NOVEL MONOCLONAL ANTIBODIES TO A NEUROPEPTIDE PRECURSOR, PROENKEPHALIN A

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Submitted for the degree of Ph.D.

December 1989

ABSTRACT

The biological relevance of the mammalian opioid precursor proenkephalin A, and indeed of any neuropeptide precusor, has remained elusive since their discovery from the cloning of neuropeptide genes. In order to address this issue, I have generated a panel of monoclonal antibodies to recombinant human preproenkephalin A fused to β-galactosidase. I screened hybridoma supernatants by immunoblotting against identical preproenkephalin sequences fused to alternative prokaryotic gene products. This novel approach to the generation of antibodies to a complex neuropeptide precursor molecule should now be widely applicable to other propeptides. Two of the antibodies, PE-1 and PE-2, have provided evidence for novel processing intermediates in isolated bovine adrenomedullary chromaffin granules. All of the antibodies recognise the intact precursor, some more strongly than others.

I have transformed rat phaeochromocytoma PC12 cells with human preproenkephalin cDNA expressed from the RSV LTR. The human proenkephalin molecule is not proteolytically processed in these cells, despite apparently correct targeting to secretory vesicles. Processing cannot be stimulated by treatment with differentiation promoters or with reserpine. This approach will provide a means of investigating the processing of proenkephalin in relation to its compartmentalisation

within the cell. One of the anti-proenkephalin monoclonal antibodies cross-reacts with naturally occurring and recombinant chromogranin A, another molecule destined for dense core secretory granules. I have performed preliminary epitope mapping of this shared antigenic determinant by immunoblotting of β-galactosidase fusion peptides incorporating overlapping regions of chromogranin.

I have used indirect immunofluorescence to examine the distribution of enkephalin precursor peptides within brain. This may not necessarily parallel that of the mature enkephalin peptides due to regional differences in proteolytic processing. Indeed, my antibodies have revealed proenkephalin within subpopulations of cerebellar neurons and non-neuronal astroglial cells, in the absence of mature enkephalin peptides. I have confirmed proenkephalin gene expression by in situ hybridisation. In dissociated glial culture, the proenkephalin molecule has a striking intracellular distribution, apparently in association with subsets of filamentous cytoskeletal elements. This raises the possibility of entirely novel intracellular pathways of protein trafficking within glial cells. The elucidation of the function of the precursor within these cells will be a challenging prospect for the future.

ACKNOWLEDGEMENTS

I wish to express sincere thanks to David Glover for all his encouragement, patience, and incisive intellectual guidance. Thanks also to Conrad Lichtenstein and David Lane for taking me in as a refugee from the very different world of clinical research. I am very grateful to all my friends on the 6th Floor, especially Will, for all their help. Latterly, I am grateful to Rory Curtis and Graham Wilkin for their help and advice in initiating me to the glial cell field.

In the more distant past, I am grateful to a clinical teacher, Professor George Alberti, for instilling in me the need to achieve a Ph.D. to gain scientific credibility. I thank also Professor David Weatherall for advising me that if I wished to embark on molecular biology I should even at the outset apply it to an area in which I already had an interest. I am sure this has sustained me through the ups and downs.

Very special thanks are due to my mother, and to my late father, for their unfailing support in all my ventures. Finally - the other big thank you, David, for all your love and support, and for having faith in me.

TABLE OF CONTENTS

	Page
Abstract	2
Acknowledgements	4
List of Tables	10
List of Figures	11
List of Abbreviations	13
Chapter 1 - Introduction	14
1.1 Background to opioid peptides	15
1.2 The post-translational processing of neuropeptide precursors	19
1.3 The intracellular pathway of neuropeptide gene products	19
1.4 Enzymes involved in proteolytic processing	21
1.5 Neuropeptide processing in relation to subcellular compartmentalisation	23
1.6 Neuropeptide processing in relation to subcellular "sorting"	25
1.7 Proteolytic processing of the "enkephalin precursor", proenkephalin A	27
Chapter 2 - Materials and Methods	32
2.1 Recombinant DNA techniques	33
2.1.1 Isolation of restriction endonuclease fragments from agarose gels	33
2.1.2 Adding linkers to DNA fragments	34
2.1.3 Dephosphorylation of DNA ends	35

2.2 Production of β-galactosidase fusion proteins 35

2.3 Induction of *cII*-proenkephalin fusion protein 37

2.4 Induction of *gene 10*-preproenkephalin fusion protein 37

2.5 Immunisation of rabbits and mice 38

2.6 Generation of hybridoma lines 38

2.7 Immunoblot analysis 41

2.8 Preparation of *E. coli* lysate coupled to CNBr activated sepharose 42

2.9 Preparation of chromaffin granule lysates 43

2.10 Eukaryotic cell transfection 45

2.11 Preparation and immunostaining of cerebellar cryostat sections 46

2.12 *In situ* hybridisation combined with anti-GFAP immunofluorescence 48

2.13 Preparation and immunostaining of dissociated cerebellar glial cultures 50

Chapter 3 - Generation of monoclonal antibodies to human proenkephalin A-β-galactosidase fusion proteins 53

3.1 Introduction 54

3.2 Generation of monoclonal antibodies to a central segment of proenkephalin 54

3.2.1 Production of β-galactosidase-preproenkephalin A-(69-207) fusion protein

for immunisation 54
3.2.2 Production of *cII*-preproenkephalin A-(69-207) fusion protein for screening of sera and monoclonal supernatants 63
3.2.3 Generation of two monoclonal antibodies, PE-1 and PE-2 64
3.2.4 Characterisation and preliminary epitope mapping of PE-1 and PE-2 67
3.2.5 Demonstration that PE-1 and PE-2 recognise naturally occurring high molecular weight proenkephalin-derived peptides 72
3.2.6 PE-1 and PE-2 immunoreactive peptides appear to be partially membrane associated 75
3.2.7 Discussion 76
3.3 Generation of monoclonal antibodies to the full-length human preproenkephalin sequence 81
3.3.1 Epitope mapping of PE-13 to PE-25 84
3.4 Determination of cross-species reactivity of the proenkephalin antibodies 86

Chapter 4 - The generation of stable transformant lines of PC12 cells which express high levels of unprocessed human proenkephalin 91
4.1 Introduction 92
4.2 Generation of stable proenkephalin transformants of PC12 cells 94
4.3 Stable PC12::hPE transformants express high levels of the uncleaved human enkephalin precursor 95

4.4 Human proenkephalin is apparently targeted to secretory granules within the transformed cell line 99

4.5 Differentiation promoters and reserpine fail to stimulate proteolytic processing of proenkephalin within PC12::hPE transformants 102

4.6 Discussion 104

Chapter 5 - A proenkephalin Mab which cross-reacts with chromogranin A 108

5.1 Introduction 109

5.2 PE-15 cross-reacts with chromogranin A 112

5.3 PE-15 recognises recombinant chromogranin A 119

5.4 Discussion 120

Chapter 6 - Proenkephalin in cerebellar neurons and astrocytes suggests biological significance of the intact precursor 123

6.1 Introduction 124

6.2 PE Mabs used in this study 126

6.3 Immunofluorescent detection of proenkephalin within subpopulations of cerebellar neurons and astrocytes in cryostat sections 133

6.4 Confirmation of proenkephalin gene expression by *in situ* hybridisation 140

6.5 Proenkephalin expression by cerebellar astrocytes in culture 144

6.6 Discussion 154

6.6.1 Enkephalin precursor forms within cerebellar neurons 154
6.6.2 Proenkephalin in a subpopulation of astrocytes 155
6.6.3 Implications 157

Chapter 7 - Discussion 161
7.1 Fusion proteins 162
7.2 Application of the Mabs in the investigation of the cell type specific processing of proenkephalin 166
7.2.1 The investigation of intracellular trafficking by transfection of proenkephalin cDNA into neuroendocrine cell lines 169
7.2.2 Immunological approaches to the investigation of protein trafficking 174
7.2.3 Astrocytes: a novel type of neurosecretory cell? 178
7.3 Concluding remarks 183

References 185

LIST OF TABLES **Page**

Table 3.1 Cross-species reactivity of the PE Mabs 88

LIST OF FIGURES **Page**

Figure 3.1 Schematic representation of the binding domains of the monoclonal antibodies PE-1 and PE-2 in relation to the primary sequence of proenkephalin A 57

Figure 3.2 Fusion protein expression plasmids 59

Figure 3.3 Appearance of β-galactosidase-preproenkephalin A-(69-207) fusion protein on a Coomassie stained SDS-polyacrylamide gel 62

Figure 3.4 *cII*-preproenkephalin A-(69-207) fusion protein 66

Figure 3.5 Preliminary epitope mapping of PE-1 and PE-2 by immunblot analysis of a series of β-galactosidase-preproenkephalin A fusion proteins 71

Figure 3.6 Immunoblot analysis of bovine adreno-medullary chromaffin granule lysates 74

Figure 3.7 Comparison of human, rat and bovine preproenkephalin amino acid sequences. 90

Figure 4.1 Immunoblot analysis of acidic lysates of transfected and non-transfected PC12 cells treated with differentiation promoters 97

Figure 4.2 Indirect immunofluorescence of PC12::hPE transformants 101

Figure 4.3 Immunoblot analysis of transfected and non-transfected PC12 cells after 106

treatment with reserpine

Figure 5.1 PE-15 cross-reacts with chromogranin A in bovine chromaffin granule lysates (A) and when expressed in *E. coli* (B) 114

Figure 5.2 Map of human and bovine chromogranin sequences which were fused to β-galactosidase 118

Figure 6.1 β-galactosidase-human preproenkephalin fusion proteins used for immunisation and epitope mapping 128

Figure 6.2 (A) Differential recognition of intact enkephalin precursor
(B) Cross-species reactivity of the PE Mabs 131

Figure 6.3 Proenkephalin-like immunofluorescence in cerebellar neuronal and astroglial cells 135

Figure 6.4 Proenkephalin expression in subpopulations of grey and white matter astrocytes 138

Figure 6.5 Confirmation of proenkephalin synthesis by *in situ* hybridisation 142

Figure 6.6 Proenkephalin expression by process-bearing astrocytes in primary cerebellar glial cultures 147

Figure 6.7 Co-staining of astrocytes in primary culture with anti-proenkephalin monoclonal antibodies and anti-GFAP 149

Figure 6.8 Proenkephalin does not appear to be associated with microtubules 153

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CNBr	Cyanogen bromide
CRGA	Chromogranin A
DMEM	Dulbecco's Modification of Eagles medium
DMSO	Dimethyl sulphoxide
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic-acid
EM	Electron microscopic
FCS	Fetal calf serum
GFAP	Glial fibrillary acidic protein
IGSS	Immunogold silver staining
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase
Kda	Kilodalton
Mab	Monoclonal antibody
PBS	Phosphate buffered saline
PMSF	Phenyl-methyl-sulphonyl-fluoride
PPE	Preproenkephalin
RNase	Ribonuclease
RSV LTR	Rous sarcoma virus long terminal repeat
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T-E	Tris 10mM-EDTA 1mM, pH 8

CHAPTER 1

INTRODUCTION

1.1 Background to opioid peptides

Opium poppy seeds were used medicinally for many hundreds of years for their analgesic, calming and euphoric effects. Pharmacological derivatives of opium, such as the alkaloid morphine, were found to have similar analgesic and psychotropic properties. Opiate drugs were also shown to produce a marked inhibition of smooth muscle activity which provided the basis for the classical opiate bioassays - the mouse vas deferens and guinea pig ileum assays. In these systems, the ability of a compound to inhibit electrically evoked contractions, which can in turn be reversed by the opiate antagonist naloxone, is tested.

In the early 1970's, characterisation of opiate binding sites in the central nervous system (Pert and Snyder,1973), led to an intensive search for the endogenous ligand(s). This culminated in 1975 with the isolation of two pentapeptides from brain with the following structures: Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu which were named Met- and Leu-enkephalin respectively. Both peptides were found to have potent opiate agonist activity in the guinea pig ileum and mouse vas deferens assays, and their inhibitory effect could be reversed by naloxone. They therefore satisfied the necessary criteria to be classed as endogenous opioid peptides.

It was subsequently shown that endogenous opioid peptides were derived from much larger precursor

molecules. In 1979, the primary structure of the precursor to the pituitary hormones adrenocorticotrophin (ACTH) and β-lipotrophin (β-LPH) - proopiomelanocortin (POMC) - was elucidated by cloning and sequencing a cDNA copy of its mRNA isolated from bovine pituitary intermediate lobe (Nakanishi *et al.*, 1979). The POMC precursor contains one Met-enkephalin motif within the β-LPH sequence which is liberated not as free enkephalin but as an extended opioid peptide, β-endorphin, instead. In 1982, the primary structure of the precursor which gives rise to free Met- and Leu-enkephalin, proenkephalin A, was deduced from the nucleotide sequence of its cDNA. The cDNA was generated from mRNA isolated from human phaeochromocytoma tissue, a tumour of the adrenal medulla which is known to be rich in enkephalin peptides (Comb *et al.*, 1982). Since proenkephalin A gives rise to free enkephalins, it is often referred to as the "enkephalin precursor". Later in 1982, DNA complementary to porcine hypothalamic mRNA encoding the precursor to the Leu-enkephalin-containing peptides, neo-endorphin and dynorphin, was cloned and sequenced. This third opioid precursor was initially termed proenkephalin B, but is now more often called prodynorphin (Kakidani *et al.*, 1982).

The study of opioid gene expression has largely centred on the preproenkephalin A gene. It is now well established that proenkephalin gene transcription can be regulated by transynaptic events involving signal transduction at cell surface receptors resulting in

activation of both protein kinase A (Eiden *et al.*, 1984) and protein kinase C (Kley, 1988). Indeed, molecular analysis of the proenkephalin gene regulatory elements has revealed a cyclic AMP and phorbol ester-inducible enhancer upstream from the gene. The enhancer sequence contains two functionally distinct elements which interact with 4 different transcription factors - the previously identified AP-1, AP-2 and AP-4, together with a novel factor, ENKTF-1 (Comb *et al.*, 1988). Cyclic AMP response elements have also been identified upstream of the somatostatin and vasoactive intestinal peptide (VIP) genes (for review see Comb *et al.*, 1987). Interestingly, such an element has also been identified upstream from the tyrosine hydroxylase gene. This may lead to co-ordinate regulation of the biosynthesis of catecholamines and enkephalins which are known to be co-stored within chromaffin granules of the adrenal medulla and which are co-released in pathophysiological stress states.

There is a considerable degree of controversy within the opioid receptor field as to the number of opioid receptor subtypes. The hope is that cloning of the genes will clarify the picture, but so far the field has been beset with conflicting and inconclusive data with no clear evidence so far. It is however fairly widely accepted that there are three major receptor classes - mu, delta and kappa (for review see Civelli *et al.*, 1987). The mu and kappa receptors were so named because of their preferential binding of morphine and ketocyclazocine,

another opiate drug, respectively. The delta receptor was named after the mouse vas deferens bioassay in which enkephalin peptides were found to be far more potent than morphine. The delta receptor is therefore often loosely referred to as the enkephalin receptor, the kappa receptor as the dynorphin receptor, to which both dynorphins and neoendorphins bind preferentially, and the mu receptor as the morphine receptor. Their separation into 3 main types has been reinforced by the use of synthetic analogues that are largely specific for one receptor type. For example, prototypic enkephalin analogues for the delta receptor are DADLE (Tyr-DAla-Gly-Phe-DLeu-OH) and DPDPE (Tyr-DPen-Gly-Gly-Phe-DPen-OH). It is interesting that the octapeptide metorphamide, which is a COOH-terminally amidated Met-enkephalin-containing peptide (Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly) exhibits its highest affinity at mu sites (Weber *et al.*, 1983), whereas Met and Leu-enkephalin bind most strongly at delta sites (Lord *et al.*, 1977). One theory is that the specific COOH-terminal extension re-routes metorphamide to a different "address", the enkephalin sequence being the identical "message" to be delivered. The mechanism of signal transduction at opiate receptor sites in all cases seems to involve coupling to inhibitory G protein, G_i, which explains their negative coupling to adenylate cyclase (Koski and Klee, 1981).

1.2 The post-translational processing of neuropeptide precursors

The primary translation product of a neuropeptide gene is very often a large precursor molecule containing within its primary sequence single or multiple copies of potentially bioactive peptide motifs. Liberation of the free, mature neuropeptides involves proteolysis at specific cleavage sites, usually pairs of basic residues, which flank the neuropeptide domains (Docherty and Steiner, 1982). The initial hydrolysis at peptide bonds within the polypeptide chain is performed by endoproteases. Subsequent "trimming" is carried out by exopeptidases which remove residual basic amino acids from the amino or carboxyl termini. In the case of complex neuropeptide precursors such as the opioid prohormones, where there are many potential cleavage sites, processing events proceed in a cascade of sequential cleavages at selected sites. Such ordered rather than random proteolysis, as would occur in non-specific degradation, may be at least partly due to the conformation of precursor molecule which exposes cleavage sites in a progressive manner as proteolysis proceeds and tertiary folding changes.

1.3 The intracellular pathway of neuropeptide gene products

All gene products destined for the secretory pathway possess a "signal" or "leader" sequence involved in

translocation across the membrane of the rough endoplasmic reticulum (RER). This "pre" sequence, usually located at the extreme NH_2 terminus of the pre-pro-protein, is composed of 15-30 largely hydrophobic amino acids. The "pre" sequence is cleaved off prior to completion of protein synthesis, that is to say it is cleaved co-translationally, liberating the pro-protein within the lumen of the RER (Blobel and Dobberstein, 1975). Cleavage of the leader sequence is carried out by a metallo-endopeptidase, a so-called "signal peptidase", associated with the inner membrane of the RER cisternae (Jackson and Blobel, 1977). Within the ER, disulphide bonds are formed and primary NH_2-linked glycosylations occur. The prohormone is then transported by small vesicles to the *cis* aspect of the Golgi apparatus where further modifications occur such as the generation of complex carbohydrate chains. Additional modifications, such as phosphorylation and tyrosine sulphation also occur in the Golgi stacks (Eipper and Mains, 1982; Huttner, 1988). There is now good evidence that endoproteolytic cleavage can sometimes be initiated within the Golgi apparatus, although the majority of proteolytic cleavage events occur within the secretory vesicles which bud off from the *trans*-Golgi and transport the protein to the cell surface. The relationship of proteolytic cleavage to subcellular compartmentalisation will be discussed in more detail later.

1.4 Enzymes involved in proteolytic processing

Endoproteases usually act at paired basic residues (Docherty and Steiner, 1982), most commonly Lys-Arg, although Arg-Lys, Arg-Arg, and Lys-Lys are also found. In addition, tri- and tetra-basic sites can be used, as for example in the pro-egg laying hormone (pro-ELH) of Aplysia (Newcomb and Scheller, 1987), and proopiomelanocortin. Single basic residues occur commonly in polypeptide precursors but cleavage at these sites is infrequent and strictly selective. Cleavage at such sites can occur in the processing of both proenkephalin to liberate amidorphin (see Figure 3.1) and metorphamide, and also of prodynorphin (Lynch and Snyder, 1986). In addition to the "core" site of basic residues, conformation of the molecule within the vicinity of the cleavage site is also thought to play a role in substrate recognition by endoproteases. In 1980, Geisow and Smyth proposed that basic residues in an α-helical structure are resistant to proteolytic attack. As a corollary of this, Rholam and co-workers (1986) suggested that cleavage may require the basic residues to be situated in or immediately adjacent to a β-turn. Evidence for this hypothesis was provided in a recent study in which site-directed mutagenesis was used to change amino acids in the immediate vicinity of prosomatostatin cleavage sites. (Gomez *et al.*, 1989). The substitutions were chosen to disrupt β-turn structures in those regions. In some cases, prosomatostatin processing was drastically reduced. Thus, higher orders of protein

structure may be involved in substrate recognition by endoproteases.

Endoproteases have been difficult to isolate, and definitive evidence that the purified enzymatic activity is involved in prohormone conversion has been elusive. However, broadly speaking, candidate endoproteases (prohormone converting enzymes) can be classified as serine or metalloproteases, which have optimal activity at neutral or basic pH; or, cysteinyl (thiol) or aspartyl proteases which generally have acidic pH optima (for review see Loh, Brownstein, and Gainer, 1984).

Evidence so far suggests that prohormone converting enzymes exhibit fairly broad substrate recognition. For example, enzymic activity purified from bovine neural lobe secretory vesicles is able to cleave *in vitro* not only provasopressin and prooxytocin - the "indigenous" precursors - but also proopiomelanocortin and proinsulin (Parish *et al.*, 1986). However, it is possible that a number of enzyme molecules with similar characteristics have been co-purified. It is tempting to speculate that there may be gene families encoding similar enzymes, but with different substrate specificities. Indeed, the glandular kallikrein gene family, products of which are involved in growth factor maturation, appears to encode such an enzyme family (Mason *et al.*, 1983). Cloning of several of these genes revealed homologous regions responsible for serine protease activity. In addition, there were non-homologous regions which included those

that had been previously identified as lining the substrate-binding pocket of the enzyme. Such differences imply specific substrate binding. It is possible that prohormone converting enzymes may turn out to belong to analogous gene families.

Endoproteases usually cleave either to the COOH-terminus of, or between, pairs of basic residues. Residual basic amino acids are removed by the action of exopeptidases. COOH-terminal residues are removed by carboxypeptidases. One such enzyme, carboxypeptidase E (enkephalin convertase) is involved in the maturation of enkephalin peptides. Sequencing of cDNA encoding this enzyme reveals a strong homolgy with other carboxypeptidase enzymes (Fricker *et al.*, 1986). Carboxypeptidase action in some cases reveals a COOH-terminal glycine residue which can be removed by peptidyl-glycine-α-amidating monooxygenase (PAM) which exposes an amide group from the peptide bond (Eipper et al., 1983). Such amidation of carboxyl termini of peptides is often necessary for biological activity. NH_2-terminal basic residues remaining after endoproteolytic cleavage are removed by aminopeptidases (Gluschankof *et al.*, 1987).

1.5 Neuropeptide processing in relation to subcellular compartmentalisation

The pH within mature secretory vesicles is acidic, whereas that within the Golgi apparatus is neutral to basic. Thus, the differing pH optima of candidate

prohormone converting enzymes may lead to activation in different subcellular compartments. This is supported by analysis of purified enzymic activity from different subcellular compartments. For example, prohormone-converting enzyme activity with an acidic pH optimum has been purified from mature secretory vesicles of bovine neural lobe (Parish *et al.*, 1986). In contrast, putative opioid precursor converting enzyme activity has been identified in association with Golgi membranes from rat brain (Demmer and Brand, 1986). In this case the activity is optimal at around pH 9 and is inhibited by serine protease inhibitors. Thus, the enzyme characteristics are in accordance with the expected local environment *in vivo*.

An immunoEM analysis demonstrated that proteolytic liberation of insulin from proinsulin is commenced within clathrin-coated immature secretory vesicles and continued to completion within mature, non-coated vesicles (Orci *et al.*, 1987a). A more recent study described two distinct proinsulin cleavage enzyme actvities, with different pH optima, suggesting that one would be active in the Golgi with the other active within secretory vesicles (Davidson *et al.*, 1988). This is consistent with the previous morphological data, since a preliminary cleavage within the Golgi apparatus will not liberate free insulin. Fisher and co-workers (1988) provided immunoEM evidence for the initiation of proteolytic cleavage of the Aplysia egg laying hormone precursor within the *trans*-Golgi. Thus, one might expect that, particularly in the case of complex

neuropeptide precursors, primary cleavage events are commenced within the Golgi apparatus, with completion of proteolysis within secretory vesicles.

1.6 Neuropeptide processing in relation to subcellular "sorting"

In neurosecretory cells which express more than one prohormone, different gene products may or may not be separately packaged. In somatomammotrophic cells of the anterior pituitary, prolactin and growth hormone are generally sorted into different vesicles (Fumagilli and Zanini, 1985). However, a subclass of vesicle within these cells contains both prohormones. Interestingly, the products are segregated into different domains within these dense cored vesicles. This may arise either from fusion of vesicles after sorting from the Golgi has occurred, or from "self-aggregation" of the different prohormones into separate foci of coalesced material within the Golgi.

Another consideration relevant to sorting in all neurosecretory cells is, how targeting of the prohormone to the "regulated" pathway is achieved. There are essentially two main routes of exit for secretory products from the cell. Products within "regulated" vesicles are discharged after receipt of an appropriate stimulus, whereas the "constitutive" compartment is unresponsive to external influence, and changes in the rate of secretion are effected solely by change in the rate of protein

synthesis (for review see Kelly, 1985). Evidence suggests that trafficking via the regulated pathway requires active sorting whereas the constitutive pathway is effectively a "default" route. Prohormones and primary cleavage products are often seen as coalesced material within the Trans-Golgi network (Pfeffer and Rothman, 1987), which may be triggered by a falling pH as the protein moves distally (Orci *et al.*, 1987a). Such "self-aggregation" is likely to be critical to the formation of the core within the dense core type of regulated vesicle. Whether a more "active" mode of sorting is require as well, for example by binding of a sorting domain on the prohormone or cleavage product to a "receptor" molecule on Golgi membranes, has been the subject of vigorous debate. Recent evidence from Chung and co-workers (1989) indicates that active intermolecular interaction may indeed be involved. They have identified prohormone binding proteins, "sortases", in association with Golgi membranes. It is possible that these proteins may be "carrier" molecules involved in aggregation of the prohormone or that they may be membrane- bound receptor molecules. Protein sorting is critical to prohormone processing, since targeting to the regulated pathway is required to achieve exposure to the full complement of processing enzyme activity. Indeed, prohormone molecules which "default" to the constitutive route, exit from the cell in an uncleaved state (Moore *et al.*, 1983). Furthermore, different primary cleavage products of the same precursor can undergo active sorting. After

preliminary cleavage of the egg laying hormone precursor within the *trans*-Golgi, different high molecular weight products are sorted into distinct subpopulations of secretory vesicles of differing morphological appearance (Fisher *et al.*, 1988). This could be a mechanism for generating even greater proteolytic diversity within a single cell type.

