes PC1 and PC2, which have been implicated in the differential processing of PE, were detected by immunoblot analysis in these cells. PC1 was found within the cell extracts of all three cell lines. PC2 was only observed in the SC115 cell line, which may account for the higher percentage of processed PE measured. The cDNA of PC2 has been transfected into ZR-75–1 cells and this was accompanied by an increase in the level of processed PE from 30 to 76%. These breast tumour cell lines may provide a useful insight into the function of enkephalin-containing peptides in breast cancer.

Journal of Endocrinology (1999) **161,** 475–484

Introduction

The induction and growth of human breast cancer appear to be directly related to the endocrine status of the host. The implication of ovarian hormones in breast cancer has been extensively reported (Sirbasku *et al*. 1980). In addition to these steroid hormones, various other factors, such as glucocorticoids, thyroid hormones, vitamin D, insulin, prolactin, growth hormone, prostaglandins, epidermal growth factors and endogenous neuroendocrine peptides have also been shown to affect breast tissue (Medina *et al*. 1987). One such family of peptides are the opioids, which include the enkephalins, endorphins and dynorphin. These peptides are derived by enzymic processing of three separate precursors; proenkephalin A (PE), proopiomelanocortin (POMC) and prodynorphin. Enzymic processing of the prohormone precursors usually occurs at selected dibasic or monobasic amino acid cleavage sites by specific prohormone-processing enzymes with which they are co-localised.

These opioid peptides and their precursors are widely distributed throughout the body but are generally localised within the tissues of the brain and the central and peripheral nervous system (Cooper *et al*. 1996). The co-expression of endogenous opioid peptides and the putative opiate receptors, through which their wide spectrum of effects are mediated, have been described in several benign and malignant tumours from humans and other animals by immunohistochemical techniques (Zagon *et al*. 1987). Immunoreactive opioid peptides were also identified in 56% of invasive ductal carcinomas of the breast from 61 premenopausal women (Scopsi *et al*. 1989). A recent immunocytochemical study of β-endorphin and enkephalin expression in primary breast cancers, adrenofibromas and peritumoral non-neoplastic tissue showed around 90% of all tumours to be positive (Chatikhine *et al*. 1994). Leu-enkephalin and met-enkephalin occurred predominantly in the epithelial cells of both benign and malignant tumours, whereas β-endorphin was found mainly in the stroma. In non-neoplastic tissues all three opioids were predominantly expressed in the stroma. Although the role of opioids in the neoplasms is unclear, they have been implicated in the controlling of analgesic, behavioural (Morley 1986) and endocrine responses (Olson *et al*. 1986) and are also thought to have effects on the humoral and cellular immune system (Chang 1984, Teschemacher & Schweigerer 1985). With regard to the action of opioids on the growth of tumour cells, reports in the literature are contradictory, since they

Online version via http://www.endocrinology.org

exert both stimulatory and inhibitory effects on the growth of experimental tumours *in vivo* and *in vitro* depending on the type of opioid peptide, the dosage of the opioid and the type of cancer cell and tissue (Zagon & McLaughlin 1981, 1984, Lewis *et al.* 1983, Murgo 1985, Scholar *et al.* 1987, Maneckjee *et al.* 1990). Neuropeptides may therefore act as endocrine, paracrine or autocrine stimulatory growth factors on the cancer cell (neoplasia) or on the surrounding stroma (desmoplasia).

Although much work has been performed on the effects of opioid peptides on breast cancer cell growth, the mechanisms by which the bioactive peptides are enzymically released from their precursors in this tissue have not yet been investigated. The prohormone-processing enzymes responsible for processing opioid prohormone precursors were first identified in mammalian tissues at the end of 1989 (Fuller *et al.* 1989) and the first half of 1990 (Seidah *et al.* 1990, 1991a). In these early studies three enzymes were recognised as the mammalian Kex-2-like convertases, namely furin (Van den Ouweland *et al.* 1990), PC1 (Smeekens *et al.* 1991) and PC2 (Smeekens & Steiner 1990). This was followed by the complete elucidation of the cDNA sequence of mouse and human PC1 (Seidah *et al.* 1992). Furin cleaves proproteins that *in vivo* are normally expressed in cells devoid of secretory granules (constitutively secreting cells). In contrast, PC1 and PC2 demonstrate a selectivity of cleavage at paired basic residues that is best suited to the activation of precursors synthesised in cells containing secretory granules (Seidah *et al.* 1991a,b). This functional specialisation of these enzymes correlates with their observed cellular and tissue distribution. Thus furin is ubiquitously expressed in constitutive secreting cells whereas PC1 and PC2 are mostly found in neuroendocrine and endocrine cells and tissues that are endowed with a regulatory secretory pathway (Smeekens & Steiner 1990, Seidah *et al.* 1991a,b, 1992, Smeekens *et al.* 1991). In addition to the differential subcellular distribution of the subtilisin-like enzymes, the levels of PC1 and PC2 transcripts are often co-regulated with those of their substrates (Day *et al.* 1992), whereas furin does not exhibit such regulation.

PC1 and PC2 are implicated in the processing of PE in selected tissues of the central and peripheral nervous system (Seidah *et al.* 1990, 1991b, Schafer *et al.* 1993) (Fig. 1). In a preliminary study we had observed different processing patterns of PE and enkephalin-containing peptides (ECPs) in MCF-7 and ZR-75–1 human metastatic cells and the mouse androgen-responsive breast tumour cell line known as SC115. The present study was designed to investigate whether the distinct processing patterns of the ECPs were the result of co-localisation and activity of specific prohormone-processing enzymes such as PC1 and PC2 in these breast tumour cells, which could alter the spectrum of activity of the final products and thus tumour pathogenicity.
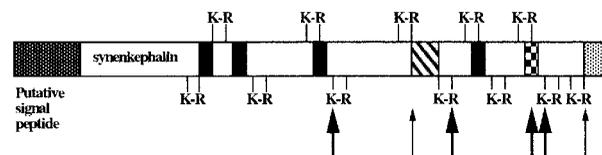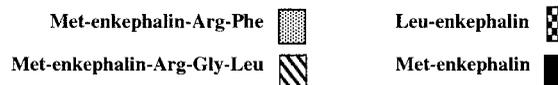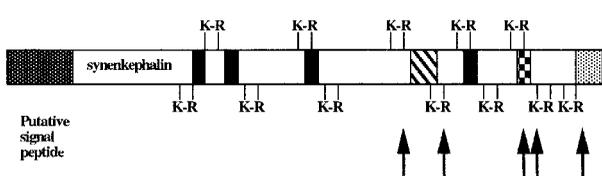

Figure 1 Representation of PE and some of the processing sites of PC1 and PC2. The main processing sites are indicated by thick arrows and the minor by thin. Adapted from Breslin *et al.* (1993).

Experimental procedures

Cell culture

Culture media, gentamicin, trypsin–EDTA solution, trypan blue, fetal calf serum (FCS) and horse serum were purchased from Gibco-BRL, Uxbridge, Middlesex, UK. Tissue culture flasks and apparatus were obtained from Falcon Marathon Lab. Supplies, London, UK.

The human MCF-7 breast carcinoma cell line was originally isolated from a pleural effusion of a primary breast cancer patient (Soule *et al.* 1973). MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with gentamicin (50 µg/ml), 1×10^{-8} M 17β-estradiol (Steraloids Ltd, London, UK) and 10% (v/v) heat-inactivated FCS.

The ZR-75–1 human breast cancer cell line was derived from a pleural effusion from a breast cancer patient (Engel *et al.* 1978) and was maintained in the supplemented DMEM medium (as the MCF-7 cells) with the addition of 0·1 unit/ml insulin (Sigma Chemical Co. Ltd, Poole, Dorset, UK).

The SC115 tumour originated spontaneously in a female mouse of the DD/S strain (Minesita & Yamaguchi 1965). Cells were maintained in DMEM supplemented with $3·5 \times 10^{-8}$ M testosterone (Steraloids), gentamicin (50 µg/ml) and 10% heat-inactivated FCS.

A Chinese hamster ovary (CHO) cell line stably transfected with the cDNA of rat preproenkephalin (DL1/50) was kindly donated by Dr I Lindberg, Department of Biochemistry and Molecular Biology, Louisiana State

University Medical Center, New Orleans, LA, USA (Lindberg *et al.* 1991). The DL1/50 cells were grown in alpha modified Minimal Essential Medium, without nucleotides or ribonucleotides, with 10% (v/v) well-dialysed FCS, 50 µg/ml gentamicin and 50 µM methotrexate (Sigma). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and were grown in 10 cm culture dishes at an initial density of $\sim 45 \times 10^3$ cells per dish. Growth medium was replaced every 2 days until the cells reached 80% confluence. At this point the cells were ready for transfection or experimentation.

Antibodies

The sheep polyclonal anti-PE antiserum (W346) was raised to recombinant full-length rat PE (purified from DL1/50 cell media by Q-Sepharose anion exchange chromatography (Hannah 1993)). Purified rat PE (3 mg) was conjugated to purified protein derivative (PPD) of avian tuberculin (MAFF, Central Veterinary Laboratory) at a ratio of 2:1 (w/w) of PPD to PE. Sheep were immunised with 400 µg rat PE conjugate added to 2 ml Freund's incomplete adjuvant (Sigma). Thereafter, 200 µg of peptide conjugate in 2 ml of incomplete adjuvant were given every 4 weeks and the sheep were bled at similar time intervals.

The anti-rat-PC2 antiserum was generously supplied by Dr J Hutton of the Department of Clinical Biochemistry, University of Cambridge, UK and was raised to a bacterial fusion protein containing rat PC2 amino acids 168–380 (Bennett *et al.* 1992). Anti-PC1 antiserum, developed against peptides representing the segment of amino acids 84–100 at the amino terminus of mouse PC1 was a generous gift from Dr N G Seidah of the Clinical Research Institute Montreal, Montreal, Quebec, Canada (Benjannet *et al.* 1993).

Secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-sheep and anti-rabbit IgG (DAKO A/S, Glostrup, Denmark). The SM2–2 antiserum, used for the met-enkephalin sulphoxide (MEO) RIA (a gift from Dr S Medbak of the Department of Chemical Endocrinology, St Bartholomew's Hospital, London, UK) was raised against MEO and has a similar specificity to that described previously (Clement-Jones *et al.* 1980).

A monoclonal antiserum that was raised previously in this laboratory to human PE (PE-1) was used at a dilution of 1:1000 for SDS-PAGE immunoblot detection of PE within breast tumour cells (Spruce *et al.* 1989). This antiserum was generated to a chimeric peptide of *Escherichia coli* β-galactosidase fused to the amino acid sequence 69–207 of human preproenkephalin. Sera from BALB/c mice immunised with the β-galactosidase–preproenkephalin A (69–207) hybrid polypeptide were tested for anti-PE activity and the binding domains of PE-1 were broadly located with respect to the primary

translation product, within amino acid sequences 152–207. The anti-PE-1 antiserum was used to confirm the specificity of the W346 antiserum in detection of PE.

Transfection of human PC2 (hPC2) into the ZR-75–1 cells

All the buffers and most of the methods used during this work were taken from Maniatis (Sambrook *et al.* 1988). Genetic manipulation reagents and restriction endonucleases were obtained from Promega (Southampton, Hants, UK) and New England Biolabs (Bishop's Stortford, Herts, UK). Full-length cDNA for hPC2 was a generous gift from Drs Steiner and Smeekens at the Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, University of Chicago, IL, USA. The multiple cloning site from Bluescript (Stratagene, Cambridge, Cambs, UK) (19 restriction sites) was ligated into the BamHI site (blunt ended ligation) of pSRα (obtained from the Department of Biochemistry, Imperial College, London, UK). pSRα possesses a promoter which is a fusion between the simian virus (SV40) early promoter and part of the long-term repeat of type 1 human T-cell leukaemia virus (Takebe *et al.* 1988) and additionally incorporates a pBr322 origin of replication and an ampicillin resistance gene. The full-length cDNA for hPC2 was subcloned from the cloning vector Bluescript II (Stratagene) into the multiple cloning site of the pSRα expression vector. The expression vector containing the cDNA of hPC2 was stably transfected by calcium phosphate co-precipitation into the ZR-75–1 cells and transfected cells were selected on the basis of neomycin resistance (G418 sulphate, geneticin, Schering, purchased from Gibco). Expressing transfectants of PC2 were assessed using SDS-PAGE immunoblot analysis.

Cell lysate preparation

The cells were lysed with 1 ml ice-cold lysis buffer (50 mM HCl, 0·1% (v/v) 2-mercaptoethanol, 0·001% (v/v) Triton X-100). The lysate was then sonicated (3×20 s bursts), heat treated (85 °C for 15 min) and subsequently centrifuged (12 000 **g** for 15 min). The supernatant was retained and the processed enkephalin peptides were analysed by the MEO RIA and by SDS-PAGE immunoblot analysis (see below). An aliquot (50 µl) of cell lysate was retained for cell protein content determination using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK).