1.7 Proteolytic processing of the "enkephalin precursor", proenkephalin A

The primary structure of preproenkephalin A, deduced from the nucleotide sequence of its cloned cDNAs, shows a high degree of sequence homology in different mammalian species between opioid and non-opioid regions of the molecule (Comb *et al.*, 1982; Legon *et al.*, 1982; Noda *et al.*, 1982; Yoshikawa *et al.*, 1984). The operation of such evolutionary constraints upon the overall structure of the precursor suggests possible biological significance of the uncleaved molecule. A single proenkephalin molecule contains several enkephalin motifs each flanked by pairs of basic amino acid residues which represent potential sites of proteolytic cleavage, as discussed earlier. Thus, "complete" proteolytic processing of a single precursor molecule at all dibasic cleavage sites will generate four copies of Met-enkephalin, together with single copies of Leu-enkephalin and Met-enkephalin sequences with short C-terminal extensions, namely, Met-enkephalin-Arg^6-Gly^7-Leu^8 and Met-enkephalin-Arg^6-Phe^7 (see Figure 3.1). However,

incomplete processing at selected cleavage sites has the potential to generate an array of extended opioid and non-opioid peptides, an increasing number of which are being identified (for review see Eiden, 1987; also Spruce et al., 1988). There is evidence that some of the extended enkephalin-containing polypeptides may be even more biologically active than the originally described pentapeptides. For example, peptide E, Bam-22P, and Bam-20P (Figure 3.1) have been shown to be many more fold active in the guinea pig ileum opioid bioassay than Met- or Leu-enkephalin (Kilpatrick *et al.*, 1981; Mizuno *et al.*, 1980). In addition, a non-opioid, amidated proenkephalin-derived peptide, amidorphin(8-26), has been isolated from brain (Liebisch *et al.*, 1986). Its amidated state is an indication of biological activity. Thus, proenkephalin may be giving rise to bioactive non-opioid as well as opioid peptide products. The biological significance of higher molecular weight cleavage products, such as synenkephalin (Liston *et al.*, 1984a) and the peptides of predicted molecular mass 8.6kDa, 12.6kDa (Jones *et al.*, 1982), 18.2kDa, and 23.3kDa (Patey *et al.*, 1984) is even more unclear. It is possible that the role of such "precursor forms" is quite distinct from that of the enkephalin peptides themselves. Indeed, evidence presented in Chapter 6 of this thesis strongly suggests this possibility.

Proenkephalin-derived peptides have been reported in many tissues including brain, adrenal medulla, spinal cord, gastrointestinal tract, lung, pancreas, in

sympathetic nerve fibres and ganglia, and in parasympathetic preganglionic neurons. Remarkably, preproenkephalin mRNA has been detected in mammalian testis and ovary at levels comparable to that in brain (Kilpatrick *et al.*, 1985), and in rat heart at even higher levels (Howells *et al.*, 1986). Thus, the range of possible functions for proenkephalin-derived peptides is likely to encompass far more than the classical neural roles of neuromodulation and neurotransmission ascribed to the enkephalin pentapeptides. Of particular interest, there is now evidence pointing to a developmental role for proenkephalin gene products. In 1975 Zagon and co-workers described small enkephalin peptides only in germinative cells of the developing cerebellum and not in the adult cerebellum when opioid binding activity has also been down-regulated. Furthermore, the proenkephalin gene may also be important in the development of non-neuronal tissues. Interestingly, preproenkephalin mRNA is expressed at high levels in mesodermal lineages during mouse development and is down-regulated upon terminal differentiation (Keshet *et al.*, 1989). In the developing rat heart, preproenkephalin mRNA levels show a biphasic pattern. There is a non-sustained elevation in early development, and a second later rise which peaks in the terminally differentiated adult heart muscle cell (Springhorn and Claycomb, 1989). Thus, products of the proenkephalin gene may have a fundamental biological purpose, since they are expressed in many different cell

types.

There is accumulating evidence of specific patterns of proteolysis in different proenkephalin-expressing cell types. In brain, processing is traditionally thought to be more complete than in the adrenal medulla, where incomplete processing leads to an accumulation of high molecular weight cleavage products (for review see Udenfriend and Kilpatrick, 1983). More recently, regional differences in the processing of proenkephalin within brain have been reported (White *et al.*, 1986; Birch and Christie, 1986), which may indicate that high molecular weight proenkephalin-derived peptides, previously regarded merely as processing intermediates, may be acting as neuropeptides in their own right. Indeed, my own work on the cerebellum, described in Chapter 6 of this thesis suggests that the intact enkephalin precursor may have a biological role which is quite unrelated to that of its mature products, the enkephalin peptides themselves.

The biological significance of the intact precursor and these high molecular weight cleavage products has remained elusive largely owing to a lack of antibodies which would enable their reliable identification. Antibodies to the small enkephalin peptides exhibit minimal or no cross-reactivity with the full-length precursor, presumably due to the conformation of the intact molecule before proteolytic cleavage. Immunohistochemical studies in particular have been restricted therefore to a search for small enkephalin

peptides, and assays for the high molecular weight forms have in general required chromatographic separation followed by trypsinisation to liberate the smaller peptides. The essential aim of this project was to generate a novel series of monoclonal antibodies (Mabs) which would reliably identify the mammalian enkephalin precursor, in which I was successful. I am now using these antibodies to study the cellular and molecular basis for the cell type specific processing of proenkephalin. Furthermore, the antibodies will provide an unprecedented means of investigating a functional role for proenkephalin. The possibility of bioactivity of an uncleaved neuropeptide precursor is a novel concept, but which now has credibility in the light of my data so far.

CHAPTER 2

MATERIALS AND METHODS

2.1 Recombinant DNA techniques

All basic procedures, including restriction endonuclease digestion, ligation of DNA ends, plasmid DNA preparation, transformation into *E. coli* and colony hybridisation were performed as described in Maniatis *et al.*, 1982. Radiolabelling of DNA for colony hybridisation was performed either by nick translation (Rigby *et al.*, 1977) or oligolabelling (Feinberg and Vogelstein, 1984).

2.1.1 Isolation of restriction endonuclease fragments from agarose gels

This was performed most successfully using the Geneclean^{TM} kit (obtained from Stratagene Scientific Ltd., London). This is based on the principle of DNA binding to ground glass particles. Digestion mixtures were run out on an ethidium stained gel. (Low melting point agarose gave optimal results although this is not strictly necessary). Bands were excised under long wave UV light. 2 to 3 "vols" (100mg gel slice = 100μl) of sodium iodide solution was added, then the tube placed in a water bath at 45 to 55^{o}C for about 5min (contents mixed after 1-2min). After chilling on ice (to prevent too avid binding of DNA), 3-5μl of glassmilk suspension was added, and the mixture incubated on ice for 5min to allow binding of DNA to the silica matrix. The glassmilk was pelleted in a microfuge for 5sec and the supernatant decanted off. The pellet was washed 3 times with 200μl of ice-cold ethanol-containing "NEW" buffer. After the final spin, the remaining drops of

wash buffer were carefully removed, then the pellet resuspended in 10μl of T-E buffer. DNA was eluted from the glassmilk by placing the tube in a 45 to 55^{o}C bath for 3min, then spun in a microfuge for 30sec. This was repeated once to achieve optimal recovery. The fragments were stored at -20^{o}C until required.

2.1.2 **Adding linkers to DNA fragments**

Restriction sites were chosen to generate either flush ends or cohesive ends with 5' overhangs. Protruding 5' ends were filled in as follows: 400ng of purified restriction fragment was concentrated using acetate/ethanol precipitation then taken up in 10μl of TE. To this was added 10μl of ddH_2O, 1μl of 2mM dNTPs (all four), 10x nick translation buffer (Maniatis recipe) and 1μl (3u) of the Klenow fragment of *E coli* DNA polymerase I. The reaction was carried out at 22^{o}C for 30min, then terminated by placing at 70^{o}C for 5min. 2μg of 5' phosphorylated linkers were added to the filled-in fragment, then precipitated with acetate/ethanol to de-salt and concentrate. The mixed DNA pellet was taken up in 5μl of T-E, to which was added 1μl of 500mM Tris pH 7.8 / 100mM $MgCl_2$, 1μl of 500μg of BSA, 1μl of 200mM DTT, 1μl of **100**mM ATP, plus 1 unit of T4 DNA ligase. The blunt end ligation reaction was performed at 22^{o}C overnight. The reaction was terminated by the addition of EDTA to at least 10mM. The enzyme was removed by phenol / chloroform extractions, then the DNA precipitated with acetate /

ethanol. The pellet was resuspended in 90μl of T-E, then subjected to appropriate restriction enzyme digestion to remove excess linkers. An excess of enzyme (about 20 units) was used owing to the large molar excess of linkers over DNA fragment. The final step was to separate the linkered fragment from free, digested linkers. This was achieved by agarose gel electrophoresis followed by Geneclean extraction of the fragment.

2.1.3 **Dephosphorylation of DNA ends**

In some cases, linear vector DNA was dephosphorylated to improve the efficiency of the subsequent ligation reaction. After linearisation of vector DNA at 37°C for 60min, 1μl (20u) of calf intestinal alkaline phosphatase (BCL) was added, and the reaction continued at 37°C for a further 60min. The reaction was terminated by heating to 70°C for 5min.

2.2 Production of β-galactosidase fusion proteins

1) Analytical scale

Recombinant vectors were transformed into the *E. coli* strains BMH 71-18 or TG2. Both strains are *lacZ*$^-$ but carry the mutation *laci*^{Q} on the F′ plasmid. Transformants were cultured overnight at 37°C on a shaking platform, in 2ml L-broth (per litre: 10g Bacto-tryptone, 5g yeast extract, 5g NaCl, 1g glucose) containing 100μg/ml ampicillin and 500μM IPTG (Sigma Chemical Co.). Cells were pelleted in a microfuge, washed in ice-cold PBS, then sonicated for

20sec in 300-500μl of SDS-PAGE sample buffer (20% glycerol, 0.125M Tris pH 6.9, 2% SDS, 5% β-mercaptoethanol) and boiled for 5min. Samples were subjected to SDS-PAGE (Laemmli, 1970) using a 7.5% separating gel at 150V for 4 to 5h. Gels were stained in a solution containing 0.2% Coomassie blue, 10% acetic acid and 25% isopropanol, and destained using 7.5% acetic acid, 25% methanol.

2) Preparative scale

In order to produce sufficient quantities of fusion protein for immunisation, transformants were cultured overnight in 100ml of L-broth containing 100μg/ml ampicillin and 500μM IPTG. Cells were harvested at 6Krpm, 4°C for 10min in a Sorvall GSA rotor. The bacterial pellet was washed with ice-cold PBS, re-centrifuged, then resuspended first in 10ml of dH_2O to which was added an equal volume of 2x concentrated SDS-PAGE sample buffer plus 1mM PMSF and 2μg/ml leupeptin. The sample was subjected to several short bursts of sonication at 4°C then placed in a boiling water bath for 5min. Aliquots of lysate were stored at -20°C.

Prior to SDS-PAGE, thawed samples of bacterial lysate were spun for 2min in a microfuge to pellet any insoluble material. 2 to 3ml of lysate was then loaded across the width of a 6% 3mm thick SDS-polyacrylamide separating gel overlain with a small stacking gel. Electrophoresis was carried out at 160 to 180V for 4 to 5h. The gel was placed in 0.25M KCl overnight at 4°C to visualise the fusion

protein band. This was excised and placed in a large dialysis bag containing 1x SDS-PAGE running buffer (0.1% SDS, 0.025M Tris, 0.192M glycine). The gel band was subjected to horizontal electrophoresis at 150V for about 6h to electroelute the fusion protein. The current was reversed for 2min at the end of the procedure to release any fusion protein clinging to the sides of the dialysis bag. If necessary, the protein solution was concentrated using an Amikon concentrator (aiming for around 0.2mg/ml) and stored in aliquots at -20°C.

2.3 Induction of *cII*-proenkephalin fusion protein

Plasmid DNA was transformed into the lysogenic *E. coli* strain QY-13 which carries the cI^{857} mutation, encoding a temperature sensitive repressor, on the prophage. The bacteria were grown in L-broth containing 100μg/ml ampicillin at 30°C on a shaking platform to an $O.D._{550}$ of approximately 0.6. The temperature of the cultures was then acutely raised to 42°C over a bunsen flame, the flasks were transferred to a 42°C water bath for 15min, and finally to a 42°C incubator for 2h. The cells were harvested and lysed into SDS-PAGE sample buffer as described above.

2.4 Induction of *gene 10*-preproenkephalin fusion protein

Plasmid DNA was transformed into the lysogenic *E. coli* strain BL21 (DE3). In this case, the prophage within the chromosome carries a copy of the T7 RNA polymerase

gene under the control of the inducible *lac*UV5 promoter. Bacteria were grown at 37°C on a shaking platform to an $O.D._{550}$ of 0.6, then IPTG to 500μM was added to induce the T7 RNA polymerase gene and thereby transcription of the fusion gene within the vector. Shaking incubation at 37°C was continued for a further 3h, after which the cells were lysed and harvested as described.

2.5 Immunisation of rabbits and mice

Electroeluted β-galactosidase fusion protein in 1x SDS-PAGE running buffer (0.1% SDS in Tris-Glycine) was emulsified with Freunds adjuvant. Rabbits were injected subcutaneously with 100μg of fusion protein in complete Freunds adjuvant, and then booster injections of 50μg of fusion protein in incomplete Freunds were given at 4 to 6 weekly intervals. Balb/c mice each received intraperitoneal injections of 10 to 20μg of fusion protein in complete, then incomplete, Freunds adjuvant at intervals of 4 to 6 weeks, or in some cases even longer. Intraperitoneal pre-fusion boosts, in the absence of Freunds, were carried out on 3 consecutive days - 6, 5 and 4 days prior to the fusion.

2.6 Generation of hybridoma lines

This was essentially as described by Mole and Lane (1987). Myeloma cells (either NS-1 or SP-2) were passaged so that on the day of fusion they were in mid-log growth phase (approximately 10^3-10^4 cells per 10cm dish). The

mouse was killed by cervical dislocation and its fur soaked in 70% ethanol. Using sterile instruments the spleen was dissected in the hood and transferred to a 10cm dish containing 4ml DMEM plus 10% FCS. The spleen was teased apart very thoroughly using a pair of 19 gauge needles mounted on 1ml syringes. 15ml of DMEM without serum was then added to the dish, and the cells transferred to a 50ml Falcon tube. Particulate matter was allowed to settle for 5min, then the upper cell suspension was carefully transferred to a fresh tube. The cells were washed once in serum-free DMEM and resuspended in 5ml of the same. Meanwhile, two 10cm dishes (about 10^7 cells) of subconfluent myeloma cells were prepared for fusion by washing twice in DMEM without serum, then resuspended in 5ml of DMEM.

The two cell types were mixed in a 10ml conical-bottom sterilin tube, then spun down together. The cell pellet was drained thoroughly using an aspirator, then 1ml of prewarmed polyethylene glycol solution (Boehringer Corporation Ltd.), diluted to 33% in DMEM, was added. The cell pellet was resuspended using a pipette (vigorous flicking to resuspend in a smaller vol. of 300μl may be better), then centrifuged at 1200g for 5min. The excess polyethylene glycol was gently aspirated off, then the pellet gently resuspended in 5ml of DMEM using a large bore pipette. The cells were then transferred to 45ml of DMEM containing 20% FCS plus HAZA (hypoxanthine and azaserine) selection, and plated out over 5 to 10 96-well

microtitre plates. (100x HAZA stock was made by dissolving 136mg of hypoxanthine and 10mg of azaserine in 100ml of ddH_2O, which was then sterilised by filtration and stored in 1ml aliquots at $-20^{o}C$).

Wells were fed after 7 days with pre-conditioned medium plus HAZA. This was made by preparing a splenocyte suspension from a non-immunised mouse in the way described. The suspension was then diluted to 50 to 100ml in DMEM plus 20% FCS, plated over four 10cm dishes, and placed in the incubator for 3 days. Splenocytes were spun out prior using the medium for feeding. Screening was commenced after around 10 days. HAZA can be discontinued eventually, if desired, but only when cells are well established and have been subcloned. This was carried out by limiting dilution (using preconditioned medium to prevent death of single cells) to remove any contaminating hybridomas, and to select for a genetically stable line.

Hybridomas were frozen down in liquid nitrogen in 1ml (per 10cm dish of subconfluent cells) of 92% FCS / 8% DMSO. Ascites fluid was prepared as follows: each mouse was injected intraperitoneally with 200μl of pristane 10 days prior to injection of hybridoma cells. Cells for injection (1x 10cm plate at subconfluence) were washed with serum-free medium, then taken up in ~200μl of serum-free medium and injected intraperitoneally using a 21-gauge needle.

2.7 Immunoblot analysis

Proteins were electrophoretically transferred to nitrocellulose sheets (Schleicher and Schuell) in an electroblot apparatus containing 1x transfer buffer (0.025M Tris, 0.191M glycine) plus 20% methanol. (Gel and nitrocellulose were sandwiched between pre-moistened 3MM chromatography paper [Whatman] and air bubbles removed by firmly rolling a pipette over the sandwich). Transfer was carried out either at 0.2A overnight at 4°C, or at 0.4-0.6A at room temperature for 2h. Transferred proteins were transiently visualised with Ponceau stain (0.2% in 3% trichloroacetic acid). After rinsing with PBS, blots were incubated overnight at 4°C in blocking buffer (3% BSA in PBS or 10% FCS in PBS plus 0.05% sodium azide or 0.01% thimerosal as preservative). Incubation with the primary antibody (undiluted monoclonal supernatant or ascites fluid diluted 1/500 in 10% FCS in PBS; or, polyclonal antibody usually at 1/100 to 1/200 in the same) was carried out in sealed polythene bags for either 2h at room temperature or overnight at 4°C. The strips were washed 3 times in 1% Nonidet P40 for 10 to 20min each, then rinsed several times in PBS before adding the second antibody, either peroxidase conjugated goat anti-mouse or anti-rabbit IgG(H+L) (Jackson Immunoresearch Ltd) diluted 1/1000 in 10% FCS in PBS. Incubation times and washes were as for the primary antibody. The blots were stained using either a filtered solution of 0.05% 4-chloro-1-naphthol (Aldrich Chemical Co. Ltd.) in PBS, or 0.05mg/ml

diaminobenzidene in PBS, to both of which was added 0.0002 vol of H_2O_2 (30% w/v). Immunogold silver staining (IGSS) was carried out according to the recommended protocol accompanying the **Auroprobe^{TM} BL plus-IntenSE^{TM} II** kit (Janssen Life Sciences Products).

2.8 Preparation of E. coli lysate coupled to cyanogen bromide activated sepharose

In order to remove anti-*E.coli* antibodies from polyclonal antisera prior to immunoblotting, the sera were incubated with bacterial lysate immobilised on activated Sepharose. 100ml cultures of appropriate host strains of bacteria (400 - 500ml total), transformed with plasmid vectors , were grown overnight. Cultures were pooled, the cells harvested at 4^{o}C in a Sorvall GSA rotor and resuspended in approximately 200ml of 0.1M sodium borate pH 8, 1M NaCl plus 200mg lysozyme, incubated at room temperature for 20 min. 200mg DNase 1 was added plus Triton X-100 to 0.2%. The lysate was centrifuged in a Sorvall SS34 rotor at 10Krpm, 4^{o}C for 20 min. The supernatant was coarsely filtered, and the pH adjusted to 8.5 - 9.0 with 0.1M sodium tetraborate. The supernatant was placed on ice. 5g of CNBr activated sepharose 4B (Pharmacia) beads were swollen in 10^{-3}M HCl and washed with approximately 500ml of 10^{-3}M HCl using a sintered glass funnel. The beads were rinsed with 25ml of 0.1M borate buffer pH 8 (5ml per g of dry gel), then the ligand solution was added immediately such that there was 5 -

10mg of protein per ml of gel with a ligand solution : gel volume ratio of 2 : 1. The gel suspension was mixed end over end overnight at 4°C. The remaining active groups were blocked with 1M ethanolamine pH 9, then the excess protein washed away with 4 - 5 cycles of alternate 0.1M sodium borate pH 9 and 0.1M sodium citrate pH 3 washes. The gel was stored in NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris pH 7.4 plus 0.05% sodium azide) at 4°C. Sera for immunoblotting were preabsorbed on the column for either 2h at room temperature or overnight at 4°C.

2.9 Preparation of chromaffin granule lysates

Bovine adrenal glands were obtained fresh from the slaughterhouse and transported on ice. Chromaffin granule isolation was carried out essentially according to the method of Bartlett and Smith (1974). Adrenal medullae were dissected out into ice-cold 0.3M sucrose (5ml per g of tissue), then chopped until the consistency of thick soup in a Kenwood blender. Further homogenisation was carried out in a glass / Teflon homogeniser using a wall-mounted motor. The plunger was pushed down a total of 5 times. The homogenate was spun in a Sorvall GSA rotor at 1900rpm for 20min at 4°C, to remove general cell debris. The supernatant was pipetted off, then spun in a Ti45 rotor at 10,400rpm for 20min at 4°C. This supernatant was discarded (by pipetting) and the pellet resuspended in 0.3M sucrose to the same as the starting volume, avoiding erythrocytes. The resuspended pellet was transferred to a fresh tube

and re-spun in a Ti45 rotor at 10,400rpm for 20min at 4°C. The supernatant was gently pipetted off until the fluffy layer was reached. This was removed by brief inversion (no longer than 2sec). The pellet was resuspended in 0.3M sucrose (2ml per 25ml of starting material), again avoiding erythrocytes. The resuspended pellet was layered over 50ml of 1.8M sucrose, then spun in a Ti45 rotor at 31,500rpm for 60min at 4°C. This final centrifugation step was carried out through 1.8M rather than 1.6M sucrose in order to minimise lysosomal contamination (Evangelista *et al.*, 1982). The supernatant was decanted and the inside of the tube wiped with tissues. 5ml of 1.8M sucrose was added, and the tube shaken slightly, to wash briefly. This was repeated once. The isolated granules were then frozen until required.

Lysates of isolated granules were prepared in one of two ways. 1) Granules were sonicated in 0.1M HCl plus 1mM PMSF, 2ug/ml leupeptin and 0.3mg/ml iodoacetamide. In order to separate membrane from contents, the lysates were centrifuged in a Beckman air-driven ultracentrifuge. The membrane fraction was resuspended in an equivalent volume of 0.1M HCl. Immediately prior to SDS-PAGE the pH of the samples was adjusted to 7 using 1M Tris base. An equal volume of two times concentrated sample buffer was added and the samples boiled for 5min before fractionating on a 12% SDS polyacrylamide gel. 2) Granules were subjected to hypotonic lysis by the addition of approximately 3ml of 5mM Tris succinate pH 5.9 per adrenal gland, with protease

inhibitors as above (TSB). Lysis was facilitated by three cycles of freeze-thaw using a dry ice-ethanol bath, thawing to 4°C. Lysed granules were subjected to centrifugation in a Ti50 rotor at 40Krpm (100,000g), 4°C. The supernatant was removed and frozen. The membrane pellet was washed in TSB, recentrifuged and finally taken up in a volume of TSB equal to the contents fraction. Both fractions were lyophilised then taken up in SDS sample buffer and boiled for 5min prior to SDS-PAGE.

2.10 Eukaryotic cell transfection

To generate stable transformant lines, vector DNA containing human preproenkephalin cDNA was co-transfected with the vector pSV2neo (Southern and Berg, 1982) which encodes the neomycin resistance gene. This allows selection of transformants with the neomycin analogue G418.

On the day prior to transfection, cells were replated at a density of $3x10^6$ per 10cm dish. On the day of transfection, a calcium phosphate precipitate was prepared as follows: into one Eppendorf tube (tube 1) was pipetted 750μl of 2x Hebs buffer (2x Hebs: 274 mM NaCl, 10mM KCl, 11mM glucose, 1.6mM Na_2HPO_4, 42mM HEPES, pH 7.05). To another tube (tube 2) was added 100μg of vector DNA containing human preproenkephalin cDNA (pRSVBam::hPE), 20μg of the plasmid pSV2neo, 94μl of 2M$CaCl_2$, and the volume made up to 750μl with ddH_2O. Using an autopipetter, air was bubbled steadily through tube 1, whilst

simultaneously adding the contents of tube 2, dropwise, which resulted in the formation of a fine precipitate. This was left at room temperature for 40min. Cells for transfection were rinsed twice with 1x Hebs, then the precipitate dripped on and left at room temperature for 20min, with occasional rocking. 10ml of serum-containing medium was then added, and the cells transferred to the incubator for 5 to 7h. The medium was aspirated and the cells washed once with serum-free medium. 1ml of serum-free medium containing 25% glycerol was then added and left on for 1min exactly at room temperature. 10ml of PBS was added, aspirated, and then the cells washed once aagin with PBS. Serum-containing medium was added, and the cells returned to the incubator. After 2 days, cells were split 1:2 into 0.5mg/ml G418, and fed every 2 days for a week. The G418 concentration was reduced to 0.25mg/ml and continued. After approximately 4 weeks, visible colonies were seen which were picked with the aid of cloning rings, and then expanded for analysis.

2.11 Preparation and immunostaining of cerebellar cryostat sections

Rats were anaesthetised with sodium pentobarbitone (0.5 to 0.7ml of Pentobarbital solution per adult) then the thoracic cavity opened. The left ventricle was pierced with a "butterfly" cannula and the right atrium opened

with scissors. Pre-warmed PBS was run into the left ventricle until full exsanguination had occurred, then the infusate changed to 4% paraformaldehyde in PBS, and continued until fixation of tissues had occurred (50-100ml of paraformaldehyed solution required per adult animal). Cerebella, or other brain regions, were dissected and placed in 4% paraformaldehyde in PBS for 2 to 3h at room temperature. The tissue was then transferred to 30% sucrose in PBS and left overnight at 4^{o}C, or longer, until equilibrium had occurred (when the tissue had sunk). The tissue was entombed in Tissue-Tek II O.C.T. compound (Raymond A. Lamb, London) on cork discs, and plunged into isopentane on dry ice. The tissue was then equilibrated to -25^{o}C in a Reichert-Jung cryostat and sections of 5 to 10 micron thickness were cut onto gelatine coated slides.

Prior to immunostaining, aldehyde quenching was achieved by two 5min soaks in sodium borohydride (0.5mg/ml in PBS), followed by a blocking step with 10% fetal calf serum (FCS) in PBS. Primary antibodies were as follows: PE Mabs ascites fluid diluted 1/500 in 10%FCS in PBS; Met and Leu-enkephalin polyclonal antisera (Immunonuclear Corporation) diluted 1/100 as above; polyclonal anti-GFAP antiserum (DAKO Ltd.) diluted 1/500 as above. Secondary antibodies were FITC conjugated F(ab')2 fragment goat anti-mouse IgG (heavy and light chain), with or without Texas Red conjugated F(ab')2 fragment goat anti-rabbit IgG (heavy and light chain), both diluted 1/500 in 10% FCS in PBS. All second antibodies used throughout the study were

from Jackson Immunoresearch. Primary antibody incubations were performed overnight, secondary incubations for 1 to 2h at room temperature. After PBS washes, sections were mounted under cover slips in medium consisting of 2.5% DABCO (Sigma Chemical Corporation) in glycerol : PBS 9:1.

2.12 In situ hybridisation combined with anti-GFAP immunofluorescence

All procedures were carried out under strict RNAse free conditions. Diethylpyrocarbonate (DEPC) to 0.1% was added to solutions prior to autoclaving. All washing chambers etc. were rinsed in 0.1% DEPC in water. Slides were handled with gloves. The vectors pYSEA 1 and pYSEC 1, which were kind gifts from Dr. S. Sabol (Yoshikawa *et al.*, 1986b), were used to generate rat proenkephalin anti-sense and sense RNA probes respectively. Template DNA was linearised with either Sma I (pYSEC) or Sac I (pYSEA), downstream of the insert. The preparation of the riboprobe and the *in situ* hybridisation were essentially as described by Ingham and co-workers (1985). *In vitro* transcription reactions were performed in the presence of ^{35}S labelled uridine triphosphate using SP6 polymerase and SP6 transcription buffer. Limited hydrolysis to 50 to 200 bases was carried out in sodium carbonate buffer then the probe resuspended in 50% formamide, aiming for $5x10^5$ counts per min / μl, and stored at -20^oC until use.

Cryostat sections from paraformaldehyde perfused animals were cut under RNase free conditions onto poly-

L-lysine coated slides. The sections were subjected to limited permeabilisation using 0.2M HCl for 20min at room temperature, but protease treatment had to be omitted to preserve GFAP antigenicity. After re-fixation in 4% paraformaldehyde, an acetylation step was performed (0.5% acetic anhydride in 0.1M triethanolamine pH 8 for 10min at room temperature in a fume hood), to reduce non-specific binding of the probe. Sections were dehydrated through an ethanol series, then air dried. The riboprobes were boiled for 2min then chilled on ice and 4vols of RNase free hybridisation buffer added (50% formamide, 12.5% dextran sulphate, 0.375M NaCl, 12.5mM Tris pH7.5, 12.5mM sodium phosphate buffer pH 6.8, 6.25mM EDTA, 1.25x Denhardt's and 12.5mM DTT). This was mixed well, then spun for 30sec. Eight to 15μl was placed near the edge of each section and the coverslip gently placed over, without sealing. The hybridisation was performed overnight in a humid chamber with 50% formamide in the base, in a 50^{o}C oven.

Washing steps consisted of 4 hour-long soaks in wash buffer (50% formamide, 10mM β-mercaptoethanol, 0.3M NaCl. 0.01M Tris, 0.01M $NaPO_4$, 50mM EDTA, pH6.8) in a 50^{o}C oven, then a short 5min soak in 1x NTE buffer (0.5M NaCl, 10mM Tris pH 7.5, 1mM EDTA) at 37^{o}C. The slides were then incubated in 20μg/ml of RNase A in NTE at 37^{o}C for 30min, then soaked in NTE at 37^{o}C for one hour with 4 changes. The RNase treatment digests any unbound RNA and thereby reduces background. A GFAP immunostain was then performed. To do this, the sections were re-fixed with 4%

paraformaldehyde in PBS for 20min at room temperature, followed by a sodium borohydride quenching step. After completion of immunostaining, as described in the previous section, the slides were finally dehydrated through an ethanol series in 0.3M ammonium acetate, air dried, then dipped in Kodak NTB2 emulsion. Exposure time was 8 days to 5 weeks.

2.13 Preparation and immunostaining of dissociated glial cultures

Cerebella were dissected from postnatal day 8 rat pups. The tissue was chopped using a McElwain tissue chopper (400 micron setting), and repeated after turning through 90^{o}. The chopped tissue was rinsed into 7ml of solution B (Solution B: 12.5mg trypsin, 2mg DNase I in 50ml of solution A; Solution A; 0.3g BSA, 2mg Dnase I in 100ml Earle's balanced salt solution) in a small sterile conical flask. Large lumps were broken up by mild trituration, then the flask placed in a shaking water bath at 37^{o}C for 15min. The contents were transferred to a 15ml Falcon tube and an equal volume of DMEM plus 10% FCS added. This was spun for 5min at 1Krpm. The pellet was resuspended well in 1ml of solution A, then a further 10ml of solution A was added, and mixed. This was allowed to stand for 10min, then the supernatant removed and retained (the cell suspension). This was spun at 1Krpm for 5min, then the pellet resuspended in 10ml DMEM plus 10% FCS, and plated out onto polylysine coated coverslips in 24-well

plates at a density of 1-2 x 10^5 per well. After 5 days, the medium was changed to DMEM containing D-valine only, and dialysed serum minus L-valine, to discourage fibroblast growth.

Immunostaining was performed using one of four methods, depending on the primary antibody being used. PE Mab, antiGFAP and anti-Metenkephalin staining was carried out using the first two methods.

1) Cells were fixed and permeabilised using methanol then acetone (pre-chilled to -20^{o}C, 4min each). As a blocking step, cells were incubated in 10% FCS, 0.2% saponin in PBS for 1h at room temperature. Primary antibody dilutions were identical to those used for cryostat sections. Incubation with primary antibody, diluted in blocking buffer, was carried out overnight at 4^{o}C and followed by several washes in 1% bovine serum albumin, 0.2% saponin in PBS. FITC conjugated goat anti-mouse IgG (heavy and light chain), with or without Texas Red conjugated goat anti-rabbit IgG (heavy and light chain), diluted 1/500 in blocking buffer, were then added for 1 to 2 h at room temperature, and followed by washes as above. After final rinses in PBS, cover slips were mounted over slides in medium as for cryostat sections.

2) Cells were fixed with 4% paraformaldehyde in PBS for 20min at room temperature, followed by aldehyde quench. After PBS rinses, a brief methanol permeabilisation was performed (methanol pre-chilled to -20^{o}C, left on for 2min). This was followed with blocking

and antibody incubations as above.

3) For labelling of GD_3 on the cell surface, live cells were incubated with LB_1 antibody (ascites fluid diluted 1/500 in Earle's balanced salt solution [EBS] plus 5% normal goat serum) at room temperature for 30min. Cells were washed with EBS plus 0.3%BSA, then incubated with class specific second antibody (Texas Red goat anti-mouse IgM (mu chain) 1/500 in EBS plus 0.3% BSA) for 30min at room temperature. These steps were followed by 4% paraformaldehyde fixation then brief (1min) methanol permeabilisation. Subsequent co-staining with PE Mabs was as described, except in the absence of saponin and using an anti-IgG specific second antibody (goat anti-mouse IgG(Fc)).

4) For dual labelling with PE and anti-tubulin antibodies (YL1/2 rat Mab supernatant from Seralabs, diluted 1/5), cells were methanol / acetone fixed, as described. Sequential antibody incubations were carried out thus: PE Mab followed by Texas Red conjugated goat anti-mouse IgG (heavy and light chain); then YL1/2 followed by FITC conjugated mouse anti-rat IgG (heavy and light chain).

CHAPTER 3

GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN PROENKEPHALIN A-β-GALACTOSIDASE FUSION PROTEINS

3.1 Introduction

A conventional approach to the generation of antibodies to proenkephalin would have required purification of the intact precursor. However, this would have been extremely difficult owing to instability of the naturally occurring molecule. I chose therefore to express the gene product within *E. coli* as a chimaeric protein, fused to β-galactosidase. This method is particularly suitable when a molecule is unstable as it could be anticipated that the hybrid molecule would be afforded at least some protection from degradation. Furthermore, because of its covalent conjugation to a highly immunogenic bacterial protein, coupling to a conventional carrier molecule is not required. Fusion proteins had previously been used as a means of synthesising peptide hormones within *E. coli*, such as the β-lactamase-growth hormone fusion construct generated by Seeberg and co-workers (1978). However, the notion of using fusion peptides to generate antibodies to an intact neuropeptide precursor was a novel concept.

3.2 Generation of antibodies to a central segment of proenkephalin

3.2.1 Production of β-galactosidase-preproenkephalin A-(69-207) fusion protein for immunisation

I initially utilised a human proenkephalin cDNA clone, isolated at Imperial College in 1982 (Legon *et al.*), which, although it did not contain the entire coding

sequence, did include a large central segment of the molecule. I cloned a 411-base pair *Pst*I restriction fragment, encoding the amino acid sequence 69 to 207 of the primary gene product [Figure 3.1, PPE-(69-207)], into the pUR series of *E. coli* expression plasmids. These vectors possess a series of unique restriction enzyme sites at the 3' end of the *E. coli* *lac* Z gene, the expression of which is driven by its natural promoter and operator (Figure 3.2; Ruether and Mueller-Hill, 1983). The cloning sites are in different reading frames with respect to the *lac* Z gene in the different vectors. The central segment of preproenkephalin A cDNA was inserted into the *Pst*I site of pUR 291, in translational phase with the COOH-terminus of β-galactosidase. Plasmid DNA was introduced into competent cells of the *E. coli* strain BMH 71-18. Transformants were selected on L-agar plates containing 50μg/ml of ampicillin. The bacterial strain used lacks the endogenous *lac* Z gene but carries the *lac* i^{Q} mutation which confers phenotypic overproduction of the lac repressor. Thus, despite the high copy of the plasmid, expression of the fusion peptide should be suppressed until addition of the synthetic inducer isopropyl thiogalactoside (IPTG).

To identify which colonies contained insert in the correct orientation and translational phase, a random selection of transformants were cultured overnight in L-broth containing 100μg/ml ampicillin and 500μM IPTG. IPTG was added at the time of inoculation of the cultures.

Figure 3.1

Schematic representation of the binding domains of the monoclonal antibodies PE-1 and PE-2 in relation to the primary sequence of preproenkephalin A.

The central boxed region illustrates the intact human enkephalin precursor. The signal sequence is located at the NH_2 terminus and enkephalin sequences are shown as shaded boxes. Each of these is flanked by pairs of basic residues which represent potential sites of proteolytic cleavage. Below the precursor, some of the high to intermediate molecular weight proteolytic cleavage products of proenkephalin A are shown. The upper panel illustrates the preproenkephalin amino acid sequences which were expressed as β-galactosidase fusion proteins for immunisation or epitope mapping.

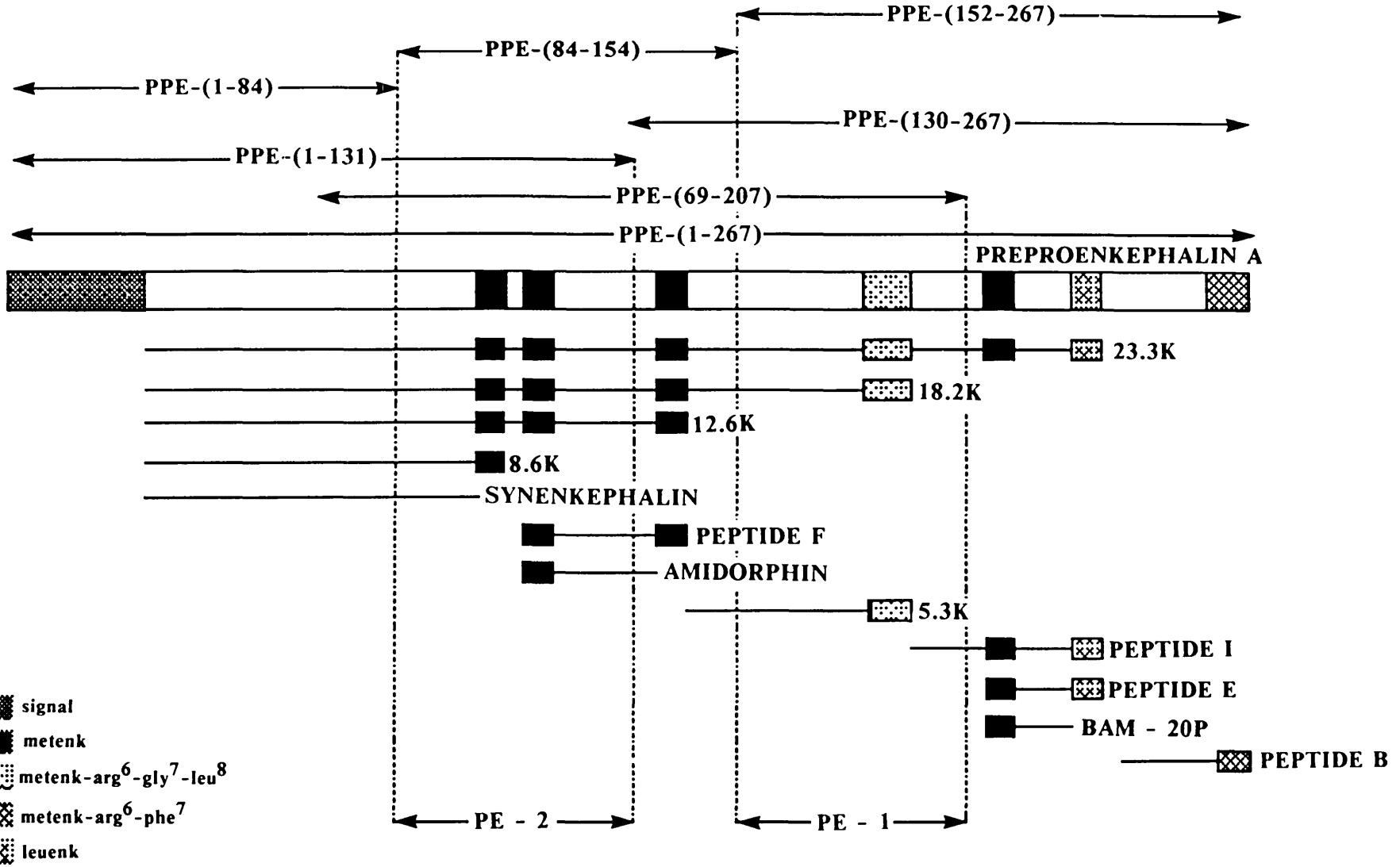
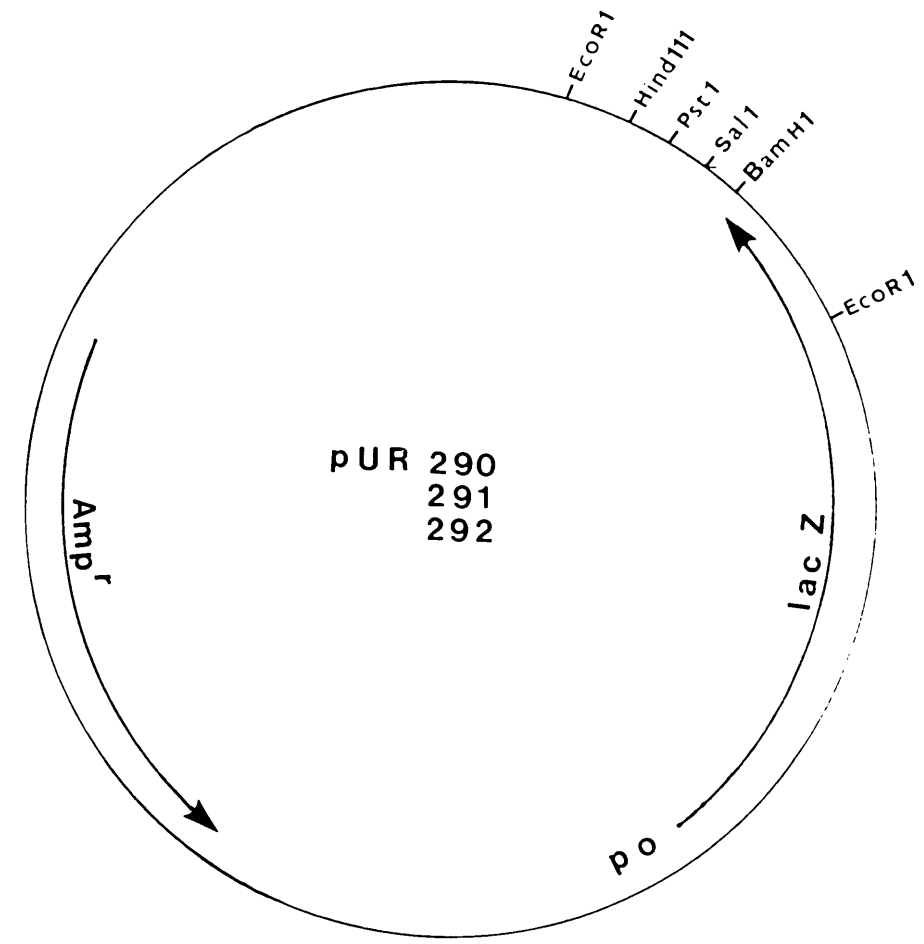

PPE-(152-267)
PPE-(84-154)
PPE-(1-84)
PPE-(130-267)
PPE-(1-131)
PPE-(69-207)
PPE-(1-267)
PREPROENKEPHALIN A
23.3K
18.2K
12.6K
8.6K
SYNENKEPHALIN
PEPTIDE F
AMIDORPHIN
5.3K
PEPTIDE I
PEPTIDE E
BAM - 20P
PEPTIDE B
PE - 2
PE - 1
signal
metenk
metenk-arg6-gly7-leu8
metenk-arg6-phe7
leuenk

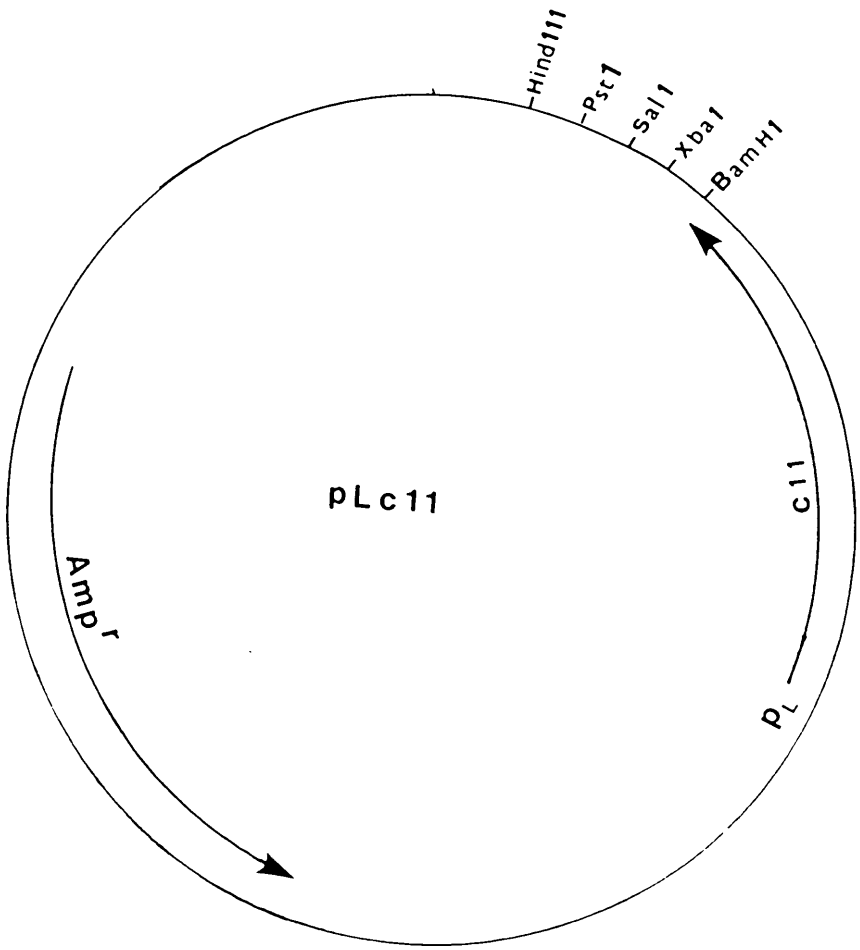
Figure 3.2

Fusion protein expression plasmids

The vector series pUR 290, 291 and 292 was used to generate β-galactosidase-PPE fusion proteins. The polylinker sequence at the 3' end of the *lac*Z gene contains a selection of restriction enzyme sites, which are not repeated elsewhere in the plasmid, in different reading frames in the 3 vectors. This allows placing of any open reading frame in translational phase with the COOH-terminus of β-galactosidase. The *lac*Z gene is driven by its own promoter and operator (p o).

The vector pLcII was used to generate the fusion protein *cII*-PPE-(69-207) which was used to screen polyclonal sera and monoclonal supernatants. The *Bam*HI site in this vector is in the same reading frame as the *Bam*HI site in the vector pUR 291. This allowed subcloning of the fragment PPE-(69-207) between the *Bam*HI and *Hin*dIII sites of pLcII. The *cII*gene is driven by the lambda leftwards promoter, p_L. Details of fusion protein induction are given in the text.


EcoR1
Hind111
Pst1
Sal1
BamH1
EcoR1
pUR 290
291
292
Amp r
lac Z
p o


Hind111
Pst1
Sal1
Xba1
BamH1
pLc11
Amp r
c11
p L

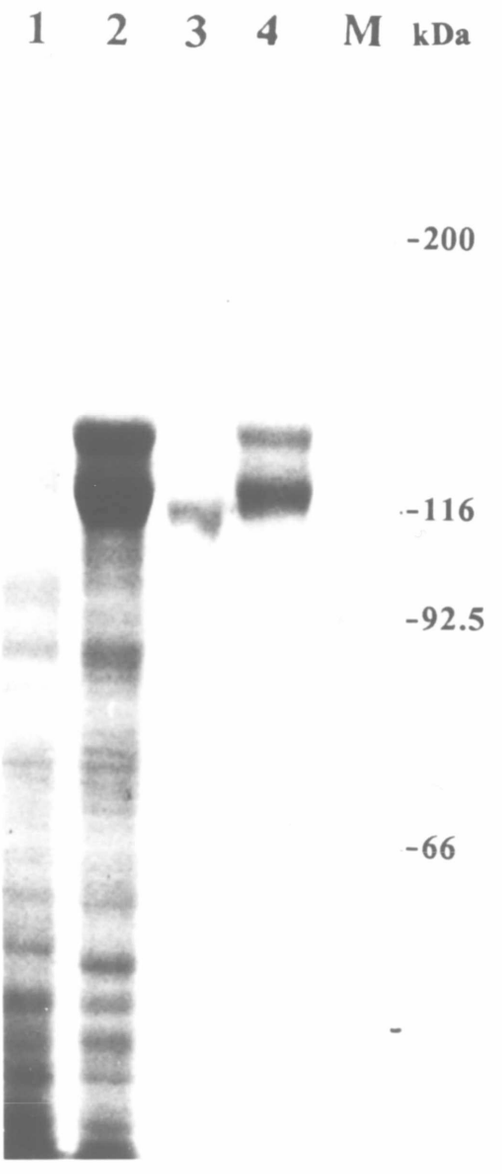
Crude lysates of saturated cultures made by sonicating the cells in sodium dodecyl sulphate (SDS) sample buffer were electrophoresed on 7.5% denaturing polyacrylamide gels. Lysates containing β-galactosidase-proenkephalin A (β-gal-PPE) fusion protein were easily identified by Coomassie staining of the gels, the hybrid polypeptide visible as the dominant, uppermost band on the gel (Figure 3.3, Lane 2). Restriction endonuclease cleavage analysis of plasmid DNA confirmed the presence of the enkephalin insert in the correct orientation (not shown).