Trichloroacetic acid (TCA) extraction of proteins for SDS-PAGE immunoblot analysis

To extract cellular proteins, 50% (w/v) of ice-cold TCA was added to 1 ml cell lysate at a final dilution of 10% (w/v) TCA and incubated overnight at 4 °C. The protein precipitate was pelleted by centrifugation

(12 000 *g* for 10 min) and traces of TCA were removed by ether extraction (3 × 1 ml). Precipitated proteins were resuspended in 100 µl SDS-PAGE sample treatment buffer (8% (w/v)) with an equal volume of sterile water. The pH of the samples was then adjusted to pH 7·6 with 1 M Na_2HCO_3. Proteins were analysed with 16% poly-acrylamide gel using the 0·75 mM mini gel Tris/Tricine Schagger and Von Jagow buffer systems (Schagger & Von Jagow 1987). The electrophoresed proteins were subsequently transferred onto polyvinyldifluorine Flourotrans transfer membrane (PALL Europe Ltd, Portsmouth, UK) using a semi-dry blotter (Biotech Instruments Ltd, Luton, UK). Non-specific protein binding sites were blocked using 0·05 M PBS (0·8% (w/v) NaCl, 0·02% (w/v) KCl, 0·181% (w/v) $Na_2HPO_4.2H_2O$, 0·024% (w/v) KH_2PO_4, pH 7·4) containing 1% (w/v) BSA and 1% (w/v) non-fat dry milk and then probed with W346 sheep anti-PE antiserum and the mouse anti-human PE (PE-1) antiserum. The cell lysate proteins of the breast tumour cells were additionally probed with anti-PC1 and anti-PC2 antiserum. PE immunoreactive bands were visualised using the enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL, USA). The immunoblot was exposed to pre-flashed Fuji RX film in a film cassette (Genetic Research Instrumentation, Gene House, Feltsted, Essex, UK) and the film was developed using Kodak D-19 and fixed with Kodak Unifix.

RIA for MEO

MEO, TPCK-treated trypsin, carboxypeptidase B (CPB) and other miscellaneous chemical reagents were purchased from Sigma. One millilitre samples of both cell lysates were digested for 2 h at 37 °C with 1 mg/ml N-tosylphenyl-alanine chloromethyl-ketone (TPCK)-treated trypsin that was dissolved in RIA buffer which contained 0·2% (w/v) human serum albumin (HSA), 0·05 M phosphate pH 7·4, 0·01% (w/v) sodium azide. Trypsin digestion was terminated by heat inactivation (85 °C for 15 min) and has been described previously (Hannah *et al.* 1993). A series of control digestions of 0 to 1 mg/ml of TPCK-treated trypsin and 0·2% (w/v) HSA (i.e digestion buffer alone) failed to detect measurable quantities of met-enkephalin immunoreactive peptides using the MEO RIA following a 2 h digestion at 37 °C. Samples were treated with CPB (40 µg/ml diluted in RIA buffer) for 30 min at 37 °C and CPB activity was then terminated by heat inactivation (85 °C for 15 min). Met-enkephalin released from PE and ECPs in samples was converted to the immunoreactive sulphoxide by oxidation with H_2O_2 (100 volumes, Fisons Scientific Co., Crawley, Sussex, UK) by the addition of a final concentration of 1% (v/v) H_2O_2 and incubation at room temperature for 2 h. MEO standards were serially diluted from 2000 to 1·9 pg/ml in RIA buffer. The samples were assayed in triplicate 200 µl aliquots and were incubated overnight at 4 °C with

50 µl rabbit polyclonal anti-MEO, SM2–2 antiserum at a final dilution of 1:64 000 in 300 µl with 50 µl radiolabelled MEO (^{125}I-MEO, 12 000 c.p.m.). MEO immunoreactivity was pelleted following a 1 h incubation at 4 °C with 200 µl pre-precipitated sheep anti-rabbit fragment crystalline (10% (v/v) in 1% (v/v) normal rabbit serum, 4% (w/v) polyethylene glycol in (0·05 M phosphate buffer (pH 7·4)). Ice-cold wash buffer (2 ml 9% (w/v) NaCl, 0·001% (v/v) Triton X-100) was added to each tube. Following centrifugation (4200 *g*, 30 min at 4 °C), the supernatants were aspirated and the MEO radioactivity retained in the pellets was assessed using a 1261 Multigamma counter (LKB, Malmo, Sweden). The lower sensitivity limit of the assay was between 5 and 10 pg/ml MEO. To assess the intracellular proteolytic activity of prohormone-processing enzymes in the processing of PE, not all samples were treated with trypsin or CPB. Trypsin and CPB treatment (treatment I), measures total met-enkephalin and by inference total PE. CPB treatment only (treatment II) measures PE partially processed by trypsin-like endogenous enzymes to met-enkephalin containing carboxy-terminal basic amino acid residues and PE that has been completely processed to met-enkephalin by endogenous trypsin-like and CPB-like enzymes. 'No treatment' (treatment III) measures PE that has been processed to met-enkephalin by specific trypsin-like and CPB-like endogenous proteolytic enzymes. To calculate the percentage of processed PE by endogenous trypsin-like enzymes, the CPB treatment (treatment II) is calculated as a percentage of the cellular PE content (treatment I).

Statistical analysis

Values of MEO content were corrected to cell protein content and expressed as mean ± s.e.m. pg per µg cell protein ($n=3$). Student's *t*-test was used when applicable.

Results

SDS-PAGE immunoblot analysis detected PE within the cell lysates of the ZR-75–1, MCF-7 and SC115 breast tumour cell lines (Fig. 2a and b). Figure 2a and b show the immunoblot detection of PE using the sheep anti-rat (W346) and mouse anti-human PE (PE-1) antisera respectively. Both antisera detected a 46 kDa form of PE in the breast tumour cell lines, suggesting that this protein is PE. In contrast to the breast tumour cell lines, the recombinant rat PE expressed in the Chinese hamster ovary CHO/Dl150 cells migrated with molecular masses of 30 and 32 kDa. The 32 kDa form of PE has been previously characterised by Lindberg *et al.* (1991) as a glycosylated form of the 30 kDa PE.