In order to generate sufficient fusion protein for immunisation, large-scale bacterial lysates were fractionated on preparative SDS-polyacrylamide gels. The fusion protein was extracted from the gel by horizontal electrophoresis of the excised gel band. A 100ml bacterial culture yielded approximately 10 to 15mg of purified fusion protein (Figure 3.3, Lane 4). The lower band of the doublet represents proteolytically liberated β-galactosidase. The hybrid polypeptide was used directly as an immunogen in rabbits and Balb/c mice. Rabbits received 100μg of purified fusion protein in complete Freunds adjuvant subcutaneously, followed by 100μg booster injections in incomplete Freunds at 4 to 6 weekly intervals. Balb/c mice each received intraperitoneal injections of 10 to 20μg of fusion protein in complete, then incomplete, Freunds at 4 to 6 weekly intervals.

Figure 3.3

Appearance of β-galactosidase-preproenkephalin A-(69-207) fusion protein on a Coomassie stained SDS-polyacrylamide gel.

Lanes 1 and 2 represent total lysates of bacteria transformed with the recombinant plasmid pβ-gal-(PPE-69-207), cultured in the absence (lane 1) or presence of IPTG (lane 2). The fusion protein can be seen as the uppermost band in lane 2. The lower band of the doublet represents proteolytically liberated β-galactosidase. Lane 4 shows the appearance of the fusion protein after electrophoretic purification. Lane 3 shows unfused β-galactosidase for comparison. Positions of molecular mass markers are shown on the extreme right.


1 2 3 4 M kDa
-200
-116
-92.5
-66
-

3.2.2 **Production of *cII*-preproenkephalin A-(69-207) fusion protein for screening of sera and monoclonal supernatants**

In anticipation that the β-gal-PPE fusion protein would elicit a pronounced anti-β-galactosidase response, I generated another gene fusion which would direct the synthesis of an identical enkephalin sequence fused to a different prokaryotic gene product. This could therefore be used to test sera and hybridoma culture medium for anti-proenkephalin activity, and any anti-β-galactosidase antibodies could be disregarded.

The proenkephalin (69-207) sequence was subcloned from the recombinant pUR 291 vector, using *Bam*HI and *Hin*dIIIsites on either side of the original cloning site, into the vector pLcII (Nagai and Thogersen, 1984). This placed the proenkephalin-(69-207) sequence in translational phase with the NH_2-terminal 31 amino acid sequence of the phage lambda *cII* gene product, under the control of the lambda leftwards promoter (P_L). The recombinant plasmid was transformed into the lysogenic *E. coli* strain QY-13, in which the prophage carries the mutation cI^{857} which encodes a temperature sensitive repressor. The repressor is fully active at 30°C so that transcription is prevented, but when the temperature is raised to 42°C, the repressor becomes unstable and transcription of the fusion gene proceeds. Thus, transformants were grown in L-broth containing ampicillin at 30°C on a shaking platform to mid-log growth phase ($O.D._{550}$ approximately 0.6). The temperature of the

cultures was then acutely raised 42°C over a bunsen flame, the flask then transferred to a 42°C water bath for 15min, and finally transferred to a 42°C incubator for 2h, at the end of which the bacteria were harvested and lysed. *cII*-PPE-(69-207) fusion protein could be seen on Coomassie stained SDS polyacrylamide gels as a plasmid-specified 23kDa protein present in lysates of cultures grown at 42°C but not at 30°C (Figure 3.4, Panel a). The fusion protein was also visible when immunoblotted against a rabbit polyclonal antiserum (R139) which had been raised to the β-gal-PPE-(69-207) fusion protein (Figure 3.4, Panel b). It was also recognised by polyclonal antisera to Met-enkephalin and Met-enkephalin-Arg^6-Gly-Leu^8. Sequences encoding these peptides are present in the PPE-(69-207) cDNA segment in three and one copies respectively. Prior to immunostaining, the rabbit polyclonal antisera were cleared of anti-*E. coli* antibodies produced in response to their natural gut flora, by adsorption to a crude *E. coli* lysate coupled to a cyanogen bromide-activated Sepharose column.

3.2.3 **Generation of two monoclonal antibodies, PE-1 and PE-2**

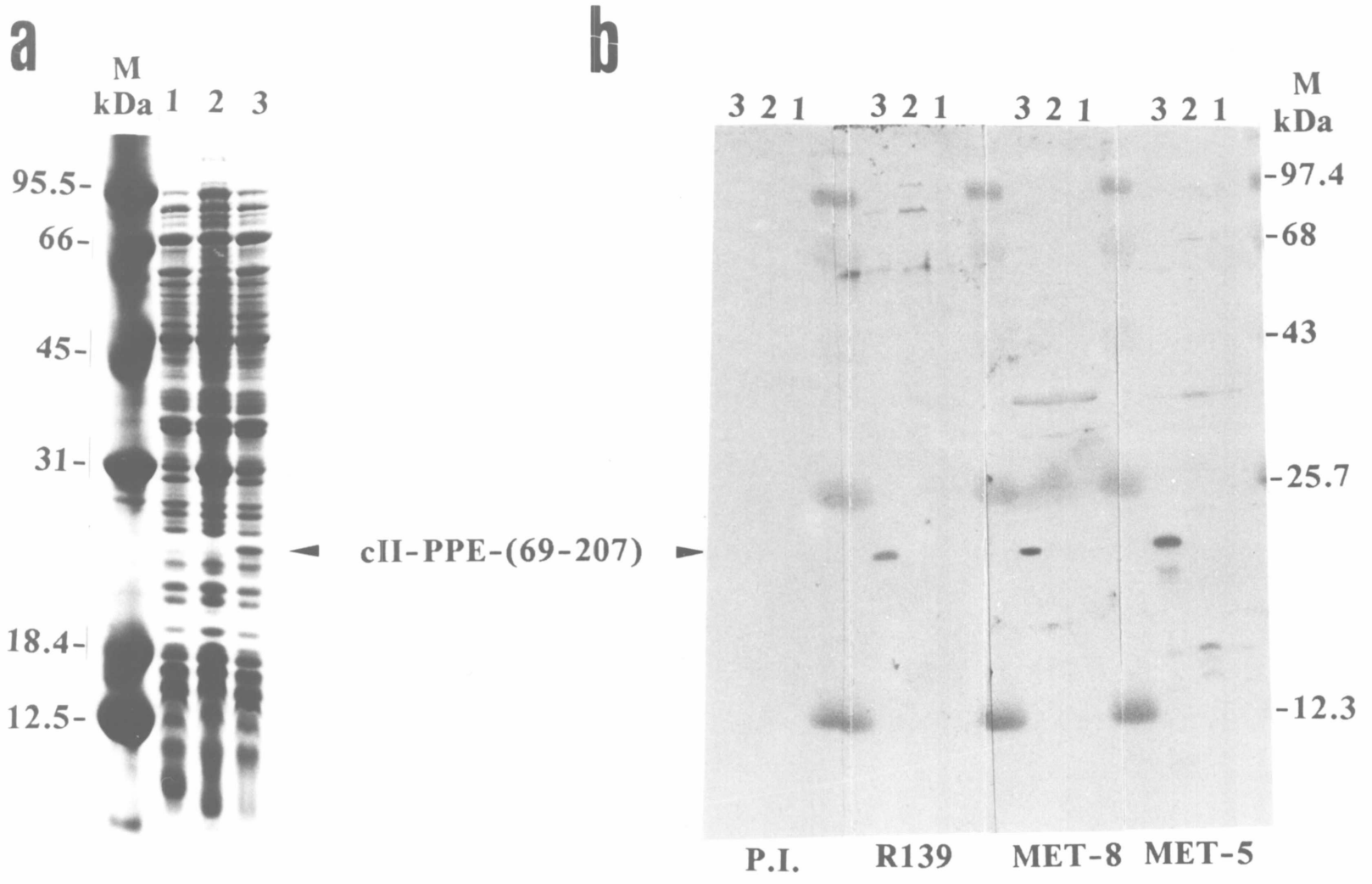
Sera from Bal/c mice immunised with the β-gal-PPE-(69-207) hybrid polypeptide were tested for anti-proenkephalin activity by immunoblotting against the *cII*-PPE-(69-207) fusion peptide. Non-specific anti-bacterial antibodies were removed by adsorption to an *E. coli* lysate

Figure 3.4

***cII*-preproenkephalin A-(69-207) fusion protein.**

Panel a, Bacterial lysates were electrophoresed on a 10% SDS-polyacrylamide gel which was stained with Coomassie blue. Lane M, molecular mass standards. Lane 1, host bacteria transformed with the parent vector pLcII, cultured at 42°C. Lanes 2 and 3, bacteria transformed with the recombinant plasmid pcII-PPE-(69-207), cultured at 30°C (lane 2) or 42°C (lane 3 -*cII*-PPE-(69-207) fusion protein visible at ~23kDa).

Panel b, immunoblot analysis of identical material (lanes 1, 2, 3 as for section a). Nitrocellulose strips were immunostained with different polyclonal antisera, using a peroxidase detection method. Antisera were as follows: P.I., a rabbit pre-immune serum as a negative control; R139, a rabbit polyclonal antiserum obtained after immunisation with the β-galactosidase-preproenkephalin A-(69-207) fusion protein; MET-8, a polyclonal antiserum to the octapeptide metenkephalin-arg^{6}-gly^{7}-leu^{8}; MET-5, a polyclonal antiserum to the pentapeptide metenkephalin. All polyclonal antisera had been preabsorbed using a crude E. coli lysate coupled to a CNBr activated sepharose column.


a
M
kDa 1 2 3
95.5-
66-
45-
31-
18.4-
12.5-
cII-PPE-(69-207)
b
3 2 1 3 2 1 3 2 1 3 2 1
M
kDa
-97.4
-68
-43
-25.7
-12.3
P.I.
R139
MET-8
MET-5

immobilised on a Sepharose column. A clear anti-proenkephalin response was observed after a total of six injections of immunogen.

The spleens of the two mice with the strongest peripheral response were used to generate hybridoma clones, as described in the Materials and Methods. Culture medium from microtitre wells was screened against nitrocellulose strips of Western blotted *cII*-PPE-(69-207) fusion peptide, using a peroxidase detection system. The strips were prepared by preparative SDS-PAGE, followed by Western blotting, of total bacterial lysate. Since the fusion protein could not be detected by a Ponceau stain, it was immunolocalised on a central strip, using the R139 anti-β-gal-PPE-(69-207) serum. The corresponding region was then excised from the remainder of the blot, cut into strips, and the screening performed in multiwell dishes. Out of several hundred clones, nine scored positive. However, owing to a technical problem with the incubator, only two clones survived on this occasion. These were designated PE-1 and PE-2. To ensure that each represented truly clonal lines, subcloning was carried out by limiting dilution.

3.2.4 **Characterisation and preliminary epitope mapping of PE-1 and PE-2**

In order to map the binding domains of PE-1 and PE-2 on the primary structure of the precursor, a series of β-gal-PPE fusion proteins incorporating overlapping regions

of sequence were constructed. For this purpose, a cDNA clone extending over the entire protein coding region, amino acids 1 to 267, was used (Comb *et al.*, 1982). The cDNA was cleaved at a variety of restriction sites to generate a series of cDNA fragments which were cloned into the pUR vectors. The products of ligation were transformed into the *E. coli* strain TG2 which has a similar genotype to the previously mentioned BMH-71-18, except that it is recombination deficient. Transformants containing proenkephalin DNA were identified by colony hybridisation using a radiolabelled preproenkephalin cDNA probe, and the orientation of the insert determined by restriction mapping of plasmid DNA. The ability of the constructs to direct the synthesis of fusion proteins was confirmed by analysing total bacterial lysates by SDS-PAGE. The following hybrid polypeptides were generated in this way: β-gal-PPE-(1-267), -(1-131), -(130-267), -(1-84), -(84-154), -(152-267) (Figure 3.1).Details of the constructs are as follows:

- The plasmid encoding the full-length preproenkephalin fusion protein, β-gal-PPE-(1-267), was made by excising a 918 base-pair *Hin*dII fragment from the human preproenkephalin cDNA clone kindly given to me by the late Dr. E. Herbert (Comb *et al.*, 1982). The *Hin*dII fragment was blunt end ligated to linear pUR 292 that had been cleaved with *Sal*I, flush ends having been generated with the Klenow fragment of DNA polymerase I.

- The plasmid encoding (p-)β-gal-PPE-(1-131) was

constructed by subcloning a *Bam*HI / *Xho*II fragment from the plasmid encoding β-gal-PPE-1-267) into *Bam*HI cleaved pUR 292.

- p-β-gal-PPE-(130-267) was made by subcloning an *Xho*II / *Hin*dIII fragment from p-β-gal-PPE-(1-267) into pUR 292 that had been cleaved with *Bam*HI and *Hin*dIII.

- In order to construct p-β-gal-PPE-(1-84), a *Dde*I fragment commencing at a *Dde*I site in the *lac* Z gene was isolated from p-β-gal-PPE-(1-267). *Bam*HI linkers were added, excess linkers were removed by digestion, and the fragment cloned into *Bam*HI cleaved and phosphatased pUR 290.

- p-β-gal-PPE-(84-154) was made by adding *Bam*HI linkers to an internal *Dde*I fragment which was inserted into pUR 290.

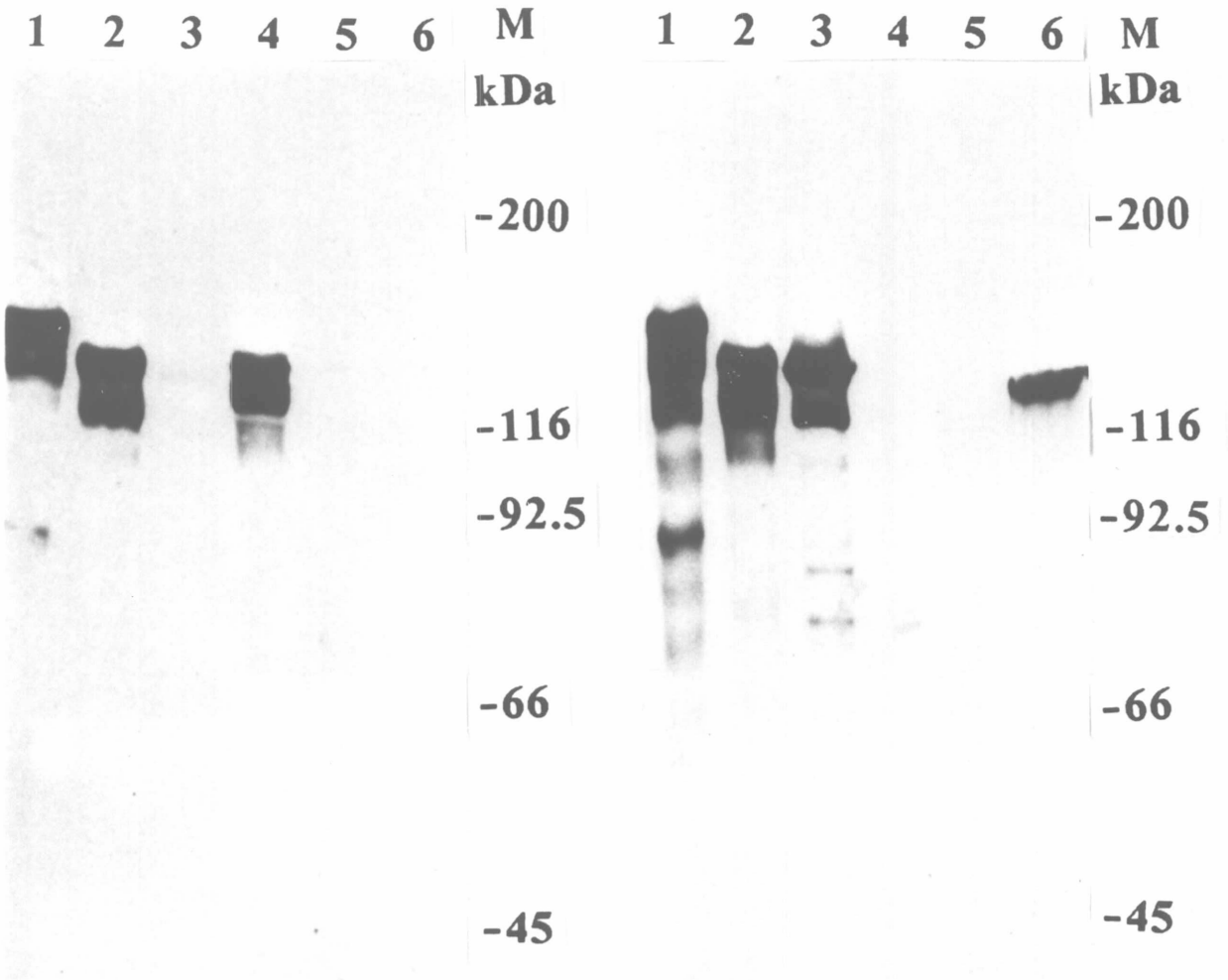
- p-β-gal-PPE-(152-267) was generated by excising a *Dde*I / *Eco*RI fragment from p-β-gal-PPE-(1-267), utilising an internal *Dde*I site and the *Eco*RI site in the pUR polylinker, to which were added *Bam*HI linkers, followed by insertion into pUR 292.

Figure 3.5 illustrates the results of immunoblot analysis of these fusion peptides. PE-1 recognises β-gal-PPE-(1-267), and -(130-267), together with the original immunogen -(69-207). PE-1 also binds β-gal-PPE-(152-267) (not shown). PE-2 recognises β-gal-PPE-(1-267), -(1-131), -(84-154), and the original immunogen -(69-207). Neither antibody recognises β-gal-PPE-(1-84). Thus, the respective binding domains of PE-1 and PE-2 can be broadly located,

Figure 3.5

Preliminary epitope mapping of PE-1 and PE-2 by immunoblot analysis of a series of β-galactosidase-preproenkephalin A fusion proteins. (see also Figure 1).

Total bacterial lysates were subjected to Western blotting followed by immunostaining with either PE-1 (left hand strip) or PE-2 (right hand strip). In each case, lanes 1 to 6 represent β-galactosidase fusion proteins in the following order: β-gal-PPE-(1-267), -(69-207), -(1-131), -(130-267), -(1-84), and -(84-154).


1 2 3 4 5 6 M
kDa
-200
-116
-92.5
-66
-45
PE - 1
1 2 3 4 5 6 M
kDa
-200
-116
-92.5
-66
-45
PE - 2

with respect to the primary translation product, within the amino acid sequences 152-207 and 84-131, (Figure 3.1).

Subclass determination of the hybridoma clones was carried out by incubation of the monoclonal supernatants with purified fusion protein spotted onto nitrocellulose, followed by immunoperoxidase detection. Using this means, PE-1 and PE-2 were both found to belong to the subclass IgG1.

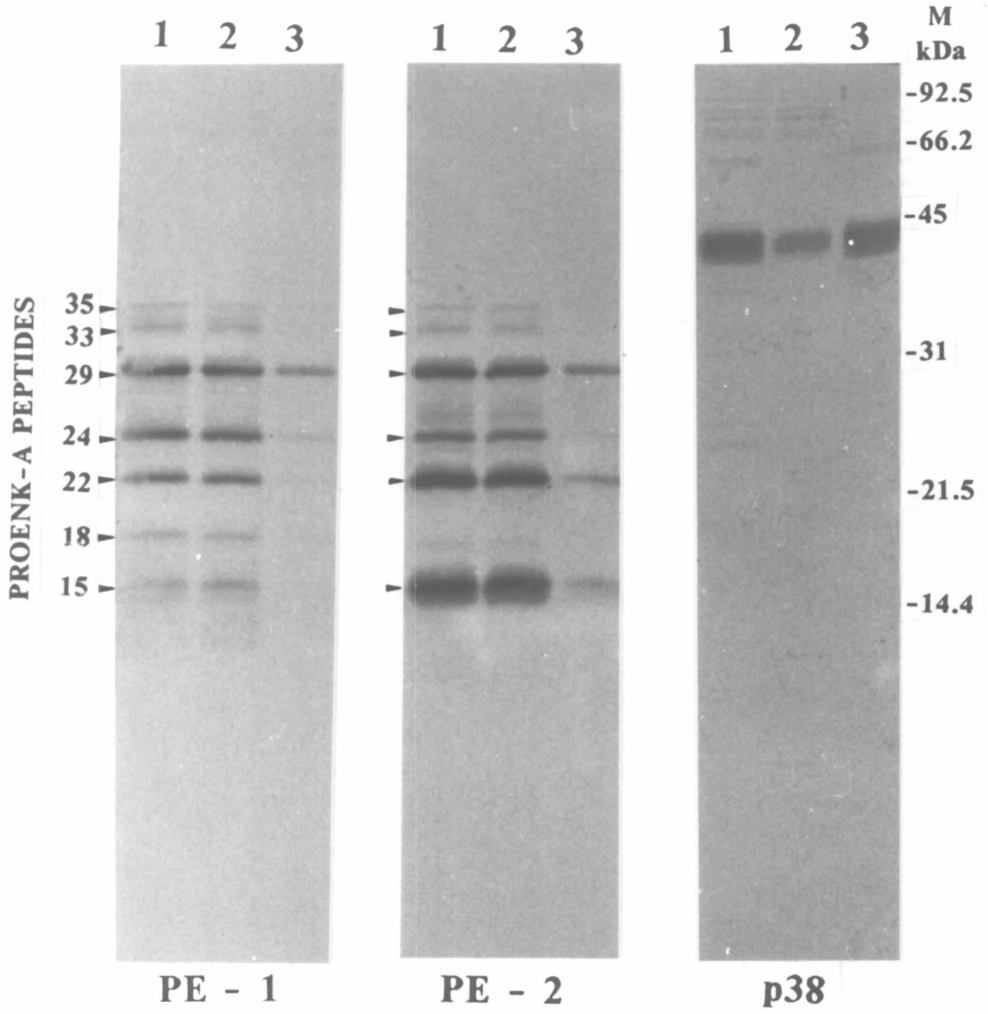
3.2.5 **Demonstration that PE-1 and PE-2 recognise naturally occurring high molecular weight proenkephalin-derived peptides**

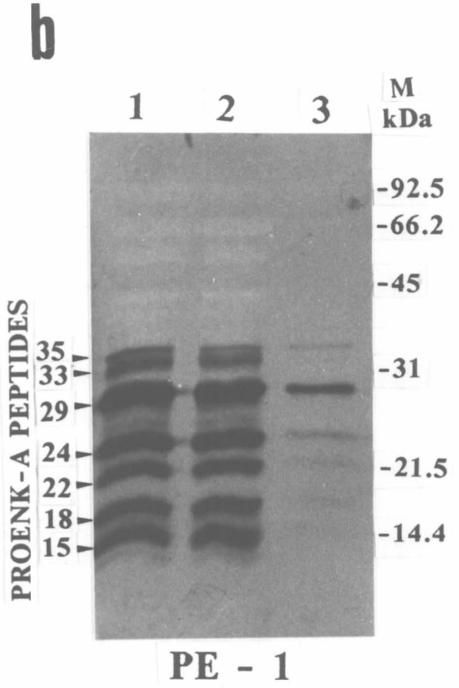
In order to determine whether PE-1 and PE-2 would recognise naturally occurring enkephalin precursor forms, I looked at isolated bovine adrenomedullary chromaffin granules which are known to be a rich source of high to intermediate molecular weight enkephalin-containing peptides. Granules were prepared by sucrose density gradient fractionation and lysed by sonication in 0.1M HCl, in order to limit artefactual proteolytic processing. Protease inhibitors were also present. The lysates were subjected to SDS-PAGE using a 12% separating gel, followed by immunoblot using a peroxidase detection method. It was found that PE-1 and PE-2 recognise proenkephalin-derived peptides of observed molecular mass 35, 33, 29, 24, 22 and 15kDa (Figure 3.6, Panel a). An 18kDa peptide is recognised by PE-1 alone, which may therefore represent a novel high molecular weight intermediate which has been

Figure 3.6

Immunoblot analysis of bovine adrenomedullary chromaffin granule lysates.

Chromaffin granule lysate proteins before and after high speed centrifugation were separated on 15% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Nitrocellulose strips were incubated with PE-1 or PE-2 supernatants, or a polyclonal antiserum to the membrane protein p38. In Panel a, a peroxidase detection system was used (diaminobenzidene stain); in Panel b, an immunogold-silver (IGSS) method was used. In all cases, lane 1 represents total granule lysate, lane 2 the granule contents and lane 3 the membrane fraction.


a
1 2 3
1 2 3
1 2 3
M
kDa
-92.5
-66.2
-45
-31
-21.5
-14.4
PROENK-A PEPTIDES
35
33
29
24
22
18
15
PE - 1
PE - 2
p38


b
1 2 3
M
kDa
-92.5
-66.2
-45
-31
-21.5
-14.4
PROENK-A PEPTIDES
35
33
29
24
22
18
15
PE - 1

proteolytically shortened from the NH_2 rather than the COOH-terminus. The 24-, 22- and 15-kDa PE-1 immunoreactive forms, which apparently co-migrate with PE-2 immunoreactive forms, may represent other novel NH_2-terminally shortened polypeptides (see Discussion section).

3.2.6 **PE-1 and PE-2 immunoreactive peptides appear to be partially membrane associated**

Studies by others have suggested that high molecular weight enkephalin peptides are associated with the granule membrane (Hook and Liston, 1987; Birch *et al.*, 1986). I was therefore interested to find which of the PE-1 and PE-2 immunoreactive peptides may be associated with the membrane. In order to separate granule contents from membranes, total granule lysates were subjected to high speed centrifugation in an air-driven ultracentrifuge. Supernatant and pellet were carefully separated, the former representing the granule contents and the latter the membrane fraction. Equivalent amounts of each fraction were subjected to SDS-PAGE followed by immunoblotting using a peroxidase detection method with diaminobenzidine as substrate. All of the forms present in the total lysate could be detected in the soluble fraction. In addition, the 29-, 22- and 15-kDa PE-2 immunoreactive forms are also associated with the granule membrane fraction, the proportion being so decreasing with diminishing molecular size. Using the same detection method, a proportion of the

PE-1 immunoreactive forms which migrate at 29 and 24kDa appear also to be in the membrane fraction (Figure 3.6, Panel a). It should be noted that the amount of protein apparently in the membrane fraction is probably an underrepresentation of the true amount, since immunostaining with an antibody to the integral membrane protein, p38, revealed significant amounts of p38 in the "contents" fraction. This suggested incomplete separation of granule membrane from contents, despite high speed centrifugation.

Since the failure to detect membrane binding of the highest molecular weight forms could have been a problem of sensitivity of the detection method, I went on to use a gold-coupled second antibody followed by silver enhancement (IGSS) to amplify the signal. This revealed that the 35-kDa form recognised by PE-1 and PE-2 is indeed found in the membrane fraction, although the 33-kDa form is not (Figure 3.6, Panel b). Furthermore, the PE-1 immunoreactive 22-, 18- and 15-kDa forms, in addition to the 29-and 24-kDa forms are also in the membrane fraction. IGSS confirmed apparent membrane association of the 29-, 22- and 15-kDa forms recognised by PE-2, and the absence of membrane association of the 24-kDa PE-2 immunoreactive form (data not shown).

3.2.7 **Discussion**

These data therefore demonstrate that antibodies raised to β-galactosidase-preproenkephalin fusion peptides

synthesised in *E. coli* are capable of recognising a wide array of naturally occurring proenkephalin-derived peptides.

Anti-enkephalin antibodies which have been available to date, exhibit either minimal or no cross-reactivity with enkephalin precursor forms, due to the tertiary conformation of the intact precursor molecule. They cannot therefore be used in direct assays for the precursor and its high molecular weight cleavage products. Assay of such forms usually requires chromatographic separation followed by trypsinisation to liberate the smaller enkephalin peptides. There are however two previously described polyclonal antibodies which do recognise high molecular weight intermediates and probably also have the capacity to recognise the intact precursor, although this has not been proven. One is an antiserum raised to a synthetic non-opiate sequence corresponding to residues 95-117 of bovine proenkephalin (Birch and Christie, 1986). Another is an antiserum raised to purified bovine proenkephalin-(1-77) (Metters and Rossier, 1987). However, the usefulness of such an antibodies when employed in isolation is bound to be limited as it has become apparent that there may not be a fixed site for the first cleavage event of the precursor. Thus, an even greater number of high molecular weight intermediates could exist than was originally envisaged. Therefore, in order to gain a full picture of the biosynthetic pathway, there is a requirement for a comprehensive range of antibodies

directed to different regions of the precursor, which will discriminate between the uncleaved prohormone and subsets of cleavage products.

The major proenkephalin A-derived peptides detected by PE-1 and PE-2 migrate with apparent molecular masses of 35, 33, 29, 22, 18 and 15kDa. To date, the highest molecular weight forms reported to exist in bovine adrenal medulla are 27kDa (Birch and Christie, 1986) and 28kDa (Metters and Rossier, 1987), as determined by SDS-PAGE. However, cross-immunoreactivity studies indicate that these forms correspond to the *23.3*kDa (*italics* indicates predicted molecular mass) peptide (Figure 3.1) rather than the intact precursor. It seems highly likely that the 29-kDa form observed by us is also this *23.3*kDa peptide. Further comparison of our immunoblot data with those of others (Birch *et al.*, 1986) suggests that the 22- and 15-kDa forms recognised by PE-2 correspond to the previously described *18.2* and *12.6*kDa peptides, respectively (Figure 3.1). The 24-kDa form may represent a post-translationally modified form of the *18.2*kDa peptide which has been observed by others to migrate as two species.

I initially assumed that the 24- and 22-kDa forms recognised by PE-1 were also likely to correspond to the *18.2*kDa peptide in modified and ummodified form. However, further data indicate that the PE-1 epitope may lie outside the *18.2*kda peptide sequence. In collaboration with Dr. Susan Jackson at Reading University I have found that PE-1 fails to bind the purified bovine 5.3K peptide

(Figure 3.1) in an ELISA system. Given their apparent molecular weights, it is therefore possible that the 24- and 22-kda PE-1 immunoreactive forms represent high molecular weight polypeptides where shortening from the NH_2-terminus of the precursor has occurred. Additionally, the PE-1 immunoreactive 15-kDa form cannot correspond to the predicted molecular mass *12.6*kDa peptide which does not contain the PE-1 epitope (Figure 3.1), and must therefore represent a polypeptide which extends further towards the COOH-terminus of the precursor. The 18-kDa form specific to PE-1 is also likely to represent another novel NH_2-terminally shortened polypeptide since it apparently does not contain the PE-2 epitope, although post-translational modification at this domain cannot at this stage be ruled out. My collaborators at Reading University (Dr. S. Jackson and Prof. P. Lowry) are currently purifying these peptides in an effort to define their structure.

The significance of these findings lies in the fact that they provide further evidence that there may not be fixed site for the first cleavage event of the precursor, since high molecular weight forms shortened from either the NH_2 or the COOH-terminus can apparently co-exist within a single cell type. There has been a certain amount of debate concerning this issue, with as yet no hard evidence on either side. Some workers maintain that the first cleavage of the precursor always occurs between the *23.3*kDa sequence and peptide B, since NH_2-terminally

extended forms of peptide B have not been detected (I. Lindberg, personal communication). However, it is possible that the anti-peptide B antibody will not cross-react with extended forms in a direct assay. Baird and co-workers (1984) found NH_2-terminally shortened forms in which the extreme COOH-terminus of proenkephalin is still present, indicating an initial cleavage towards the NH_2-terminus. Furthermore, high molecular weight proenkephalin-derived peptides without synenkephalin (NH_2-terminal) immunoreactivity were identified in hypothalamus, indicating the existence of high molecular weight carboxyl terminal fragments (Liston *et al.*, 1984b). What my data suggests is that, even within a single cell type, the enkephalin precursor may undergo proteolytic shortening initiated from opposite termini. One possible explanation at the subcellular level could be that distinct vesicular subpopulations are responsible for specific sets of cleavage events initiated from different ends of the molecule.

A number of hormone precursors, such as proopioimelanocortin, proinsulin, proglucagon and prosomatostatin have been shown to be associated with secretory granule membranes. This may provide a mechanism for correct sorting and orientation for proteolytic processing. In order to determine if this might be the case for the enkephalin precursor, I analysed the distribution of the various proenkephalin-derived peptides between the granule membrane and its contents by high

speed centrifugal separation. On subsequent immunoblot analysis with IGSS detection, I found that the 35-kDa form appeared to be membrane-associated whereas the 33-kda form was not. However, the majority of the 35-kDa form still remained in the granule contents. This suggests that this form does not correspond to the intact precursor, since other intact prohormones have been shown to be approximately 80% membrane-bound (Loh and Tam, 1985). However, these previous studies looked at newly synthesised prohormone only, and did not examine steady state levels. Furthermore, I did not achieve complete separation of granule membrane from its contents, since significant amounts of the intrinsic membrane protein, p38, remained in the supernatant (contents fraction) after centrifugation. It will require purification of the different forms, together with NH_2- and COOH-terminal sequencing, to establish their identity firmly. This is being undertaken by my collaborators at Reading University.

3.3 Generation of monoclonal antibodies to the full-length human preproenkephalin sequence

In order to extend the antibody repertoire, I generated a different chimaeric protein for immunisation comprised of the entire 267 amino acid sequence of human preproenkephalin fused to β-galactosidase. Details of this construct have been given in Section 3.2.4. After immunisation with purified fusion protein, as described

previously, screening of hybridoma supernatants was performed in the first instance by immunoblotting against a hybrid protein consisting of proenkephalin fused to 71 amino acids of the chloramphenicol acetyl transferase (CAT) gene. this was achieved by subcloning the "*Eco*RI cassette", containing the preproenkephalin insert, from pUR 292 into the natural *Eco*RI site of the CAT gene within the vector pYEJ001 (Pharmacia). In this vector, the CAT gene is driven by a synthetic promoter which is under the control of the *lac* operator, so fusion protein induction is conveniently achieved with IPTG. A potential drawback of this construct lay in the fact that one was "carrying over" the sequence encoding the extreme COOH-terminal 17 amino acids of β-galactosidase which was therefore "sandwiched" between the CAT gene product and β-galactosidase. As a result of this screening strategy, a number of positive clones were generated which were named PE-3 to PE-12. However, on subsequent epitope mapping, it was found that, remarkably, all were directed to the extreme COOH-terminus of β-galactosidase, and none were directed to the much (16x) larger preproenkephalin sequence. This demonstrates very strikingly the immunodominance of β-galactosidase over proenkephalin.

In order that preliminary screening of polyclonal antisera could be guaranteed to detect anti-proenkephalin activity, an alternative screening construct was engineered. This utilised a series of vectors centred on the phage T7 RNA polymerase (Studier and Moffat, 1986).

Subsets of these vectors were designed either to generate fused or unfused constructs. The cloning sites in all plasmids were situated downstream of a phage T7 promoter which is activated only by its own specific polymerase. The T7 RNA polymerase is supplied when one transforms the vector into a lysogenic strain of *E. coli* (DE3) which carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lac*UV_5 promoter. When the transformed cells are in log growth phase, IPTG is added to induce expression of the T7 RNA polymerase gene, the product of which drives transcription of the recombinant vector. An initial construct, in the vector pAR 2192, was designed with the aim of expressing unfused preproenkephalin, utilising the natural initiation codon of the preproenkephalin gene. However, this was unsuccessful. In the absence of any reasons why transcription should not have occurred, I attributed the failure to lack of stability of the unfused proenkephalin molecule within the bacteria. I then generated a fusion construct in which *Bam*HI linkered preproenkephalin cDNA was inserted in translational phase with the NH_2-terminal 11 amino acid sequence of the phage T7 capsid protein *gene 10*, within the vector pAR 3039. This approach was more successful, leading to detectable fusion protein on immunoblotting with PE-1 and PE-2. Presumably the protein was "stabilised" by the additional fused amino acids.

The *gene 10*-preproenkephalin [*G10*-PPE-(1-267)] fusion protein was then used to screen polyclonal antisera, prior

to initiating hybridoma lines. It took many months to establish a clear anti-proenkephalin response, using the full-length β-gal-PPE-(1-267) construct. Presumably, the proenkephalin moiety was folding in a manner "familiar" to the animal. A total of 6 to 8 injections over a period of 12 to 18 months was required before a clear serum response was achieved, when successful fusions ensued. Fusions had been attempted at earlier stages, in the absence of a serum response, but these were unsuccessful. Screening of hybridoma supernatants was performed in the same way as previously - by immunostaining of strips of Western blotted fusion protein, in this case *G10*-PPE-(1-267). Induction of fusion protein was achieved by the addition of IPTG when the cells were in log growth phase. Bacteria were harvested and lysed after 3 hours of induction. It was notable that, in those cells expressing fusion protein, there was a dramatic cessation of cell division after addition of IPTG.

A total of 13 new hybridoma clones was eventually achieved. These were designated PE-13 to PE-25. To ensure that the lines were truly clonal, subcloning by limiting dilution was performed. Subclass determination revealed that all belong to the IgG1 class, apart from PE-18 which is an IgG2a clone.

3.3.1 **Epitope mapping of PE-13 to PE-25**

Preliminary epitope mapping was carried out as for PE-1 and PE-2 - by immunoblotting against a series of β-

galactosidase fusion proteins incorporating overlapping regions of primary sequence. This established clearly that all antibodies are directed to the COOH-terminal half of a central portion of the molecule, since they recognise constructs containing the sequences PPE-(1-267), PPE-(69-207) and PPE-(130-267), but not PPE-(1-131) (Figures 3.1 and 6.1). This therefore places the recognition domain somewhere within the amino acid sequence 130 to 207 which must therefore be an immunodominant region of the molecule. The Mabs were also immunoblotted against constructs which subdivide this region, namely, PPE-(84-154) and PPE-(152-267) (Figure 3.1 for map). Most display either weak or absent cross-reactivity with these fusion proteins, suggesting disruption either of a linear epitope which spans these sequences (at ~ amino acid 152) or of a more distant conformational epitope. Three antibodies, PE-20, PE-21 and PE-24 do, however, display clear recognition of fusion proteins incorporating PPE-(152-267) but not PPE-(84-154). In these cases, the domain therefore lies between amino acids 152 and 207, within which the PE-1 epitope also lies.

I propose to carry out more detailed epitope mapping by construction of an expression sublibrary incorporating random proenkephalin cDNA fragments generated by limited sonication. Clones reactive with antibody will be subjected to DNA sequencing. A similar approach to epitope mapping has been described previously (Mehra *et al.*, 1986).

3.4 Determination of cross-species reactivity of the proenkephalin antibodies

In order to determine whether the PE Mabs would cross-react with the bovine and rat sequences, I made constructs analogous to βgal-hPPE-(69-207) using cDNAs from these species, since epitope mapping has revealed that all the antibodies are directed to this central region of the molecule. Central *Pst*I fragments of rat and bovine proenkephalin cDNAs were cloned into the β-gal expression vector, pUR 291, and the fusion proteins immunoblotted against the Mabs. Results of this experiment are given in Table 3.1. Two antibodies, PE-1 and PE-2, exhibit full cross-reactivity with bovine and rat proenkephalin. The majority (8) cross-react with rat proenkephalin only. Interestingly, 4 antibodies (PE-20, 21, 22 and 24) are human specific. Given this information, examination of computer-aligned sequences should assist in epitope mapping.

Aligned sequences of human, rat and bovine proenkephalin cDNA are shown in Figure 3.8. Within the amino acid sequence 130 to 207 of human preproenkephalin (the region within which PE-13 to PE-25 bind), residues which are human specific or shared with the rat sequence only, are indicated by arrows and stars respectively. Other residues which are shared by the human and bovine, but not the rat, sequences are also present but have not been indicated since none of the PE Mabs cross-react with the bovine but not the rat molecule. The greater degree of

sequence divergence in this region explains why the antibodies which bind here have limited cross-reactivity and could well be why it is relatively immunodominant. Preliminary epitope mapping has indicated that PE-20, 21 and 24 bind within the amino acid sequence 152 to 207. This is consistent with them being human specific since this region has a higher number of human specific residues (arrows). However, interestingly, only 3 of these residues reflect non-conservative changes which perhaps would be more likely to confer immunological divergence. These non-conservative substitutions are located at positions 162 (glutamic acid vs. glycine, human vs. rat), 171 (histidine vs. serine) and 182 (valine vs. aspartate). Thus, the degree of biologically relevant divergence may be even less than the sequence comparisons suggest.

TABLE 3.1

Cross-species reactivity of the PE Mabs

Antibody	Human PE	Rat PE	Bovine PE
PE-1	+	+	+
PE-2	+	+	+
PE-13	+	very weak	very weak
PE-14	+	+	-
PE-15	+	+	-
PE-16	+	+	-
PE-17	+	+	weak
PE-18	+	+	-
PE-19	+	+	-
PE-20	+	-	-
PE-21	+	-	-
PE-22	+	-	-
PE-23	+	+	-
PE-24	+	-	-
PE-25	+	+	-

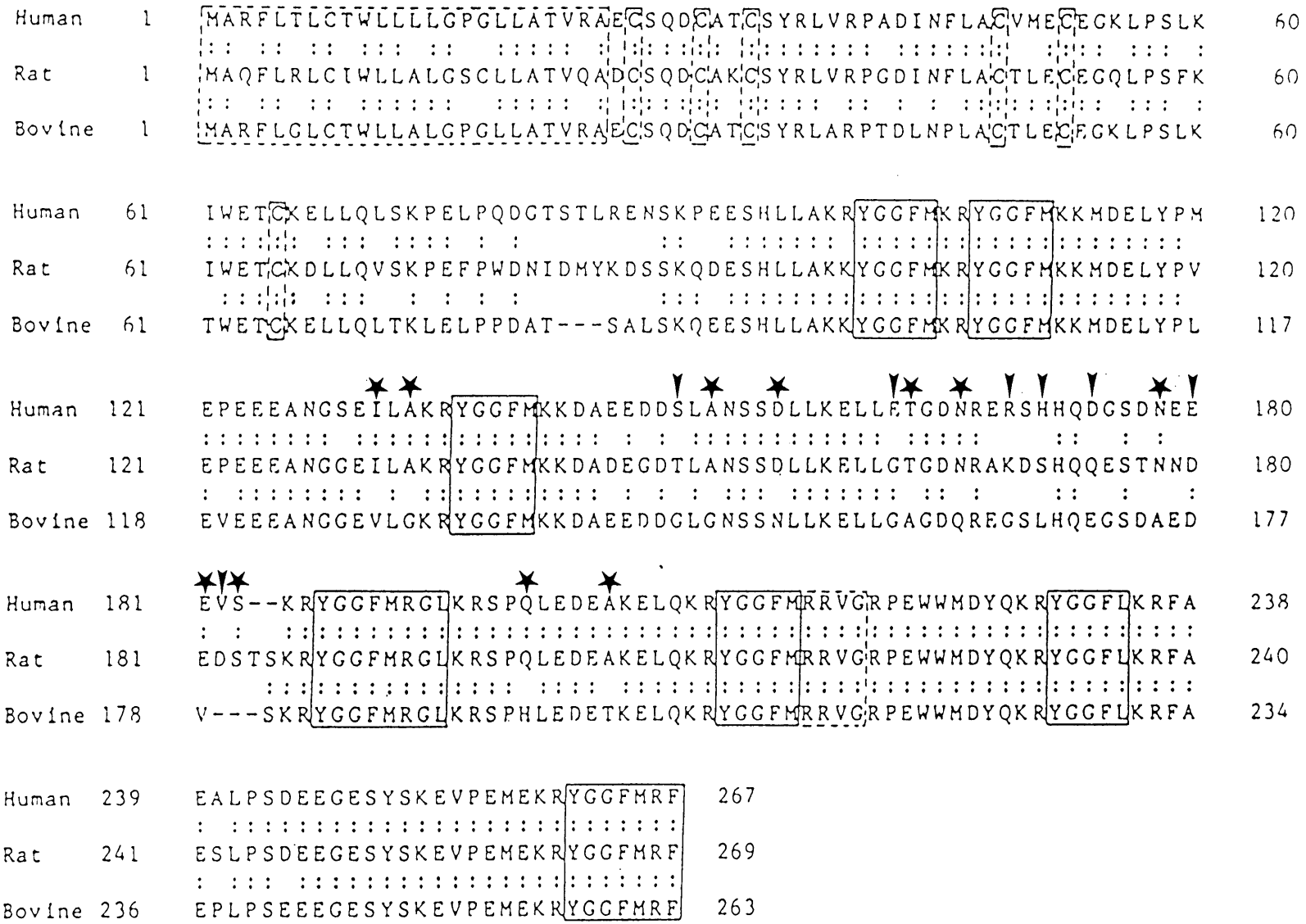
Figure 3.7

Comparison of human, rat and bovine preproenkephalin amino acid sequences.

(Taken from Yoshikawa and Sabol, 1984a)

Sequences are represented by standard one-letter amino acid abbreviations. Colons between aligned sequences indicate matched amino acids. Enkephalin sequences are shown in solid-line boxes. The putative signal peptide and cysteine residues are shown in broken-line boxes.

Within the amino acid sequence 130 to 207 (within which the epitopes of PE-13 to PE-25 lie), arrows indicate residues which are human specific and stars indicate residues shared by the human and rat sequences only.


Human 1 MARFLTLCTWLLLLGPGLLATVRAECSQDCATCSYRLVRPADINFLACVMECEGKLPSLK 60
Rat 1 MAQFLRLCIWLLALGSCLLATVQADCSQDCAKCSYRLVRPGDINFLACTLECEGQLPSFK 60
Bovine 1 MARFLGLCTWLLALGPGLLATVRAECSQDCATCSYRLARPTDLNPLACTLECEGKLPSLK 60
Human 61 IWETCKELLQLSKPELPQDGTSTLRENSKPEESHLLAKRYGGFMKRYGGFMKKMDELYPM 120
Rat 61 IWETCKDLLQVSKPEFPWDNIDMYKDSSKQDESHLLAKKYGGFMKRYGGFMKKMDELYPV 120
Bovine 61 TWETCKELLQLTKLELPPDAT---SALSKQEESHLLAKKYGGFMKRYGGFMKKMDELYPL 117
Human 121 EPEEEANGSEILAKRYGGFMKKDAEEDDSLANSSDLLKELLETGDNRERSHHQDGSDNEE 180
Rat 121 EPEEEANGGEILAKRYGGFMKKDADEGDTLANSSDLLKELLGTGDNRAKDSHQQESTNND 180
Bovine 118 EVEEEANGGEVLGKRYGGFMKKDAEEDDGLGNSSNLLKELLGAGDQREGSLHQEGSDAED 177
Human 181 EVS--KRYGGFMRGLKRSPQLEDEAKELQKRYGGFMRRVGRPEWWMDYQKRYGGFLKRFA 238
Rat 181 EDSTSKRYGGFMRGLKRSPQLEDEAKELQKRYGGFMRRVGRPEWWMDYQKRYGGFLKRFA 240
Bovine 178 V---SKRYGGFMRGLKRSPHLEDETKELQKRYGGFMRRVGRPEWWMDYQKRYGGFLKRFA 234
Human 239 EALPSDEEGESYSKEVPEMEKRYGGFMRF 267
Rat 241 ESLPSDEEGESYSKEVPEMEKRYGGFMRF 269
Bovine 236 EPLPSEEEGESYSKEVPEMEKRYGGFMRF 263

CHAPTER 4

THE GENERATION OF STABLE TRANSFORMANT LINES OF PC12 CELLS WHICH EXPRESS HIGH LEVELS OF UNPROCESSED HUMAN PROENKEPHALIN

4.1 Introduction

Transfection of prohormone genes into cell lines of neuroendocrine origin can be used to study the nature of tissue specific processing. Several groups have shown that transfection of a propeptide gene into a heterologous cell line, that is one that does not express the endogenous gene, can result in apparently normal post-translational processing of the exogenous gene product. For example, proinsulin transfected into the murine anterior pituitary cell line AtT20 is proteolytically cleaved to insulin (Moore *et al.*, 1983); and preprovasopressin (Cwikel and Habener, 1987) and preprosomatostatin (Sevarino *et al.*, 1987) are cleaved in both RIN 5F insulinoma cells and AtT20 cells. AtT20 cells, which express endogenous proopiomelanocortin but not proenkephalin A, can process transfected human proenkephalin to Met-enkephalin. However, the absence of the octapeptide Met-enkephalin-Arg^6-Gly^7-Leu^8 and the heptapeptide Met-enkephalin-Arg^6-Phe^7, which are normal terminal products, suggests aberrant processing (Comb *et al.*, 1985). Proenkephalin transfected into GH4CI cells, a somatomammotrophic pituitary cell line, is not processed, despite the presence of storage granules (Thomas *et al.*, 1986).