To measure the intracellular processing of PE in each of the breast tumour cell lines, the cell lysates were subjected
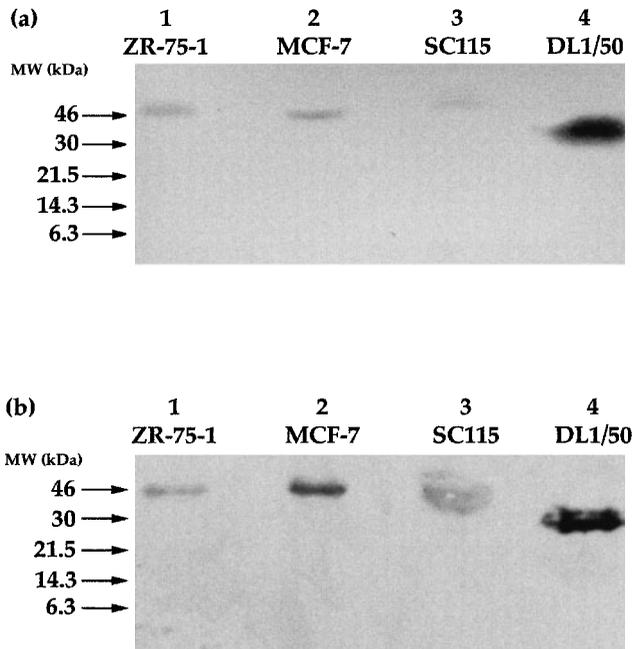
Figure 2 (a) Immunoblot of the TCA-precipitated protein extracts from the cell lysates of the human breast tumour cell lines ZR-75–1 (lane 1) and MCF-7 (lane 2), the mouse breast tumour cell line SC115 (lane 3) and CHO cells transfected with the cDNA encoding rat PE (CHO/DL150 cells (lane 4)), using a 1:1000 dilution of W346 sheep anti-PE antiserum. The methods and reagents for detection of PE were by ECL using an HRP-conjugated secondary antibody. PE that migrated with a molecular mass of 46 kDa was detected in all three breast tumour cell lines (lanes 1–3) in contrast to the rat PE expressed by the DL1/50 cell line that migrated with molecular masses of 30–32 kDa. (b) The same experiment as in (a) but using a 1:1000 dilution of mouse anti-human PE (PE-1) antiserum. The methods, reagents and results were the same as in (a).

Figure 3 Histogram of the percentage of intracellular processed PE (calculated by MEO RIA analysis) contained within the cell lysate extracts of the MCF-7, ZR-75–1 and SC115 breast tumour cells. The percentage of PE processed by the SC115 cell line is significantly greater than that by both the MCF-7 and ZR-75–1 wild type cell lines ($*P<0.05$). The percentage of PE processed to met-enkephalin immunoreactive peptides is significantly greater within the cell lysates of the ZR-75–1/PC2 cells when compared with the ZR-75–1 wild type cells ($***P<0.01$).

to three different enzymic treatments (as described in the Experimental procedures), and the resultant peptides were analysed by the MEO RIA. Treatment I corresponds to the total amount of met-enkephalin and by inference is the total amount of PE. Treatment II (an addition of CPB only) corresponds to the amount of PE processed to met-enkephalin and carboxy-terminally elongated met-enkephalins which are formed by the action of cellular trypsin-like enzymes on the precursor. The total amount of PE processed to peptides containing the met-enkephalin sequence is calculated by expressing treatment II as a percentage of treatment I. As shown by Fig. 3 the MCF-7, ZR-75–1 and SC115 cells processed 28 ± 5.0, 29 ± 11 and $78 \pm 17\%$ of the total cellular PE respectively, indicating that the human and mouse breast tumour cell lines differentially process PE. As shown by Fig. 4, the amount of met-enkephalin (pg per µg cell protein) varies between each breast tumour cell line. The majority of all the enkephalin peptides present in the SC115 cell extracts were detected as processed met-enkephalin.
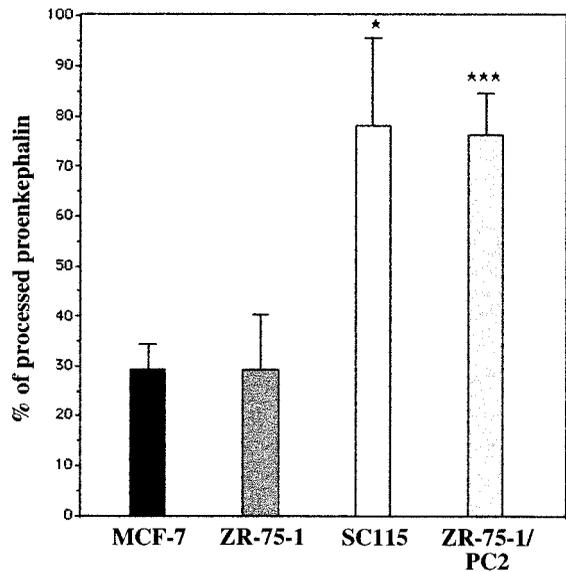
The differential PE processing patterns between the MCF-7, ZR-75–1 and the SC115 breast tumour cells suggested that these cell lines may contain different complements of the PE-processing enzymes. To determine whether the differential processing profiles of PE were due to the co-expression of prohormone-processing enzymes, such as the prohormone-converting enzymes PC1 and/or PC2, TCA-precipitated cell proteins were separated by SDS-PAGE and the immunoblots were probed with anti-PC1 and anti-PC2 antisera. PC1 was detected in all three breast tumour cell lines; however, variant molecular mass forms of PC1 were observed in each of the cell lines (see Fig. 5). The MCF-7 and the ZR-75–1 breast tumour cells both contained a 68 kDa form of PC1. The most predominant form of PC1 detected within the SC115 cells exhibited a molecular mass of 69 kDa. In contrast to the SC115 cells the MCF-7 cells contained multiple forms of the PC1 enzyme. The five forms of PC1 detected within the cell lysates of the MCF-7 cells exhibited molecular masses of 100, 96, 88, 68 and 52 kDa. It is possible that the 100 kDa form of PC1 can be identified as the unactivated mature proenzyme. The 52 kDa form of PC1 is not noticeable within the cell lysates of the ZR-75–1 and SC115 cells. Two 96 and 68 kDa forms of PC1 were found within the cell lysates of ZR-75–1 cells.
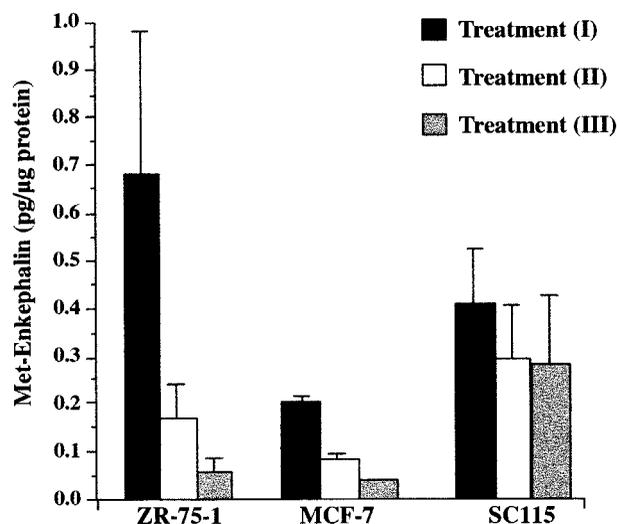
Figure 4 MEO RIA analysis of the relative amounts of PE and ECPs contained within the cell lysates of the MCF-7, ZR-75–1 and SC115 breast tumour cells. The cell lysates were subjected to three CPB or trypsin treatments. Trypsin and CPB treatment (treatment I), measures total met-enkephalin and by inference total PE. CPB treatment only (treatment II) measures PE partially processed by trypsin-like endogenous enzymes to met-enkephalin containing carboxy-terminal basic amino acid residues and PE that has been completely processed to met-enkephalin by endogenous trypsin-like and CPB-like enzymes. 'No treatment' (treatment III) measures PE that has been processed to met-enkephalin by specific trypsin-like and CPB-like endogenous proteolytic enzymes. The values of PE-derived peptides and PE (pg per µg cell protein) varies significantly between each of the breast tumour cell lines. Statistical significance ($P<0.05$) between the three treatments was achieved with ZR-75–1 and MCF-7 cells. There was no difference between the three treatments in the case of the SCll5 cells.