It is my ultimate aim to generate a number of stable transformant cell lines with varying capacities to process human proenkephalin, in order that I can analyse proteolytic processing of the molecule in relation to its

subcellular compartmentalisation. As an extension of this, the generation of cell lines expressing mutant proenkephalin molecules will enable me to study processing in relation to intracellular trafficking of the molecule. In the preliminary study outlined here, I chose to introduce human preproenkephalin cDNA into the PC12 cell line, a phaeochromocytoma line of New England Deaconess Hospital (NEDH) rat adrenomedullary chromaffin cell origin (Greene and Tischler, 1976). This strain of rat is unusual in that the adult adrenal medulla is a rich source of high to intermediate molecular weight proenkephalin A-derived peptides, whereas adrenals from common laboratory strains of rat are a poor source of these peptides. Some workers have been unable to detect endogenous enkephalin in PC12 cells (Greene and Tischler, 1982), although more recently another group (Byrd *et al.*, 1987) has detected extended enkephalin peptides, albeit at much lower levels than in the adult NEDH rat adrenal. Interestingly, the latter study also shows that the content of endogenous proenkephalin-derived peptides in PC12 cells can be stimulated by treatment with the differentiation promoter sodium butyrate. Furthermore, the chromatographic profile changes from a non-specific heterogeneous array of intermediate molecular size peptides to a pattern resembling that in the adult adrenal. This could indicate stimulation of processing enzyme activity with some degree of specificity for the enkephalin precursor. I was therefore optimistic that the

processing machinery of these cells might recognise human proenkephalin as a familiar protein and cleave it in an appropriate fashion.

4.2 Generation of stable proenkephalin transformants of PC12 cells

The expression plasmid used was pRSVBamHI, (supplied by R. Kelly, UCSF), which is a hybrid of the expression plasmids pRSVCAT (Gorman *et al.*, 1982) and pSV2CAT. The promoter sequence is provided by the Rous sarcoma virus long terminal repeat (RSV LTR) downstream of which is a *Bam*HI cloning site created with linkers. The polyadenylation site from pSV2CAT has been retained, after removal of the CAT gene. To construct the recombinant vector, *Bam*HI linkers were added to the 918 base pair *Hin*dII fragment of human preproenkephalin cDNA, described in the previous chapter. The linkered fragment was inserted in the correct orientation into the *Bam*HI cloning site of pRSVBamHI, downstream of the RSV LTR, to create pRSVBam::hPE.

Calcium phosphate-mediated transfection, in combination with glycerol shock, was carried out (described in more detail in the Materials and Methods section). Stable transformants were generated by co-transfection with the plasmid pSV2neo, followed by selection with the neomycin analogue G418. Approximately 20 clones were obtained, of which 10 were expanded for analysis (PC12::hPE).

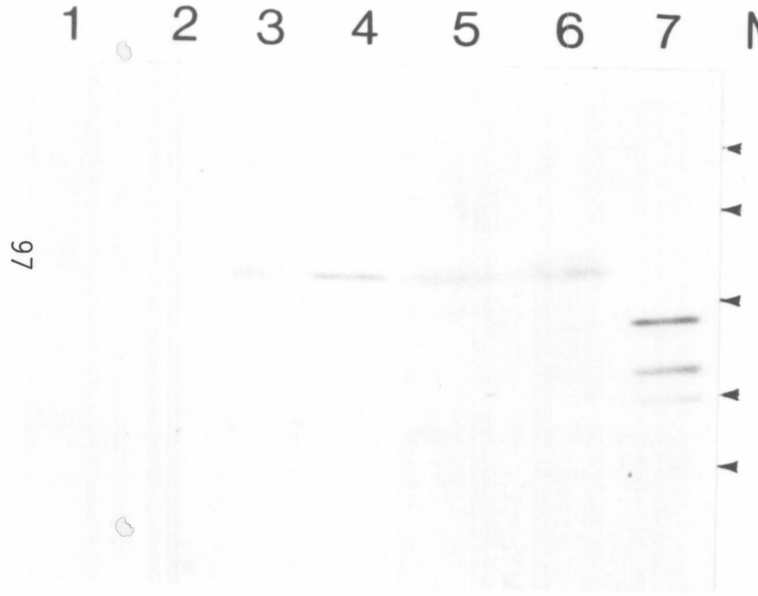
4.3 Stable PC12::hPE transformants express high levels of the uncleaved human enkephalin precursor

The ten clones selected were screened for proenkephalin expression by immunoblot analysis of acidic cell lysates, initially using the monoclonal antibodies PE-1 and PE-2. As described in Chapter 3 (and Spruce *et al.*, 1988), these antibodies recognise all the major high molecular weight cleavage products of proenkephalin A. Immunoblots revealed that two out of the ten clones expressed high levels of the uncleaved enkephalin precursor, in the absence of high molecular weight cleavage products. Figure 4.1 illustrates an immunoblot analysis of non-transfected PC12 cells and one of the transformed lines, PC12::hPE. Both transformed lines gave an identical immunoblot result. Lanes 1 and 2 represent whole cell lysates of non-transformed PC12 cells. Not surprisingly, the low levels of endogenous enkephalin peptides are below the sensitivity of the immunoblot system. In lane 3, which represents untreated PC12::hPE transformants, a single band which migrates at 36kDa is visible, although PE-2 clearly exhibits much stronger recognition of this band than PE-1. The newer series of antibodies generated to the full-length preproenkephalin sequence, PE-13 through PE-25, also recognise this 36kDa band, in some cases even more strongly than PE-2 (see Chapter 6 for illustration and further discussion). Thus, I am confident that the 36kDa form represents a proenkephalin molecule and is not an artefact induced by

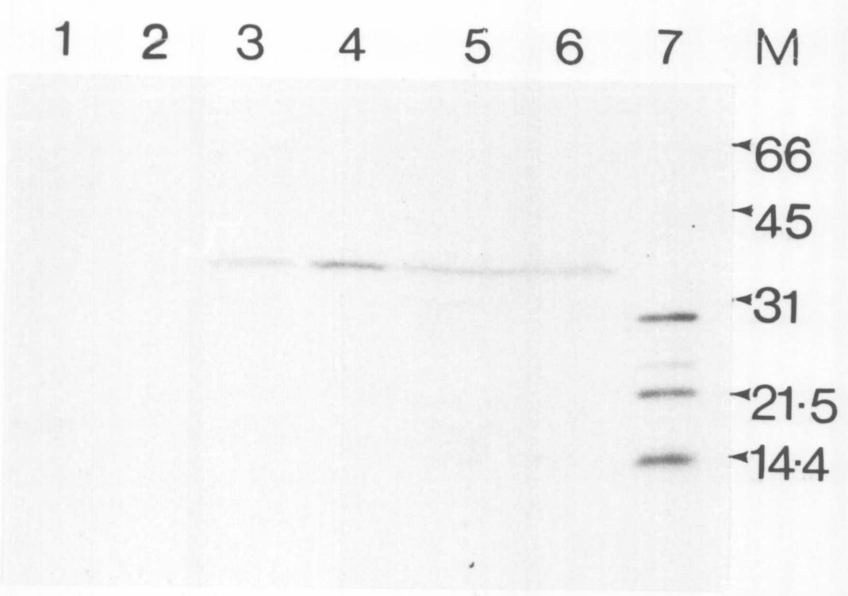
Figure 4.1

Immunoblot analysis of acidic lysates of transfected and non-transfected PC12 cells treated with differentiation promoters.

Lane 1, untreated non-transfected PC12 cells; lane 2, non-transfected PC12 cells after 48h treatment with 6mM sodium butyrate; lane 3, untreated PC12::hPE transformants; lane 4, PC12::hPE transformants after 48h treatment with 6mM sodium butyrate; lane 5, PC12::hPE transformants after ten days treatment with 50ng/ml NGF; lane 6, PC12::hPE transformants after six days treatment with 1uM dexamethasone; lane 7, bovine adrenomedullary chromaffin granule lysate showing the major high molecular weight proenkephalin-derived peptides; lane M, positions of molecular mass standards (kDa). Blots were incubated with either PE-1 or PE-2 monoclonal supernatant. Within the transformed cell lysate tracks (lanes 3 to 6), a band at ~36kDa is visible, compatible with unprocessed proenkephalin.



PE – 1



PE – 2

the transfection, since it is recognised to greater or lesser degrees by 15 anti-proenkephalin Mabs, some to widely separated epitopes. Furthermore, it is reasonable to presume that it represents the intact prohormone, since it migrates more slowly than any of the highest molecular weight cleavage products of proenkephalin (Spruce *et al.*, 1988). For comparison,lane 7 in Figure 4.1 shows the major proteolytic cleavage products recognised by PE-1 and PE-2 in bovine adrenomedullary chromaffin granule lysates, a rich source of proenkephalin peptides. There are no equivalent bands in the transformed cell lysate tracks. Thus, given the high level of unprocessed proenkephalin, it has to be concluded that there is markedly reduced proteolytic processing in this transformed line. A remaining, but unlikely, possibility would be that small enkephalin peptides, undetectable on the immunoblot, are present in the absence of high molecular weight intermediates. However, this could only occur if a proportion of the uncleaved proenkephalin had been diverted into a parallel subcellular compartment within which proteolytic processing is completed efficiently, without the accumulation of processing intermediates. To address this possibility, I have recently performed preliminary Met-enkephalin assays, in collaboration with Dr. Susan Jackson of Reading University, on whole cell lysates of transfected and non-transfected cells. We find that levels of free Met-enkephalin in transformed cells are no higher than in non-transfected cells. The vast

majority of the Met-enkephalin activity in the transformed cells is detectable only after trypsinisation, which liberates free peptides from precursor forms. This therefore confirms that the exogenous human proenkephalin molecule does not undergo processing to Met-enkephalin.

4.4 Human proenkephalin is apparently targeted to secretory granules within the transformed cell line

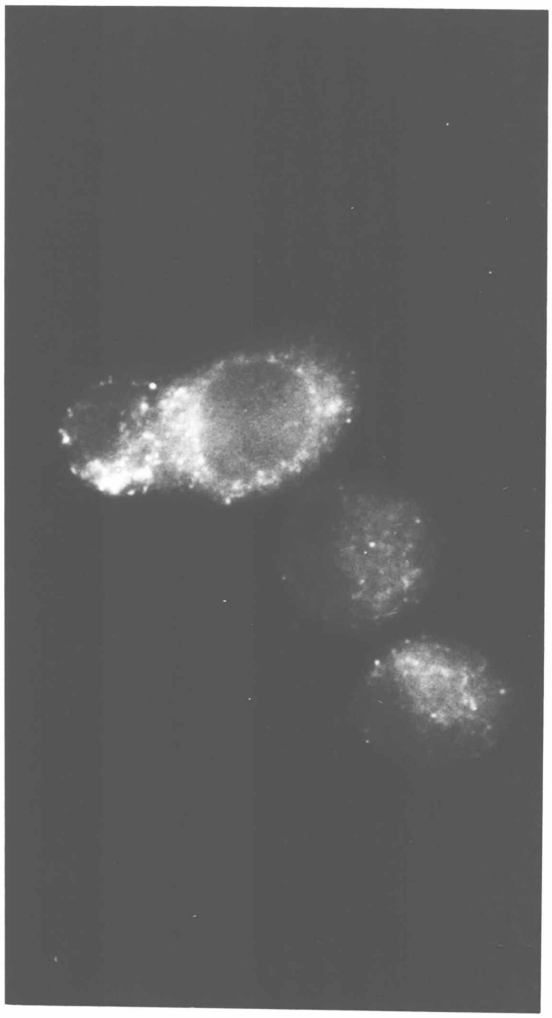
Indirect immunofluorescence of transfected and non-transfected cells was carried out with several of the proenkephalin Mabs. Figure 4.2 shows the immunofluorescent staining pattern of PC12::hPE transformants using the Mab PE-18 which exhibits strong, apparently preferential, recognition of the intact precursor (Chapter 6). The proenkephalin molecule has a clearly punctate distribution. There was no significant immunofluorescent staining of non-transfected cells (not shown) due presumably to the very low levels of endogenous intact precursor in these cells.

The punctate distribution of the proenkephalin molecule could be due to lysosomal targeting or compartmentalisation within secretory granules. The former possibility seems most unlikely as lysosomal enzymes would quickly degrade this ordinarily highly unstable molecule. The latter possibility therefore seems the most likely, although ultrastructural analysis will be required to confirm this.

Figure 4.2

Indirect immunofluorescence of PC12::hPE transformants.

Cells were fixed and permeabilised with methanol and acetone, then incubated with PE-18 ascites (1/500 in 10% FCS/PBS) followed by FITC conjugated goat anti-mouse IgG. Cells were viewed using a Nikon Microphot FX with epifluorescence optics. A punctate pattern of fluorescence was seen which was not present in non-transformed cells.



4.5 Differentiation promoters and reserpine fail to stimulate proteolytic processing of proenkephalin within PC12::hPE transformants

Byrd and co-workers (1987) found that treatment of PC12 cells with the differentiation promoter sodium butyrate induced endogenous enkephalin gene expression, and appeared to stimulate specific processing enzyme activity also. Sodium butyrate has been shown to induce differentiation of PC12 cells along a chromaffin cell-like pathway (Byrd and Alho, 1987), whereas treatment with nerve growth factor (NGF) induces differentiation along a neuronal pathway (Tischler and Greene, 1975). Another differentiation promoter, dexamethasone, has been shown to induce PC12 cells to resemble a population of intensely fluorescent cells seen in sympathetic ganglia (Chiba *et al.*, 1981).

I chose to treat PC12::hPE transformants with all 3 differentiation promoters, at separate times, in the hope that one or more of these agents would stimulate proenkephalin processing enzyme activity. I treated transformed and non-transformed cells with either 6mM sodium butyrate for 48h, 1μM dexamethasone for 6 days, or NGF at 50ng/ml for 10 days. After treatment of the cells with NGF or sodium butyrate there was a marked change in cellular morphology. The NGF treated cells became flattened with marked neurite outgrowth, which are the classical changes desribed. In contrast, the cells treated with sodium butyrate became rounded and displayed reduced

cell-substratum adhesion. This differs from the changes described by Byrd and Alho (1987) on butyrate treatment, namely, increased cell-cell and cell-substratum adhesion. However, my observation of a dramatic cessation of cell division would be in keeping with differentiation induction. I can only explain these differences on the basis of the well-known plasticity of this line. Lanes 4, 5 and 6 (Figure 4.1) illustrate the results of immunoblot analysis of lysates of PC12::hPE transformants treated with butyrate, NGF and dexamethasone respectively. In all cases, only the intact prohormone, and no cleavage products, are visible, indicating that there has been no significant stimulation of processing activity. Preliminary enkephalin assays performed in collaboration with Dr. Susan Jackson have confirmed this finding, since free Met-enkephalin levels are no higher in treated as compared with untreated transformed cells.

I also chose to examine the effect of treatment with reserpine, which is an inhibitor of catecholamine reuptake. Reserpine has previously been shown to stimulate both proteolytic processing and amidation of proenkephalin A-derived peptides in primary chromaffin cell cultures (Lindberg, 1986; Eiden and Zamir, 1986), although the mechanism is unknown. These workers noted an increase in terminal products with a concomitant reduction in intermediate sized peptides which would suggest stimulation at a relatively late stage in the proteolytic cascade. The cells were treated with 1μM reserpine for

72h. Controls were given 0.1% DMSO which was the drug vehicle used. No change in cell morphology was noted. Figure 4.3 shows the results of immunoblot analysis of cell lysates, using a mixture of PE-1 and PE-2 Mab supernatants. DMSO and reserpine treated PC12::hPE transformants (lanes 3 and 4 respectively) are shown alongside non-transformed cell lysates (lanes 1 and 2). The 36kDa band is unchanged after treatment with reserpine, showing no apparent effect on processing, at least on initial proteolytic cleavage events. Enkephalin assays have not yet been carried out.

4.6 Discussion

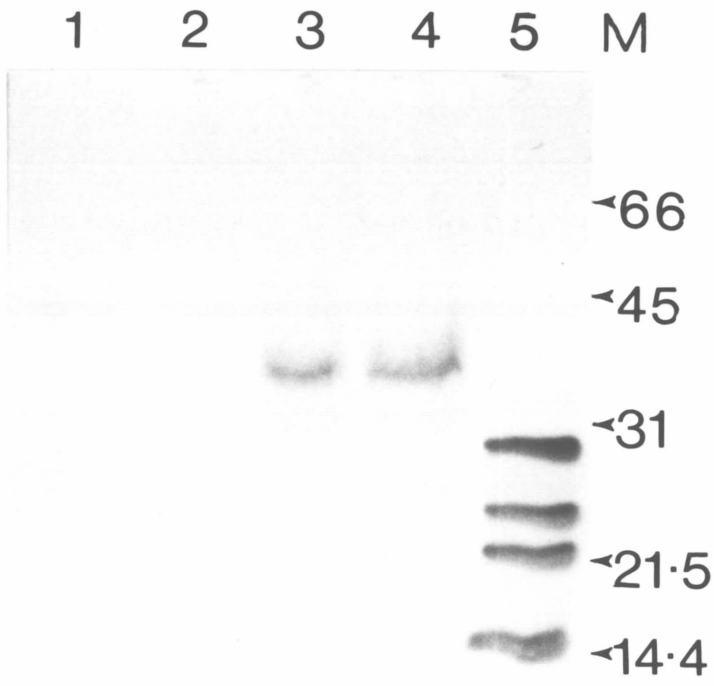
In this study I transfected a "homologous" gene into the PC12 cell line, since a familiar protein might have greater fidelity for its processing enzymes than a non-familiar substrate. In previous studies, "heterologous" polypeptide hormone genes have been transfected into PC12 cells. For example, following transfection of the gene encoding human growth hormone, the protein is packaged in secretory vesicles and exhibits release kinetics which are identical to those of endogenous catecholamines, indicating sorting to the regulated pathway (Schweitzer and Kelly, 1985; Lowe *et al.*, 1988). However, when hormones were introduced which normally undergo proteolytic processing, a defect of the cleavage machinery of the cell was indicated. For example, PC12 transformants expressing prosomatostatin (Sevarino *et*

Figure 4.3

Immunoblot analysis of transfected and non-transfected PC12 cells after treatment with reserpine.

Lane 1, non-transfected PC12 cells in the presence of 0.1%(v/v) DMSO (drug vehicle) only; lane 2, non-transfected PC12 cells after treatment for 72h with 1uM reserpine; lane 3, PC12::hPE transformants in the presence of 0.1% DMSO; lane 4, PC12::hPE transformants after treatment for 72h with 1uM reserpine. The blot was incubated with a 1:1 mixture of PE-1 and PE-2 supernatants.

Lane M = molecular mass standards (kDa)


1
2
3
4
5
M
66
45
31
21·5
14·4

al., 1987) and proinsulin (R. Kelly, personal communication) did not cleave the prohormones. Nevertheless, it remained possible that converting enzyme activity with greater affinity for a gene product normally present in that cell type may be present. However, the failure of these cells to cleave proenkephalin indicates defective proteolytic processing even when one introduces a polypeptide hormone which is familiar to the cell. This is despite the presence of specific proenkephalin converting enzyme activity, since the cells contain free endogenous Met-enkephalin. It is possible, though, that this activity is present at low levels, in keeping with the low level of endogenous proenkephalin expression. If this is so, it suggests the possibility of coordinate gene regulation of processing enzymes and their substrates, and will be interesting to study. A low level of converting enzyme activity would be quickly saturated by the high levels of exogenous proenkephalin. Another possible explanation could be that the intragranular milieu is not conducive to prohormone cleavage, which would better explain the inability of the cells to cleave a number of prohormones.

CHAPTER 5

A PROENKEPHALIN MAB WHICH CROSS-REACTS WITH CHROMOGRANIN A

5.1 Introduction

The chromogranins are polypeptide molecules which are major constituents of dense core secretory granules in a wide variety of endocrine and neurosecretory cells. The primary structures of three chromogranins - chromogranin A (Benedum *et al.*, 1986), chromogranin B (also known as secretogranin I) (Benedum *et al.*, 1987), and chromogranin C (or secretogranin II) (Gerdes *et al.*, 1989) have been determined from the nucleotide sequence of their complementary DNAs. They have several structural features in common such as an abundance of acidic residues, which confers an overall negative charge; multiple potential proteolytic cleavage sites at pairs of basic residues; thirdly, a motif extending over a stretch of 10 amino acid residues appears to represent the most significant linear sequence shared between all three molecules (Gerdes *et al.*, 1989).

Despite the ubiquitous nature of these molecules, their function has remained unclear. Both intracellular and extracellular roles have been put forward, though have yet to be proven. It has been proposed, for example, that they may be involved in the packaging of polypeptide hormones into dense core granules of the regulated secretory pathway (see Chapter 1 for discussion of the regulated and constitutive pathways). Indeed, coalescence of chromogranin molecules may be critical to the formation of the dense core. Molecules which are associated with chromogranin molecules would thereby be diverted into the

regulated pathway of secretion. Gerdes and co-workers (1989) have recently demonstrated aggregation of secretogranin II *in vitro* which is calcium and low pH dependent. These conditions are characteristic of the luminal milieu of neuroendocrine secretory granules. In contrast, molecules which are secreted in a constitutive fashion *in vivo* do not aggregate under these conditions. Thus, molecular self-aggregation which is induced by change in the ionic environment may be critical to the sorting process. The internal pH of the trans-Golgi network is also slightly acidic, though not to the same degree as within granules, and the calcium concentration is higher than within the rough endoplasmic reticulum. Thus, electron microscopic evidence for aggregation of secretory products within the trans-Golgi cisternae, in the region of incipient granules (Orci *et al.*, 1987b), would be in keeping with this mechanism.

Other workers have proposed active intermolecular interactions in the sorting process. Chung and co-workers (1989) recently identified a set of putative prohormone binding proteins in association with Golgi membranes, so-called "sortases", which they proposed may act either as carrier molecules or as cross-linking agents in the formation of aggregates. Binding of hormone molecules to these proteins is active at the neutral pH of the trans-Golgi, and dissociation of the complex occurs at lower pH, as in mature secretory granules, allowing potential recycling of the sortases. The soluble nature of these

molecules would make it unlikely that they represent membrane-bound receptor proteins. However, it is possible that some specific feature of the aggregate could be recognised by a receptor, which would explain co-purification of these proteins with the Golgi membrane. Indeed, recognition of a membrane bound receptor by some specific sorting signal on secretory proteins has been put forward as another mechanism for the sorting phenomenon. It is possible that a combination of aggregation and receptor binding may be involved, in the way described.

The possibility of an extracellular role for the chromogranins arose from the fact that they possess multiple potential proteolytic cleavage sites at pairs of basic residues, suggesting thereby that they may be precursor molecules to one or more bioactive peptides. Furthermore, they are released upon exocytosis of the secretory granule. This was given extra credence when it was discovered that within chromogranin A there is a sequence which is highly homologous to the porcine peptide, pancreastatin, so-called because it inhibits glucose-induced insulin release from the endocrine pancreas. Furthermore, this peptide sequence is bounded by potential cleavage sites. Thus, it was suggested that pancreastatin was derived either from chromogranin A, or from a related molecule (Eiden, 1987; Huttner and Benedum, 1987). However, rigorous proof for this precursor-product relationship has not been forthcoming.

It has also been suggested that chromogranins may

regulate proteolytic processing of prohormones by the diversion of endoproteases to their own cleavage sites. In support of this, chromogranin A was found to reversibly inhibit the cleavage of both proenkephalin-derived peptides and ACTH by processing enzymes *in vitro* (Seidah *et al.*, 1987). However, a reciprocal relationship between the amount of chromogranin and the degree of proteolytic processing within a given cell type has not been demonstrated. Indeed, other workers have suggested that chromogranin may facilitate cleavage by the promotion of suitable conditions within the vicinity of the prohormone.

The purpose of this chapter is to discuss the possible significance, if any, of a fortuitous finding - the cross-reaction of one of the anti-proenkephalin Mabs with chromogranin A.

5.2 PE-15 cross-reacts with chromogranin A

Immunoblot analysis of the anti-proenkephalin Mabs against bovine adrenomedullary chromaffin granule lysates revealed that one of them, PE-15, recognised a high molecular weight triplet (lane 2, Figure 5.1, Panel A). The molecular size of the highest band, 70kDa, is the apparent molecular weight of chromogranin A on SDS-PAGE. Furthermore, the triplet pattern on immunoblot, presumably due to proteolytic cleavage products, is also characteristic of the molecule. None of the other PE Mabs demonstrate this cross-reaction.

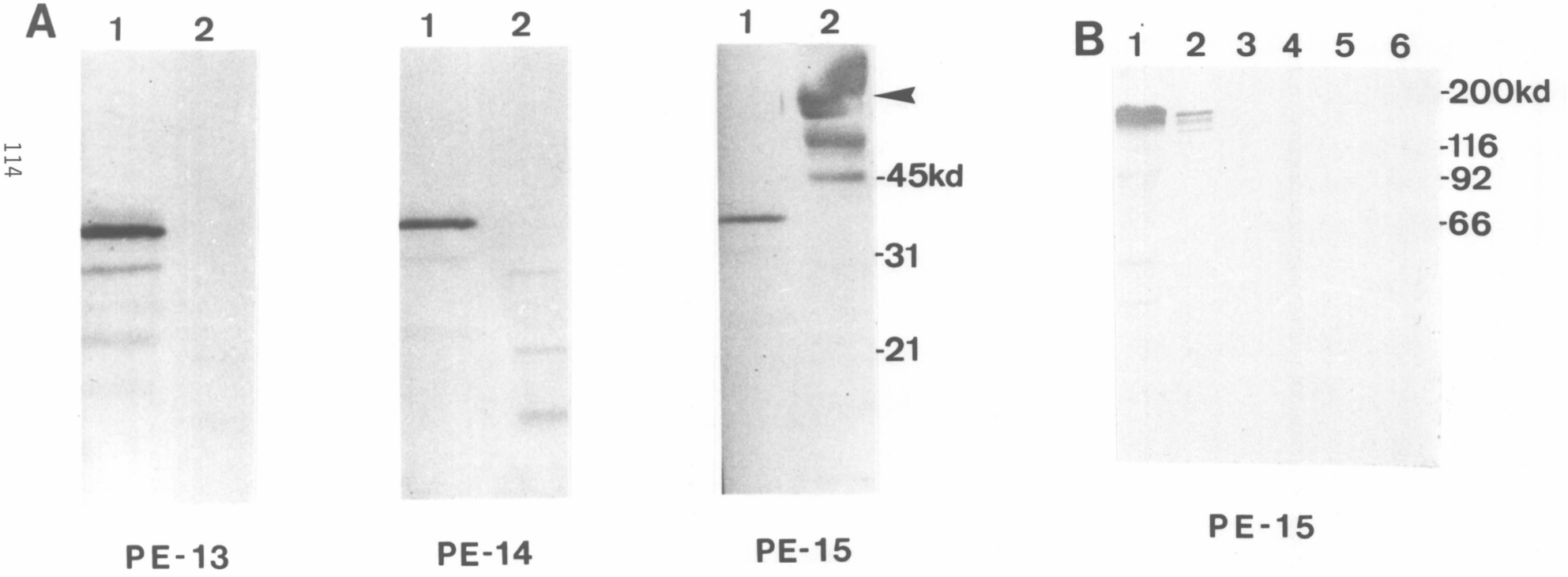
A primary sequence comparison between proenkephalin A

Figure 5.1

PE-15 cross-reacts with chromogranin A in bovine chromaffin granule lysates (A) and when expressed in *E. coli* (B).

Panel A: lysates of PC12::hPE transformants (lanes 1) and bovine adrenomedullary chromaffin granule lysates (lanes 2) were immunoblotted against PE-13, PE-14 and PE-15. A peroxidase detection system was used. All antibodies show strong reactivity for the intact human enkephalin precursor expressed in PC12 cells. PE-15 alone recognises a band migrating at ~70kDa (arrowed) with lower molecular weight products visible also. This "triplet" pattern is characteristic of chromogranin A.

Panel B: bovine and human chromogranin A - β-galactosidase fusion proteins immunoblotted against PE-15. Lanes are as follows (see also Figure 5.2): lane 1, bCRGA-(1-449); lane 2, bCRGA-(66-449); lane 3, bCRGA-(130-381); lane 4, bCRGA-(66-130); lane 5, hCRGA-(66-450); lane 6, hCRGA-(1-87). PE-15 cross-reacts with bCRGA-(1-449) and -(66-449) only.


A
1
2
PE-13
1
2
PE-14
1
2
-45kd
-31
-21
PE-15
B
1
2
3
4
5
6
-200kd
-116
-92
-66
PE-15

and chromogranin A of different species revealed no particularly striking homology apart from a short pentapeptide sequence, leu-leu-lys-glu-leu, which is present in human, rat and bovine proenkephalin A and human and bovine chromogranin A. This short homologous sequence lies between amino acids 156 and 160 of human proenkephalin A, which is therefore within the region PPE-(130-207) (Figure 3.1), to which the PE-15 epitope has been preliminarily mapped. The PE-15 epitope is disrupted by cleavage between amino acids 152 and 154, since PE-15 fails to bind PPE-(84-154) or PPE-(152-267). This could be due either to disruption of a linear epitope which spans these sequences (at ~amino acid 152), or of a more distant conformational epitope. Its failure to bind PPE-(152-267) means that the homologous pentapeptide array is in itself insufficient to elicit binding, but it remains possible that it could be involved in a more complex conformational or discontinuous epitope.

In order to confirm the cross-reaction, I decided to check if the antibody would recognise chromogranin expressed within *E. coli*. Dr. Wieland Huttner (EMBL) generously donated bovine and human chromogranin A cDNAs which had been cloned within his laboratory. I expressed these as β-galactosidase fusion proteins, using the pUR expression vector series. I initially expressed fragments which incorporated the leu-leu-lys-glu-leu sequence, situated near the NH_2 terminus of chromogranin A (CRGA), and checked for cross-reactivity with PE-15 by

immunoblotting. However, the constructs containing the amino acid sequences human CRGA (1-87) and bovine CRGA (66-130) failed to elicit binding. I then proceeded to engineer fusion proteins incorporating larger fragments of chromogranin. Details of the constructs are given below (see Figure 5.2 for map):

- **bovine CRGA(1-449)** (incorporating entire protein coding region): a 1.4kb *Nar*I restriction endonuclease fragment was cloned into the *Cla*I restriction site within the polylinker of pUR 290, since *Nar*I cohesive ends are compatible with *Cla*I ends. This required a partial enzyme digest as there is a natural *Cla*I site within the *lac*Z gene of the vector.

-bovine CRGA(66-449): *Bam*HI linkers were cloned onto the ends of the 1.4kb *Nar*I fragment, which on subsequent *Bam*HI digestion yielded a 1.19kb fragment, due to an internal *Bam*HI site within chromogranin. I cloned this fragment into *Bam*HI cleaved pUR 292, which when expressed in *E. coli* generated a fusion protein truncated at the NH_2-terminus.

-bovine CRGA(66-130): a 192bp internal *Bam*HI-*Pst*I fragment was inserted between the *Bam*HI and *Pst*I sites of pUR 290.

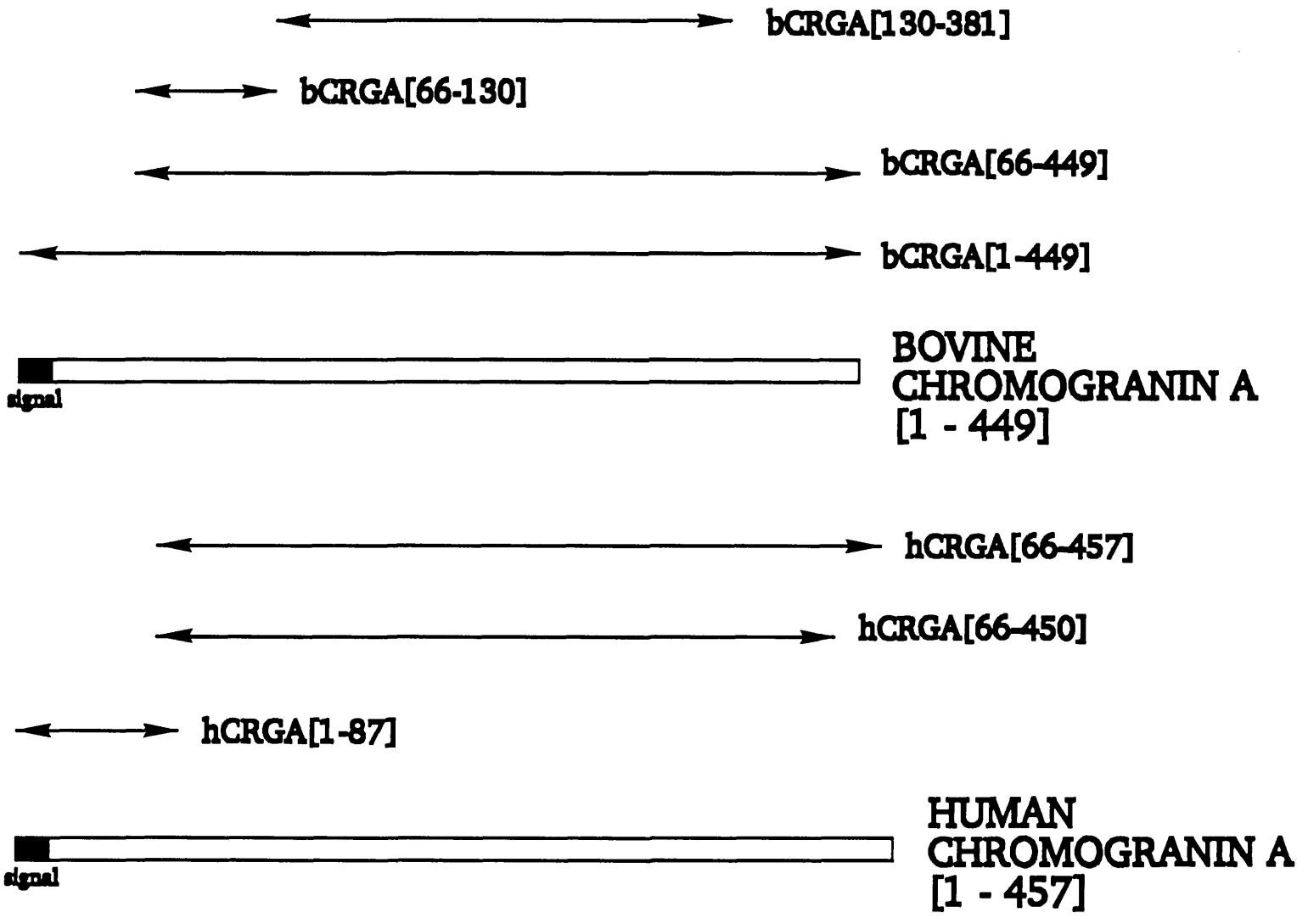
-bovine CRGA(130-381): a 752bp internal *Pst*I-*Pst*I fragment was inserted into the *Pst*I site of pUR292.

-human CRGA(1-87) (includes additional Ala-Pro-Gly-Ser-Ala from 5′ untranslated region): a 271bp *Nar*I fragment was cloned into the *Cla*I site within the

Figure 5.2

Map of human and bovine chromogranin sequences which were fused to β-galactosidase

The boxed regions illustrate the full-length bovine and human chromogranin sequences. Arrowed lines indicate the amino acid sequences of chromogranin which were expressed as β-galactosidase fusion proteins in *E. coli* to confirm the cross-reactivity of PE-15 and in an attempt to epitope map the shared domain.


bCRGA[130-381]
bCRGA[66-130]
bCRGA[66-449]
bCRGA[1-449]
signal
BOVINE
CHROMOGRANIN A
[1 - 449]
hCRGA[66-457]
hCRGA[66-450]
hCRGA[1-87]
signal
HUMAN
CHROMOGRANIN A
[1 - 457]

polylinker of pUR 291.

-human CRGA(66-450): a 1157bp internal *Bam*HI-*Pst*I fragment was inserted between the same sites within the polylinker of pUR 292.

-human CRGA(66-457): *Bam*HI linkers were cloned onto a 1.9kb *Eco*RI fragment. Subsequent *Bam*HI digestion cleaved at an internal site as well as the COOH-terminal linker, to yield a 1.18bp fragment which was cloned into *Bam*HI cleaved pUR 292. This encoded the extreme COOH-terminal amino acid sequence, missing from the previous construct.

Fusion proteins for SDS-PAGE and immunoblot analysis were prepared in an identical way to the PPE fusion proteins.

5.3 PE-15 recognises recombinant chromogranin A

By immunoblot analysis of bacterially-expressed fusion proteins, I was able to confirm that PE-15 does recognise chromogranin A. The constructs recognised by PE-15 are, the full-length bovine chromogranin fusion protein, bCRGA(1-449) (Lane 1, Figure 5.1, Panel B) and the NH_2-terminally truncated construct, bCRGA(66-449) (Lane 2). The apparently weaker signal in the latter case is due to lesser amounts of this less stable fusion protein. The antibody does not, however, recognise bCRGA(66-130) and bCRGA(130-381) and it does not recognise any of the human chromogranin fusions proteins (Lanes 3 to 6). I have yet to succeed in making a full-length human chromogranin fusion protein, however. Since PE-15

recognises bovine chromogranin fusion proteins missing the NH_2 but not the COOH-terminal region of the molecule, it is possible that the PE-15 epitope lies towards the COOH terminus of the molecule. However, disruption of a more NH_2-terminally situated conformational epitope remains a possibility.

5.4 Discussion

PE-15, an anti-human proenkephalin antibody, therefore recognises bovine chromogranin A both in chromaffin granule lysates and when expressed as a chimaeric protein in *E. coli*. The main question is, could this immunological cross-reaction be biologically significant? It should be stressed that this is a highly specific cross-reaction, since none of the other PE Mabs recognise chromogranin. Furthermore, I have not detected cross-reactivity of PE-15 with any other molecules. Finally, the cross-reactivity is still seen even in the presence of low amounts of chromogranin, such as the bCRGA-(66-449) fusion protein which is less abundant than the full-length fusion protein (see Figure 5.1, Panel b). Against biological relevance is the fact that, on the face of the data, this anti-human proenkephalin antibody recognises only bovine and not human chromogranin. However, if the epitope is conformational, I may need to express the full-length human chromogranin sequence before detecting a cross-reaction. Furthermore, it is possible that a subtle

difference in conformation due to fusion to β-galactosidase has destroyed the epitope.

There are 3 possible biological reasons for the shared determinant. Firstly, PE-15 could be recognising a region of tertiary homology in the chromogranin and proenkephalin molecules within the vicinity of one or more proteolytic cleavage sites. One has to invoke conformational similarity, since there is no noteworthy primary sequence homology, other than the "core" of basic residues, adjacent to these sites. As discussed in Chapter 1, there is now good evidence that higher orders of protein structure may be involved in the recognition of substrate binding sites by proteolytic cleavage enzymes. Furthermore, as mentioned at the beginning of this chapter, there is *in vitro* evidence for potential competition between proenkephalin and chromogranin A for cleavage enzymes, suggesting some similarity in the substrate recognition sites.

Secondly, since both chromogranin A and proenkephalin A are molecules destined for dense core secretory granules, it is tempting to speculate that the homologous region may be a "sorting" domain, responsible for directing molecules to the regulated secretory pathway. Such a sorting signal could interact possibly with a receptor molecule on the trans-Golgi membrane or perhaps with an intraluminal binding protein involved in aggregate formation. This will be discussed in more detail in the final chapter.

A third, but more remote, possibility is that PE-15 is actually a spontaneously generated anti-idiotypic antibody which has the same 3-D structure as the domain on the proenkephalin molecule to which the corresponding idiotype is directed. Its cross-reaction with chromogranin would then be explained by a domain on the chromogranin molecule which is reciprocal to one on the proenkephalin molecule. Such reciprocal domains could possibly be involved in protein-protein interaction during formation of a dense core aggregate. However, retention of binding ability of PE-15 to the proenkephalin molecule would imply the existence of a reciprocal domain also within the proenkephalin molecule.

CHAPTER 6

PROENKEPHALIN IN CEREBELLAR NEURONS AND ASTROCYTES SUGGESTS BIOLOGICAL SIGNIFICANCE OF THE INTACT PRECURSOR

6.1 Introduction

To date, immunohistochemical studies on brain have been restricted to a search for small enkephalin peptides. My anti-proenkephalin Mabs now provide the opportunity to examine the distribution of enkephalin precursor peptides within brain, which may not necessarily parallel the distribution of mature enkephalin peptides due to regional differences in proteolytic cleavage of the precursor. Indeed, data available from chromatographic studies combined with enkephalin assays have already provided evidence for regional differences in the processing of proenkephalin within brain (Birch and Christie, 1986). This suggests that high molecular weight cleavage products, and possibly even the intact precursor, may be acting as neuropeptides in their own right.

I was particularly interested to examine cerebellum, since data included in a study from Pittius and co-workers (1985) indicated a discrepancy between significant levels of proenkephalin mRNA but negligible amounts of immunoreactive mature enkephalin peptides in bovine cerebellum. One could interpret this as evidence for reduced proteolytic processing leading to a predominance of enkephalin precursor peptides which would not have been detected in the conventional enkephalin assays used. Interestingly, Zagon and co-workers (1985) had described immunocytochemical detection of Met- and Leu-enkephalin in 10 day old, but not adult, rat cerebellum, and thereby suggested a role for enkephalins in cerebellar

development. This is in keeping with a previous demonstration of opiate binding activity only in the developing, and not the adult, rat cerebellum (Tsang *et al.*, 1982).

Until recently, neuropeptide gene expression in brain has been presumed to be restricted to neurons. However, the intriguing possibility of neuropeptide expression by glial cells, generally thought of as supporting cells within the nervous system, has been recently suggested. Vilijn and co-workers (1988) detected preproenkephalin mRNA by Northern blot analysis in cultured astrocytes from different brain regions, including cerebellum. This finding was confirmed by Shinoda and co-workers (1989) who detected cyclic AMP inducible preproenkephalin mRNA in cultured astrocytes from cerebellum, cortex and striatum, indicating regulation of the preproenkephalin gene within glia in a similar fashion to that in neurons. Furthermore, cortical and striatal, but not cerebellar, astrocytes were found to contain and release immunoassayable Met-enkephalin peptide. This study also described somatostatin mRNA and peptides in cerebellar astrocytes only, which indicated regional differences in neuropeptide gene expression by glial cells.

In this study I used my PE Mabs to examine postnatal rat cerebellum by indirect immunofluorescence. Remarkably, I found that subpopulations of both neuronal and astroglial cells were apparently rich in enkephalin precursor peptides, in the absence of mature enkephalin

peptide immunofluorescence, in cryostat sections. I also examined the distribution of proenkephalin within glia in dissociated culture.

6.2 PE Mabs used in this study

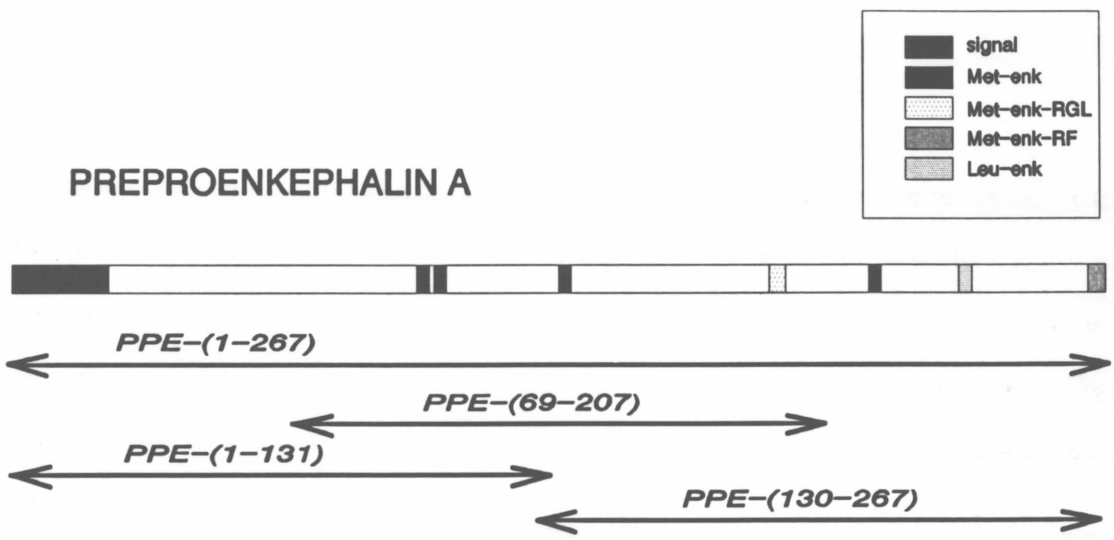
Five of the proenkephalin Mabs give an identical immunofluorescent staining pattern of cerebellar neurons and astrocytes in cryostat sections. The antibodies are, PE-14, PE-16, PE-18, PE-19, and PE-25, which were all generated to the full-length preproenkephalin sequence (see Chapter 3 for details). Reassuringly, all of these antibodies cross-react with rat proenkephalin expressed as a fusion protein (see Chapter 3). Furthermore, those antibodies which are human specific and do not cross-react with rat proenkephalin, show no staining of either neurons or glia.

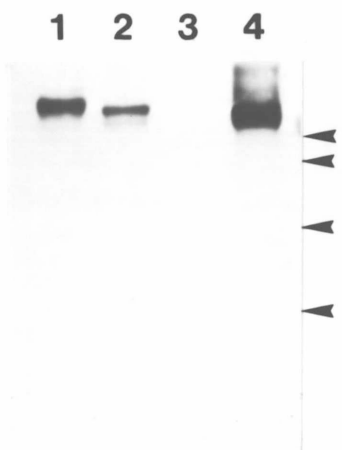
Two of these Mabs, PE-18 and PE-25, were used to generate the immunofluorescence data shown in this section. The remaining three Mabs gave equally strong staining. PE-18 and PE-25 belong to different subclasses - IgG2a and IgG1 respectively. As outlined in Chapter 3, preliminary epitope mapping of PE-18 and PE-25 has been carried out by determining cross-reactivity with a series of β-galactosidase fusion proteins. Figure 6.1 shows an immunoblot analysis of PE-18 and PE-25 against different regions of the proenkephalin sequence - PPE-(1-267), PPE-(69-207), PPE-(1-131) and PPE-(130-267). Both Mabs recognise a central region of the proenkephalin molecule

Figure 6.1

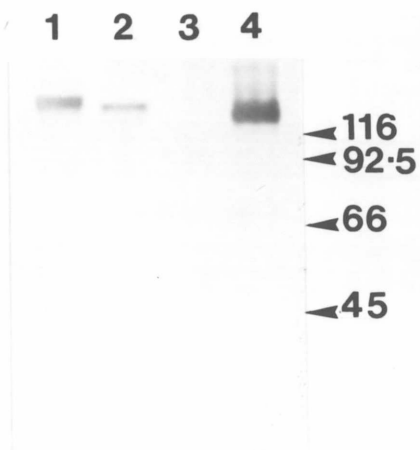
β -galactosidase - human preproenkephalin fusion proteins used for immunisation and epitope mapping.

The primary sequence of mammalian preproenkephalin A is illustrated in the upper panel. The fusion protein β-galactosidase-PPE-(1-267), incorporating the entire 267 amino acid sequence of preproenkephalin, was used for immunisation. Preliminary epitope mapping of the Mabs PE-18 and PE-25 by immunoblot analysis of the purified fusion proteins β-gal-PPE-(1-267), -PPE-(69-207), -PPE-(1-131), and -PPE-(130-267) is shown in the lower panel (Lanes 1 to 4 respectively).


signal
Met-enk
Met-enk-RGL
Met-enk-RF
Leu-enk
PREPROENKEPHALIN A
PPE-(1-267)
PPE-(69-207)
PPE-(1-131)
PPE-(130-267)


1 2 3 4

PE-25


1 2 3 4
116
92·5
66
45

PE-18

between amino acids 130 and 207, common to three of the four fusion proteins. As discussed in Chapter 3, attempts to subdivide this region to more tightly define the epitopes were unsuccessful, perhaps due to conformational dependence of the binding domains.

Panel A, Figure 6.2 illustrates the strong reactivity of PE-18 and PE-25 with the intact enkephalin precursor. This was determined by immunoblotting against total cell lysate of the human proenkephalin / PC12 transformed line which expresses high levels of unprocessed proenkephalin (Lanes 2). This strong reactivity of PE-18 and PE-25 contrasts with the very much weaker recognition of the intact prohormone by PE-1 (Lane 2 ,left hand strip). PE-1 is nonetheless able to recognise a subset of high molecular weight cleavage products in bovine chromaffin granules (Lane 1, left hand strip). At this stage, I can not comment with certainty on the relative ability of PE-18 and PE-25 to recognise primary cleavage products. Panel B, Figure 6.2 illustrates an immunoblot of PE-1, PE-18 and PE-25 against human, rat and bovine proenkephalin fusion proteins. Since PE-18 and PE-25 do not cross-react with bovine proenkephalin (Lanes 3), they can not be tested against bovine chromaffin granules. In contrast to the bovine adrenal, the normal rat adrenal is a poor source of proenkephalin-derived peptides, unless the gland is denervated which has been shown to stimulate proenkephalin synthesis (Kilpatrick *et al*; 1984). Using this means, it appears that PE-18 and PE-25 exhibit only very weak cross-

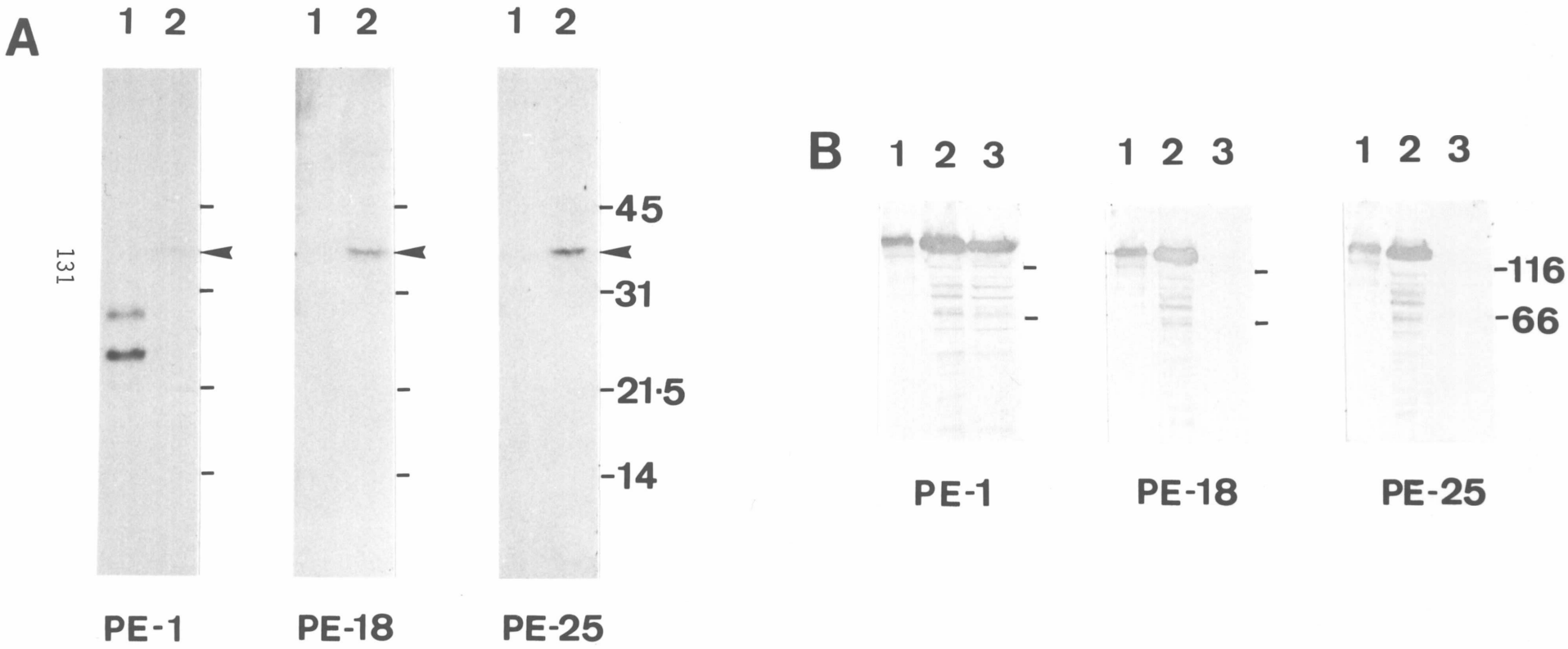
Figure 6.2

(A) Differential recognition of intact enkephalin precursor.