Both PC1 and PC2 were detected within the cell protein extracts of the SC115 cells (Fig. 6). It is possible that the co-localisation of both PC1 ($\sim$68 kDa) and PC2 ($\sim$67 kDa) in the SC115 cell line may be responsible for the high level of processed PE measured in this cell line in comparison with the ZR-75–1 and MCF-7 cell lines (Fig. 3).

To demonstrate that PC2 is more active than PC1 in processing PE, the cDNA of hPC2 was stably transfected into the ZR-75–1 cell line, which does not express this enzyme endogenously (Fig. 6). Immunoblot analysis confirmed the expression of hPC2 in this cell line (Fig. 6) and the expression of PC2 was accompanied by a significant increase in the intracellular processing of PE to met-enkephalin immunoreactive peptides from $22 \pm 5\%$ in the ZR-75–1 wild type cell line to $76 \pm 8\%$ ($P<0.01$) in the PC2-expressing ZR-75–1 cells (Fig. 3).

Discussion

In this study we have detected PE and met-enkephalin immunoreactive peptides co-localised with the
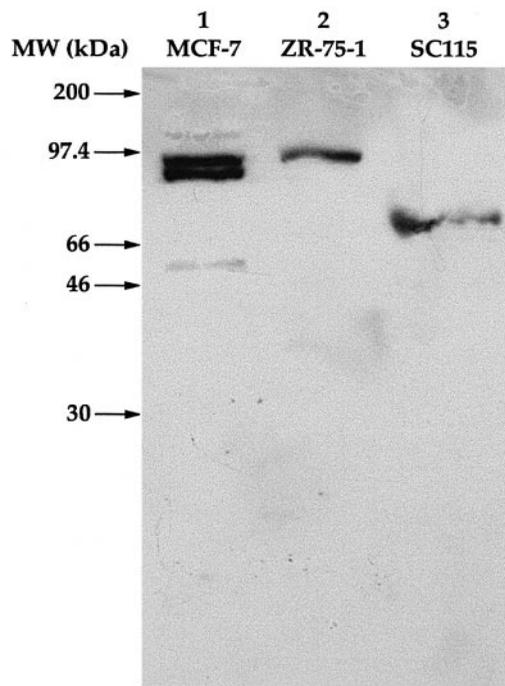
Figure 5 Immunoblots of the TCA-precipitated proteins from the cell lysate extracts of the MCF-7 (lane 1), ZR-1–75 (lane 2) and SC115 (lane 3) breast tumour cells were probed with a 1:1000 dilution of rabbit anti-PC1 amino terminus antiserum. The bands were visualised by luminescence using a 1:1000 dilution of HRP-conjugated secondary antibody. PC1 expression was detected in all three cell lines. The molecular masses of the PC1 varied in each of the cell lines. The major form of PC1 in the SC115 cells is of a lower molecular mass (68 kDa) than the predominant form of PC1 detected within the ZR-75–1 cell ($\sim$90 kDa). The major forms of PC1 in the MCF-7 cells are present in two high molecular mass forms of $\sim$90 kDa and $\sim$86 kDa.

prohormone-processing enzyme PC1 in two human breast tumour cell lines (MCF-7 and ZR-75–1) and in the mouse androgen-responsive SC115 metastatic breast tumour cell line. In addition, PC2 has been detected in the mouse SC115 cell line. The variant forms of PC1 located within the cell lysates of the MCF-7 cells may represent different post-translationally modified forms of PC1 or carboxy-terminal truncated variants. The large 100 kDa form of PC1 detected in the MCF-7 cells may represent the precursor form of the enzyme. A pro-form of PC1 has been previously characterised in AtT-20 cells (a rat anterior pituitary cell line) (Vindrola & Lindberg 1993), where it exhibited a molecular mass of 87 kDa. The 87 kDa form of PC1 is thought to be processed further to a 66 kDa form of the enzyme. Both the 66 and 87 kDa forms of PC1 are active in the processing of prohormone precursors (Benjannet *et al.* 1993, Vindrola & Lindberg 1993). These higher molecular mass forms of PC1 were not detected in the SC115 cells where only the 69 kDa
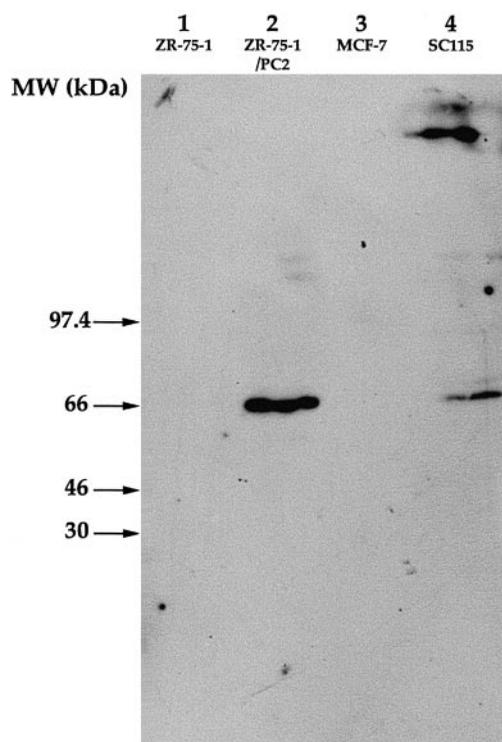
Figure 6 Immunoblots of TCA-precipitated proteins from the ZR-75–1 cell lysate extract (lane 1), ZR-75–1/PC2 cells stably transfected with the cDNA encoding hPC2 (lane 2), MCF-7 cells (lane 3) and SC115 cells (lane 4) were probed with a 1:1000 dilution of anti-PC2 antiserum. PC2 (66 kDa) was visualised only in the SC115 cell lysates (lane 4) and the ZR-75–1 cells that were stably transfected with the cDNA encoding PC2 (lane 2).

processed form of PC1 was present, co-expressed with the 67 kDa form of PC2 (Benjannet *et al.* 1993).

It was interesting to observe the 46 kDa protein in the cell lysates of the human and mouse breast tumour cells (Fig. 2), in contrast to the 32 kDa recombinant rat PE which immunoreacted with antisera to PE. Human PE has been shown to exhibit a molecular mass of 36·5 kDa in a human pheochromocytoma (Comb *et al.* 1982). As there is only a difference of two in the number of amino acids in the rat and human PE, it is possible that the larger form of PE in the breast tumour cells has been subjected to differential post-translational modifications such as glycosylation; however, initial deglycosylation and dephosphorylation experiments failed to reduce the molecular mass of the tumour PE (data not shown). The PE secreted by SK-N-MC cells (a human neuroblastoma cell line) has also been shown to be appreciably glycosylated and phosphorylated (Lindberg & Shaw 1992); however, the molecular mass for the higher molecular weight form of PE in this cell line was only 34 kDa. Met-enkephalin immunoreactivity eluting with the authentic peptide and higher molecular weight material have also been detected

in other human tumour tissues (Clement-Jones *et al.* 1982) including adrenal medullary tumours, tumours of the lung, pancreas, thymus and the gall bladder; however, an explanation for these higher molecular weight forms of PE was not given.

The detection of substantially more processed PE in the SC115 cell lysates in comparison with the human breast tumour cells (Figs 3 and 4) may be due to the co-localisation of both PC1 and PC2 with PE in this cell line (Figs 5 and 6). PE processed by both PC1 and PC2 would theoretically result in four free met-enkephalins in addition to met-enkephalin-Arg[6]-Phe[7] and met-encephalin-Arg[6]-Gly[7]-Leu[8]. The latter two peptides would not be detected by our MEO assay. Therefore the theoretical percentage of PE processed by PC1 and PC2 measured by the MEO assay would be 67% similar to the 79% that we observed in the SC115 cells. The theoretical partial processing of PE by PC1 would only give two amino-terminally extended peptides with detectable enkephalin (by the immunoassay) at their carboxy terminus and therefore a much lower theoretical value in the MEO assay of 33%, similar to the 29% of processed PE that we observed in the human breast tumour cell lines.

Such differential PE processing patterns are apparent in the tissues of the central nervous system and in the nuclei of the brain (Giraud *et al.* 1984), which may be due to the differential tissue distribution of PC1 and PC2. The distribution of PE-derived peptides varies greatly between discrete regions of the brain, but is particularly apparent between the striatum and the hypothalamus (Schafer *et al.* 1993), where there is evidence for differential PE processing. For example, the level of PC1 expression is high in the supraoptic and the paraventricular nuclei of the hypothalamus (Seidah *et al.* 1991*b*), where more selectively cleaved PE-derived peptides have been found. PC2 mRNA is also observed in these areas with moderate abundance. However, high levels of PC2 mRNA have been detected in the preoptic area and the mamilliary bodies and in other regions of the CNS including the thalamic nuclei in the hippocampus, the deep superficial layers of the cortex, the amygdala, and in the striatum where more complete cleavage of PE is noticeable. Therefore, PC2 is thought to be less selective in the cleavage of PE and where PC1 and PC2 are co-localised the processing of PE is enhanced.

The less selective proteolytic action of PC2 on PE was observed in the cell lysates of both the ZR-75–1 breast tumour cell line that had been transfected with the cDNA of hPC2 and the SC115 cells. Both PC1 and PC2 would appear to be co-localised in these cells with more completely processed PE-derived peptides. The high percentage of PE processed to met-enkephalin in the SC115 and the ZR-75–1/PC2 cells is similar to the data published by Seidah and coworkers in which PC1, PC2 and PE were transiently expressed by a vaccinia virus expression system in a rat somatotroph cell line (GH$_4$Cl) (Breslin *et al.* 1993).

The data produced from their study revealed that the major immunoreactive enkephalins formed from the activity of PC2 are met-enkephalin-Arg[6]-Phe[7], free met-enkephalin-Arg[6]-Gly[7]-Leu[8], leu-enkephalin and met-enkephalin (see Fig. 1). In contrast PC1 is shown to produce intermediate-sized processing products (3–10 kDa); the major immunoreactive ECP eluted at the position of peptide B (5·3 kDa) and free leu-enkephalin (Cullinan *et al.* 1991).

This work differed from the present study as they co-expressed PC1, PC2 and PE by the transient transfection procedure in a foreign cell line. The disadvantage in expressing proteins transiently is that the cells can only be used for short-term experiments and not for long-term cell manipulation studies. We have, however, stably transfected hPC2 into the ZR-75–1 human breast tumour cell line, which processes endogenous PE and PC1. These breast tumour cell lines additionally possess opiate receptors (Maneckjee *et al.* 1990). The distinct processing patterns of PE by PC1 and PC2, in our stably transfected human breast tumour cell lines, will need to be investigated in more detail. To our knowledge this is the first stably transfected human cell where PC2 has been shown to be active in the processing of PE.

The function of the expression of opioid peptides in breast tumour cells is yet to be determined. However, opioid peptides released from specific cancer tissues and cells have been shown to affect the growth of these cells (Zagon & McLaughlin 1981, 1983, 1984, Murgo 1985, Scholar *et al.* 1987, Maneckjee & Minna 1990, Maneckjee *et al.* 1990, Kirchimair *et al.* 1992, Schrey & Patel 1994). For example human small cell lung cancer (SCLC) cell lines express both opioid peptides and receptors; however, the growth of these cells is inhibited in response to endogenous opioid peptides (Maneckjee & Minna 1990). In contrast, the POMC peptide, β-endorphin, has been reported to stimulate the clonal growth of human SCLC cells (Murgo 1985). The chronic administration of the opioid agonist heroin has been shown to retard tumour growth and prolong survival time of mice with transplanted neuroblastoma tumours and to inhibit neuroblastoma cell growth *in vitro*. The opioid antagonist naloxone was shown to block these anti-tumour effects (Zagon & McLaughlin 1981). Met- and leu-enkephalins have been shown to produce anti-metastatic actions on B16-BL6 melanoma as well as decrease the number of metastases to the lung (Scholar *et al.* 1987). Pretreatment with naloxone and naltrexone may significantly inhibit the growth of carcinogen-induced mammary cancers in rats and cause complete regression in mice with spontaneous and transplantable mammary tumours (Zagon & McLaughlin 1983). In the human breast cancer cell line known as MCF-7, opioid peptides inhibited cell growth only in the presence of oestradiol (Maneckjee *et al.* 1990).

It is conceivable that PC1 and PC2 could additionally be involved in the activation of other prohormone precursors or growth factors that have also been detected in breast tumour cells, which could in turn affect tumour growth and development. Breast tumour cells are also known to express other prohormone precursors and POMC-derived opioid peptides. Like PE these are located in the tissues of the central and peripheral nervous system (Cullinan *et al.* 1991). POMC is completely processed in the pars intermedia, where there is a high expression of PC2 with a low expression of PC1 (Benjannet *et al.* 1991, Zhou & Mains 1994). The corticotrophs of the anterior pituitary only contain PC1, which has been shown *in vitro* and in co-expression studies to process POMC to adrenocorticotrophin (ACTH), β-lipotrophin and a 16 kDa amino-terminal fragment (pro-γ-melanocyte-stimulating hormone (MSH)). The melanotrophs of the intermediate lobe contain very little PC1 and large amounts of PC2 and hence the amino-terminal POMC fragment is processed to γ3-MSH, ACTH is processed to α-MSH and corticotrophin-like intermediate peptide and β-lipotrophin is processed to γ-lipotrophin and β-endorphin. Prosomatostatin (PSS) has also been detected in breast tumour cells (Nelson *et al.* 1989). It has been observed that PSS is proteolytically activated by furin, PC1 and PC2 in eukaryotic cell lines (Galanopoulou *et al.* 1993). Whether these enzymes are responsible for the activation of PSS in breast tumour cells, which may in turn have a direct or indirect effect on the growth and development of breast tumour cells, needs further investigation. A recent study reporting the comparative analysis of expression of furin, PACE4, PC1 and PC2 in human lung tumours suggests that these enzymes may be intimately involved in the production of many signalling molecules, when abnormally expressed in lung cells, and could lead to their neoplastic transformation (Mbikay *et al.* 1997).

In conclusion, we have established that PE is differentially processed in human and mouse breast tumour cell lines. In the mouse breast tumour cell, PE is completely processed to met-enkephalin, met-enkephalin-Arg-Phe and met-enkephalin-Arg-Gly-Leu, which may be due to the co-localisation of both PC1 and PC2 in this cell line. More limited PE processing is evident in the human breast tumour cell lines, which may be due to the expression of only PC1 and not PC2. Preliminary immunoblot analysis of total protein extracted from solid metastatic breast tumours (1·5 g) from six patients has detected PC1, PE and PC2 in all the tumours; however, the expression of these proteins varied between the subjects (data not shown). Further investigation of these tissues may reveal the function of these enzymes in the growth and development of breast tumour cells.

Acknowledgements

and helpful suggestions on cancer cell line culture from Dr P Darbre of the School of Animal and Microbial Sciences, Whiteknights, University of Reading. The authors would like to thank Dr N G Seidah, Dr J Hutton and Dr S Medbak for providing antibodies for this study.

References

Benjannet S, Rondeau N, Day R, Chretien M & Seidah NG 1991 PC1 and PC2 are pro-protein convertases capable of cleaving POMC at distinct pairs of basic residues. *Proceedings of the National Academy of Sciences of the USA* **88** 3564–3568.

Benjannet S, Rondeau N, Paquet L, Boudreault A, Lazure C, Chretien M & Seidah NG 1993 Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. *Biochemical Journal* **294** 735–743.

Bennett DL, Bailyes EM, Nielson E, Guest PC, Rutherford NG, Arden SD & Hutton JC 1992 Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryotic subtilisin family. *Journal of Biological Chemistry* **267** 15229–15236.

Breslin MB, Lindberg I, Benjannet S, Mathis PJ, Lazure C & Seidah NG 1993 Differential processing of proenkephalin by prohormone convertases 1(3) and 2 and furin. *Journal of Biological Chemistry* **268** 27084–27093.

Chang K-J 1984 Opioid peptides have actions on the immune system. *Trends in Neuroscience* **7** 234–235.

Chatikhine VA, Chevrier A, Chauzy C, Duval C, D'Anjou J, Girard N & Delpech B 1994 Expression of opioid peptides in cells and stroma of human breast cancer and adenofibromas. *Cancer Letters* **77** 51–56.

Clement-Jones V, Lowry PJ, Rees LH & Besser GM 1980 Development of a specific extracted radioimmunoassay for methionine enkephalin in human plasma and cerebrospinal fluid. *Journal of Endocrinology* **86** 231–243.

Clement-Jones V, Corder R, Smith R, Medbak S, Lowry PJ, Rees LH & Besser GM 1982 Met-enkephalin and related peptides in man. *Advances in Biochemistry and Psychopharmacology* **33** 379–386.

Comb M, Herbert E & Crea R 1982 Partial characterisation of the mRNA that codes for enkephalin in bovine adrenal medulla and human pheochromocytoma. *Proceedings of the National Academy of Sciences of the USA* **79** 360–364.

Cooper JR, Bloom FE & Roth R 1996 *The Biochemical Basis of Neuropharmacology*. New York: Oxford University Press.

Cullinan WE, Day NC, Schaffer M, Day R, Seidah NG, Chretien M, Akil H & Watson SJ 1991 Neuroanatomical and functional studies of peptide precursors and processing enzymes. *Enzyme* **45** 285–300.

Day R, Schaffer MKH, Watson SJ, Chretien M & Seidah NG 1992 Distribution of the prohormone convertases PC1 and PC2 in the rat pituitary. *Molecular Endocrinology* **6** 485–497.

Engel LW, Young NA, Talka TS, Lippman ME, O'Brian SJ & Joyce MJ 1978 Establishment and characterisation of three new continuous lines derived from human breast carcinomas. *Cancer Research* **38** 3352–3364.

Fuller RS, Brake AJ & Thorner J 1989 Intracellular targeting and structural conservation of a prohormone-processing protease. *Science* **246** 482–486.

Galanopoulou A, Kent G, Rabbani SN, Seidah NG & Patel YC 1993 Heterogeneous processing of prosomatostatin in constitutive and regulated secretory pathways; putative role of the endoproteases furin, PC1 and PC2. *Journal of Biological Chemistry* **268** 6041–6049.

Giraud AS, Williams RG & Dockray GJ 1984 Evidence for different posttranslational processing of proenkephalin in the bovine adrenal, colon and striatum indicated by radioimmunoassay using region specific anti-sera to Met-enk-arg-phe and Met-enk-arg-gly-leu. *Neuroscience Letters* **46** 223–228.

Hannah MJ 1993 Studies on the expression and processing of proenkephalin A. *PhD Thesis*. University of Reading.

Hannah MJ, Jackson S, Gillies G & Lowry PJ 1993 Met-enkephalin secretion from mixed cultures of hypothalamic neurons and astrocytes. *Neuroendocrinology* **57** 884–891.

Kirchmair R, Egger C, Gee P, Hogue-Angeletti R, Lasop A & Winkler H 1992 *FEBS Letters* **143** 143–145.

Lewis JW, Shavit Y, Terman GW, Nelson IR, Gale RP & Liegeskind JC 1983 Apparent involvement of opioid peptides in stress-induced enhancement of tumour growth. *Peptides* **4** 635–638.

Lindberg I & Shaw E 1992 Posttranslational processing of pro-enkephalin in SK-N-MC cells: evidence for phosphorylation. *Journal of Neurochemistry* **58** 448–453.

Lindberg I, Shaw E, Finely J, Leone D & Deininger P 1991 Post-translational modifications of rat proenkephalin overexpressed in Chinese hamster ovary cells. *Endocrinology* **128** 1849–1856.

Maneckjee R & Minna JD 1990 Opioid and nicotine receptors affect the growth and regulation of human lung cancer cell lines. *Proceedings of the National Academy of Sciences of the USA* **87** 3294–3298.

Maneckjee R, Biswas R & Vonderhaar BK 1990 Binding of opioids to human breast cancer cells and their effects on growth. *Cancer Research* **50** 2234–2238.

Mbikay M, Sirois F, Yao J, Seidah NG & Chretien M 1997 Comparative analysis of expression of the proprotein convertases furin, PACE4, PC1 and PC2 in human lung tumours. *British Journal of Cancer* **75** 1509–1514.

Medina D, Kidwell W, Heppner G & Anderson 1987 *Cellular and Molecular Biology of Mammary Cancer*. New York: Plenum Publishing Corp.

Minesita T & Yamaguchi K 1965 An androgen-dependent mouse mammary tumour. *Cancer Research* **25** 1168–1174.

Morley JE 1986 Neuroendocrine effects of endogenous opioid peptides on human subjects: a review. *Psychoneurological Endocrinology* **8** 361–379.

Murgo AJ 1985 Inhibition of B16-BL6 melanoma growth in mice in by methionine-enkephalin. *Journal of the National Cancer Institute* **75** 341–344.

Nelson J, Crein M & Murphy RF 1989 Synthesis of somatostatin by breast cancer cells and their inhibition by exogenous somatostatin and sandostatin. *British Journal of Cancer* **59** 739–742.

Olson GA, Olson RD & Kastin AJ 1986 Endogenous opiates. *Peptides* **7** 907-933.

Sambrook J, Fritsch EF & Maniatis T 1988 *Molecular Cloning: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Schafer MKH, Day R, Cullinan WE, Chretien M, Seidah NG & Watson SJ 1993 Gene expression of prohormone and proprotein convertases in the rat CNS: a comparative *in situ* hybridisation analysis. *Journal of Neuroscience* **13** 1258–1279.

Schagger H & Von Jagow G 1987 Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins from a range from 1 to 100 kDa. *Analytical Biochemistry* **166** 368–379.

Scholar EM, Violi L & Hexum TD 1987 The antimetastatic activity of enkephalin-like peptides. *Cancer Letters* **35** 133–138.

Schrey MP & Patel KV 1994 Role of regulatory peptides in the control of breast cancer and cell growth and function. *Endocrine-Related Cancer* **3** 41–70.

Scopsi L, Balsev E, Brunner N, Poulsen HS, Andersen J, Rank F & Larsson L 1989 Immunoreactive opioids in human breast cancer. *American Journal of Pathology* **134** 473–479.

Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M & Chretien M 1990 cDNA sequence of two distinct pituitary proteins

homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. *DNA* **9** 415–424.

Seidah NG, Day R, Marcinkiewicz M, Benjannet S & Chretien M 1991*a* Mammalian neural and endocrine pro-protein and pro-hormone convertases belonging to the subtilisin family of serine proteinases. *Enzyme* **45** 271–284.

Seidah NG, Marcinkiewicz M, Benjannet S, Gaspar L, Beaubien G, Mattei MG, Lazure C, Mbikay M & Chretien M 1991*b* Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin and Kex2: distinct chromosomal localisation and mRNA distribution in pituitary and brain compared with PC2. *Molecular Endocrinology* **5** 111–122.

Seidah NG, Hamlelin J, Gaspar A, Day R & Chretien M 1992 The cDNA sequence of the human pro-hormone and pro-protein convertase PC1. *DNA and Cell Biology* **11** 283–289.

Sirbasku DA & Benson RH 1980 Proposal of an indirect mechanism of estrogen induced mammary tumour cell growth. In *Cell Biology of Breast Cancer*, pp 289–314. Eds CM McGrath, MJ Brennan & MA Rich. New York: Academic Press.

Smeekens SP & Steiner DF 1990 Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. *Journal of Biological Chemistry* **265** 299–300.

Smeekens SP, Avruch AS, LaMendola J, Chan SJ & Steiner DF 1991 Identification of a cDNA encoding a putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proceedings of the National Academy of Sciences of the USA* **88** 340–344.

Soule HD, Vazquez J, Long A, Albert S & Brennan M 1973 A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute* **51** 1409–1416.

Spruce BA, Jackson S, Lowry PJ, Lane DP & Glover DM 1989 Monoclonal antibodies to proenkephalin A fusion peptide synthesised in *Escherichia coli* recognise novel proenkephalin A precursor forms. *Journal of Biological Chemistry* **263** 19788–19795.

Takebe Y, Seiki M, Fujisawa JI, Hoy P, Yokota K, Arai KI, Yoshida M & Arai N 1988 SRa promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R–U5 segment of human T-cell leukemia virus type 1 long term repeat. *Molecular and Cellular Biology* **8** 466–472.

Teschemacher H & Schweigerer L 1985 Opioid peptides: do they have immunological significance? *Trends in Pharmacological Sciences* **6** 368–370.

Van den Ouweland AMW, Van Duijnhoven HLP, Keizer GD, Dorssers LCJ & Van de Ven WJM 1990 Structural homology between the human fur gene product of the subtilisin-like protease encoded by yeast KEX-2. *Nucleic Acids Research* **18** 664–669.

Vindrola O & Lindberg I 1993 Release of the prohormone convertase PC1 from AtT-20 cells. *Neuropeptides* **25** 151–160.

Zagon IS & McLaughlin PJ 1981 Heroin prolongs survival time and retards tumour growth in mice neuroblastoma. *Brain Research Bulletin* **7** 25–32.

Zagon IS & McLaughlin PJ 1983 Naltexone modulates tumour response in mice with neuroblastoma. *Science* **221** 671–673.

Zagon IS & McLaughlin PJ 1984 Opiates alter tumour cell growth and differentiation *in vitro*. *NIDA Research Monograph* **49** 344–350.

Zagon IS, McLaughlin PJ, Goodman SR & Rhodes RE 1987 Opioid receptors and endogenous opioids in diverse human and animal cancers. *Journal of the National Cancer Institute* **79** 1059–1065.

Zhou A & Mains ER 1994 Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells over expressing prohormone convertases 1 and 2. *Journal of Biological Chemistry* **269** 17440–17447.