Bovine adrenomedullary chromaffin granule lysates (lanes 1) and whole cell lysates of PC12::hPE transformants (lanes 2) were immunoblotted against the PE Mabs. PE-18 and PE-25 exhibit much stronger recognition of intact proenkephalin (migrating as a 36kd band in lanes 2) than PE-1 which does, however, recognise high molecular weight proenkephalin derived peptides in bovine chromaffin granules (lane 1, first strip).

(B) Cross-species reactivity of the PE Mabs.

Human, rat and bovine proenkephalin expressed as β-galactosidase fusion proteins (lanes 1,2,3 respectively) were immunoblotted aginst the PE Mabs. PE-1 recognises all 3 fusion peptides whereas PE-18 and PE-25 cross-react with rat and not bovine proenkephalin.


A
1 2
1 2
1 2
-45
-31
-21·5
-14
PE-1
PE-18
PE-25
B
1 2 3
1 2 3
1 2 3
-116
-66
PE-1
PE-18
PE-25

reactivity with at least one high molecular weight cleavage product (migrating at 29kDa). However, proper characterisation of the molecular forms within the denervated rat adrenal has not to date been carried out. Nevertheless, if my interpretation is valid, this would suggest that the antibodies are predominantly precursor-directed. This could be explained if they were recognising conformational epitopes since even limited proteolytic cleavage might affect a complex tertiary state.

What I can say with certainty is that PE-18 and PE-25 appear significantly better able to recognise the intact enkephalin precursor than PE-1 which is nonetheless able to recognise high molecular weight cleavage products. A simple difference in antibody titre can be excluded by the fact that all three antibodies recognise identical amounts of human and rat proenkephalin fusion proteins with equivalent intensity (Panel, B, Figure 6.2). The relevance of this apparent predilection of PE-18 and PE-25 for the intact precursor , lies in the fact that PE-18 and PE-25 stain both neurons and glia whereas PE-1 stains neurons only. I interpret the presence of PE-1 immunoreactivity as indicative of the existence of high molecular weight cleavage products, indicating the operation of limited proteolytic processing. On the other hand, I provisionally interpret the absence of PE-1 immunoreactivity but presence of PE-18 and PE-25 immunoreactivity as suggestive of a predominance of uncleaved precursor, and therefore essentially absent proteolytic processing.

6.3 Immunofluorescent detection of proenkephalin within subpopulations of cerebellar neurons and astrocytes in cryostat sections

On indirect immunofluorescence of cerebellar cryostat sections from 25 and 40 day old rats, several of the PE Mabs gave identical staining of subsets of cerebellar neurons and astrocytes. However, there was no mature enkephalin peptide immunofluorescence on dual-labelling with conventional Met- and Leu-enkephalin ploYclonal antisera (Immunonuclear Corporation), indicating reduced proteolytic processing leading to an accumulation of enkephalin precursor forms. I had proven the immunohistochemical capability of the enkephalin antisera by staining striatum (caudate putamen and globus pallidus), a region rich in mature enkephalin peptides (data not shown). Control sections were incubated with PE Mab which had been preabsorbed with purified β-galactosidase-proenkephalin fusion protein, after which no astrocytes or neurons were stained.

PE-1 immunofluorescence is restricted to neurons, suggesting at least limited proteolytic processing is occurring in these cells, as discussed above. The absence of PE-1 immunoreactivity in astrocytes suggests that the uncleaved precursor predominates in this cell type.

-Neuronal expression

Figure 6.3 (Panels A and B) shows bright punctate staining of a cell type resembling Golgi cells, inhibitory interneurons, with the PE Mabs. From the numbers of cells

Figure 6.3.

Proenkephalin-like immunofluorescence in cerebellar neuronal and astroglial cells.

Cerebellar cryostat sections from 25 day old (Panels A,B) and 40 day old (Panels C,D) rats were incubated with PE-25, followed by FITC conjugated goat anti-mouse immunoglobulin. Sections were viewed using a Biorad / MRC Lasersharp Confocal microscope.

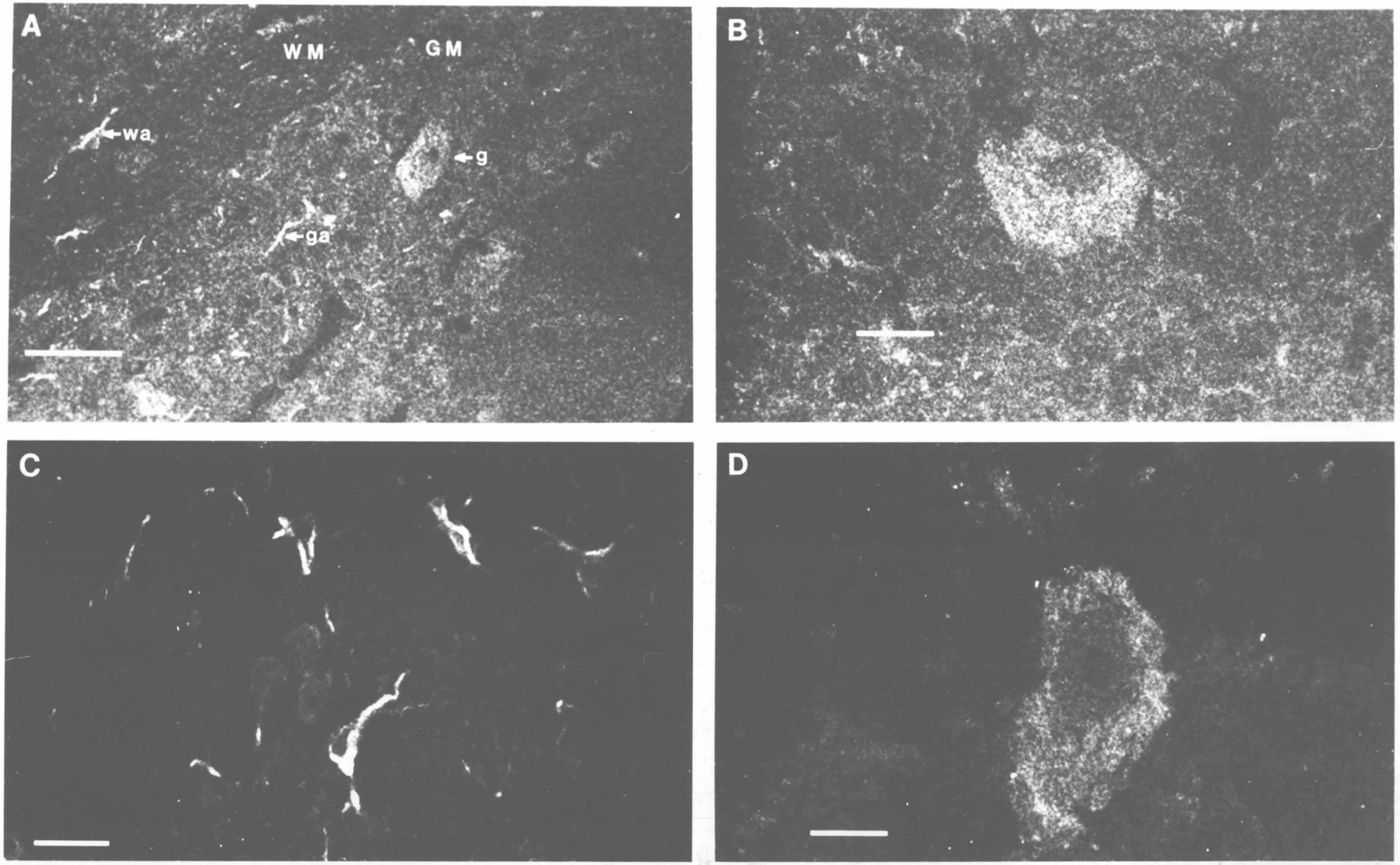
(A) White (wa) and grey (ga) matter astrocytes in a folia white matter tract (WM) and the granule cell layer (GM) are indicated. A brightly labelled Golgi cell (g) is also shown.

(B) Another Golgi cell under higher magnification.

(C) Central white matter astrocytes showing proenkephalin-like immunofluorescence.

(D) A macroneuron in a deep cerebellar nucleus labelled with PE Mab.

Scale bars are 25 (A) and 10 (B,C,D) microns.


A
WM
GM
wa
g
ga
B
C
D

stained, I estimate that the entire Golgi cell population may be expressing proenkephalin. Immunohistochemical staining, by the peroxidase-anti-peroxidase technique, of Golgi cells using an antibody to Met-enk-Arg^{6}-Gly^{7}-Leu^{8} has been described previously (Ibuki *et al.*, 1988). Furthermore, a previous in situ hybridisation study on cerebellum showed proenkephalin mRNA apparently within Golgi cells (Shivers *et al.*, 1986).

I also observed bright staining of a subpopulation of Purkinje cells. These are seen at the boundary between the molecular and granule cell layers (Figure 6.4, Panel A). A brightly stained cell, **"p"**, is flanked by non-labelled cells. The proportion of Purkinje cells which express proenkephalin varies between different folia and so is difficult to quantify. The observation that only a subset of the cells show expression accords with Shiver's finding (1986) that some Purkinje cells do not contain preproenkephalin mRNA. Interestingly, macroneurons within deep cerebellar nuclei, situated within deep central white matter, also show proenkephalin immunofluorescence, such as the cell shown in Panel D, Figure 6.3. Purkinje cell axons terminate within these cellular nuclei.

-Astrocytic expression

I also observed strong proenkephalin immunofluorescence of subpopulations of cells resembling astrocytes in both grey and white matter. Examples of PE-stained grey and white matter astrocytes (ga and wa) are shown in Figure 6.3, Panel A. White matter astrocytes

Figure 6.4

Proenkephalin expression in subpopulations of grey and white matter astrocytes.

Cerebellar cryostat sections from 25 day old (Panels A,B,C) and 40 day old (Panel D) rats were dual-labelled with either PE-18 (A,B) or PE-25 (C,D), and anti-GFAP antiserum, followed by FITC conjugated goat anti-mouse immunoglobulin and Texas red conjugated goat anti-rabbit immunoglobulin. Proenkephalin immunofluorescence (fluorescein) and GFAP fluorescence (Texas red) are shown in the left and right hand panels respectively, each pair representing an identical optical plane. All are confocal micrographs.

(A) A brightly labelled Purkinje cell (p) is indicated at the boundary between the molecular (ML) and granule cell (GL) layers. A grey matter astrocyte showing PE immunofluorescence is also shown (ga), with the corresponding cell visible in the GFAP fluorescence field.

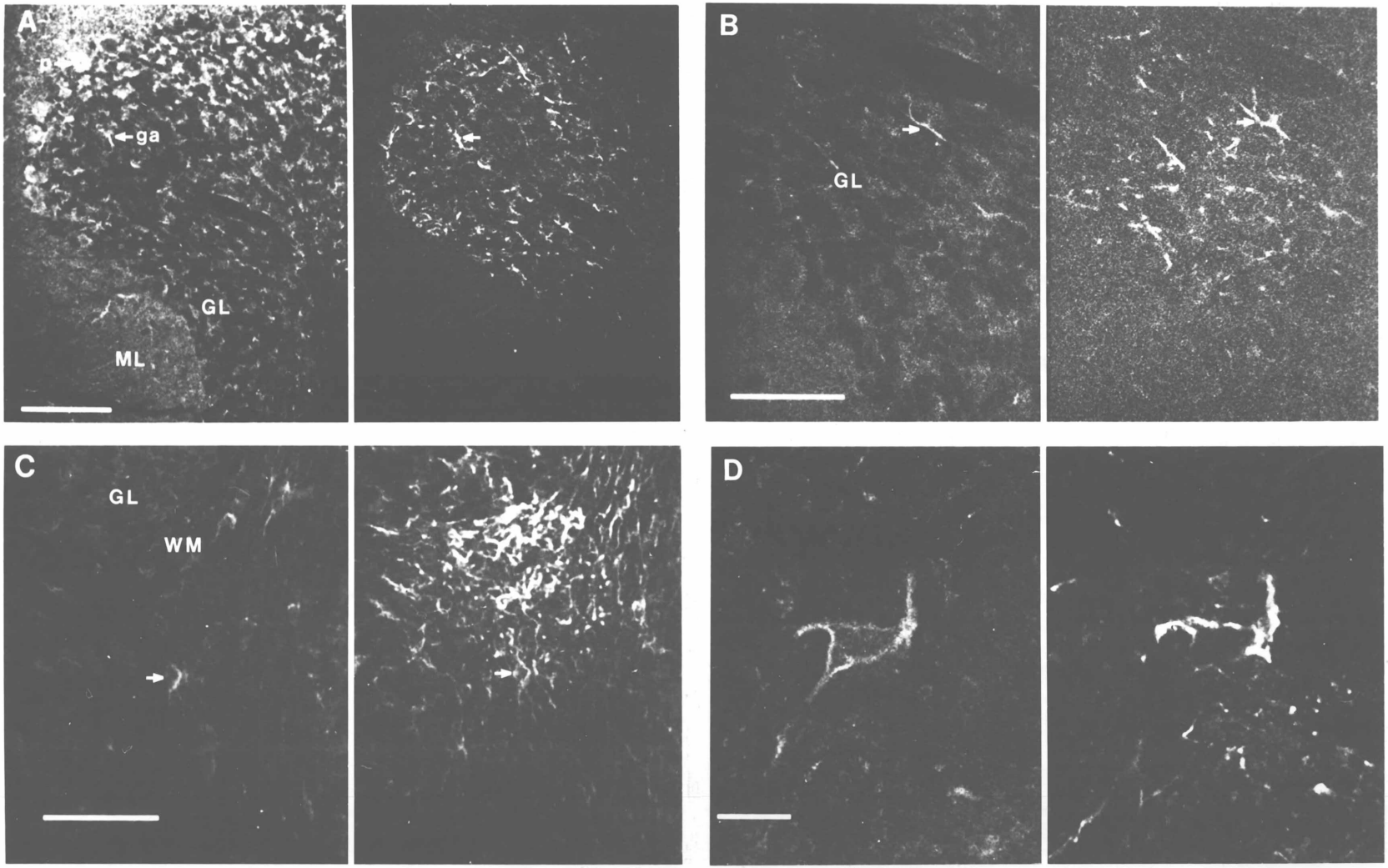
(B) Another grey matter astrocyte labelled with PE Mab (arrow), under higher magnification.

(C) An astrocyte showing PE immunofluorescence in central white matter is indicated by the arrow.

(D) A white matter astrocyte at higher magnification, showing differential PE and GFAP immunofluorescence.

Panels A and C illustrate that glia expressing proenkephalin comprise only a subpopulation of grey and white matter astrocytes.

Scale bars are 100 (A), 50 (B,C) and 10 (D) microns.


A
ga
GL
ML
B
GL
C
GL
WM
D
PE
GFAP
PE
GFAP

showing proenkephalin-like immunofluorescence are present throughout the cerebellar white matter tracts although they are more numerous in the central white matter. Panel C in Figure 6.3 shows a cluster of PE positive astrocytes in central white matter.

In order to confirm that these cells are indeed astrocytes, I performed dual labelling with a polyclonal antibody to a glial fibrillary acidic protein (GFAP) (Figure 6.4). GFAP is a major component of astrocytic intermediate filaments and so is a useful marker of this cell type in the central nervous system. All the cells which show proenkephalin immunofluorescence co-express GFAP, confirming their identity as astrocytes. This figure also demonstrates that those glia which express proenkephalin comprise only a subpopulation of both grey (Panels A and B) and white (Panel C) matter astrocytes. At low magnification the quality and distribution of the proenkephalin stain is very similar to the intermediate filament stain as revealed by the GFAP antibody. However, at higher magnification differential staining can be observed. Panel D in Figure 4 shows such a cell where there is an apparently different distribution of PE and GFAP, suggesting localisation with subsets of filaments which may be less rich in GFAP. This is a confocal micrograph, and therefore one is comparing staining at an identical optical plane.

A preliminary examination of some other brain regions reveals that proenkephalin-expressing astrocytes are not

confined to the cerebellum, since I find them in hypothalamus and striatum also, which is consistent with the data from Shinoda and co-workers (1989).

I have also undertaken a preliminary developmental profile which shows that proenkephalin immunofluorescence within astrocytes first appears between postnatal days 5 and 10, becomes maximal between 3 and 4 weeks and is maintained through to adulthood.

6.4 Confirmation of proenkephalin gene expression by in situ hybridisation

In order to confirm the existence of proenkephalin mRNA in neuronal and glial cells, I performed an in situ hybridisation upon sections from 25 day old animals using a ^{35}S labelled rat proenkephalin antisense riboprobe, combined with a GFAP immunostain. I was unable to perform simultaneous immunostaining for proenkephalin which did not withstand the vigorous hybridisation treatments. A sense riboprobe was used as a negative control.

After 8 days exposure, clusters of grains in locations consistent with presence of mRNA within neurons were seen. However, silver grain clusters within white matter did not become apparent until between 2 and 5 weeks of exposure (Figure 6.5). Each panel of this figure depicts GFAP immunofluorescence alone (left) alongside a combined bright field / fluorescence exposure. In Panel A, a rim of grain clusters is seen at the boundary between the molecular (ML) and granule (GL) cell layers,

Figure 6.5

Confirmation of proenkephalin synthesis by in situ hybridisation.

Cryostat sections from 25 day old rats were incubated with a ^{35}S labelled rat proenkephalin anti-sense riboprobe, followed by a GFAP immunostain and exposed for 5 weeks. Left hand panels show GFAP fluorescence; right hand panels show combined bright field and GFAP fluorescence.

(A) A rim of grain clusters in the Purkinje cell layer (P) at the boundary between the molecular (ML) and granule cell (GL) layers. A more deeply situated cluster may be over a Golgi cell or a grey matter astrocyte.

(B) A cluster of grains in a folia white matter tract (WM) is indicated by the arrow.

(C,D) At higher magnification, clusters of grains over a cerebellar white matter astrocyte (C) and a hypothalamic astrocyte (D) are shown.

A
GL
ML
P
B
GM
WM
C
D

compatible with expression of proenkephalin by Purkinje cells. Clusters of grains in the granule cell layer (Panels A and B) may be over either Golgi cells or grey matter astrocytes. The presence of proenkephalin mRNA in astrocytes is, however, confirmed by clusters of grains within within folial white matter tracts, where only glial cells exist, as in Panel B. The grain clusters obscure the penetration of the fluorescence signal and so give an apparent negative image on the GFAP fluorescence field. However, where the grains are less dense, it is possible to discern more clearly a signal over astrocytes, as in Panel C which shows an astrocyte in cerebellar white matter, and in Panel D which shows a hypothalamic astrocyte. However, clear dual labelling cannot be achieved due to impedance of the fluorescence signal by the silver grains. Instead, confirmation of expression is provided by the location of the grain clusters.

One initially disconcerting finding was that the number of grain clusters overall in cerebellar white matter were fewer than the apparent number of proenkephalin-immunostained astrocytes. However, on reflection this is not surprising given that the astrocytic perikarya occupy significantly less space than the sometimes widely ramifying astrocytic processes. Thus, the perikarya, potential sites of mRNA labelling, are likely to be represented in far fewer planar sections than the astrocytic processes which will nonetheless be immunostained. Furthermore, in order to preserve GFAP

immunofluorescence I was unable to use pronase treatment which may have compromised penetration of the probe.

6.5 Proenkephalin expression by cerebellar astrocytes in culture

In order to try to determine whether the subpopulation of astrocytes which express proenkephalin may correspond to a particular astrocyte subtype, I established primary cultures of cerebellar astrocytes from 8 day old rats. Cerebellar astrocytes in primary culture have previously been shown to have essentially two morphologies - irregularly epithelioid (flat) and stellate (process-bearing) (Wilkin *et al.*, 1983). The morphology and antigenic characteristics of such cells (Johnstone *et al.*, 1986; Levi *et al.*, 1986) suggest that they may correspond to the type-1 and type-2 astrocytes, respectively, first identified by Raff and co-workers (1983a) in cultures established from rat optic nerve. There is powerful evidence from the optic nerve system that type-1 and type-2 astrocytes derive from different cell lineages. *In vitro*, type-2 astrocytes originate from O-2A bipotential progenitor cells (Raff *et al.*, 1983b). O-2A cells are so named because they have the capacity to differentiate into oligodendrocytes in the absence of serum, or into type-2 astrocytes in the presence of serum. The existence in cerebellum of O-2A precursor cells and their progeny has not yet been proven, but their existence is suggested by the appearance of stellate (type-2-like)

astrocytes in culture only in the presence of serum (Levi *et al.*, 1986). These cells also bind the Mab LB_1, directed against the surface ganglioside GD_3, which does not recognise astrocytes (type-1-like) in the absence of serum.

A subpopulation of astrocytes showed proenkephalin-like immunofluorescence from 3 days in culture (from a postnatal day 8 animal) which became maximal between 9 and 12 days of culture. Dual-labelling with anti-GFAP antiserum confirmed that the PE positive cells were astrocytes and not oligodendrocytes or neurons. When the cultures are plated at a low density so that the cells are well separated, the astrocytes which clearly express proenkephalin are stellate in appearance (as in Figure 6.6). However, when the cells are more confluent, the PE positive cells are flatter in appearance and not so typically stellate (as in Figure 6.7). This tendency of stellate (type-2-like) astrocytes in cerebellar culture to assume a more epithelioid appearance in confluent conditions, presumably due to cell-cell contact, has been documented previously and does not seem to be associated with a class switch (Wilkin *et al.*, 1983). More than 95% of the proenkephalin-expressing cells co-express the surface ganglioside GD_3, as detected with the Mab LB_1 (Panels E,F; Figure 6.6), another property by which they resemble type-2-like cells of the O-2A lineage. Although the ganglioside GD_3 may be a surface antigen marker for the O-2A lineage in culture, GD_3 has not been identified

Figure 6.6

Proenkephalin expression by process-bearing astrocytes in primary cerebellar glial cultures.

Astrocyte cultures were established from postnatal day 8 rat cerebella. Cells were fixed and immunostained as described in the text, with conditions appropriate for the particular antigen. Cells shown were immunostained after either 6 or 9 days in culture and viewed with the confocal microscope.

(A) A process-bearing cell single labelled with PE-25 followed by FITC conjugated goat anti-mouse immunoglobulin. The inset shows the nuclear region of the same cell at a different optical plane, under higher magnification.

(B) Another process-bearing cell immunostained as above shows a striking axial concentration of proenkephalin.

(C,D) A cell dual-labelled with PE-18 (fluorescein) and anti-Metenkephalin (Texas red) (C,D respectively) shows proenkephalin immunofluorescence only.

(E,F) A cell dual-labelled with PE-25 (fluorescein) and LB_1 (Texas red) (E,F respectively) shows co-expression of proenkephalin and GD_3.

Scale bars are 25, 12.5 (A, A inset), 25 (B,C,D) and 50 (E,F,) microns.

A
C
D
B
E
F

Figure 6.7

Co-staining of astrocytes in primary culture with anti-proenkephalin monoclonal antibodies and anti-GFAP.

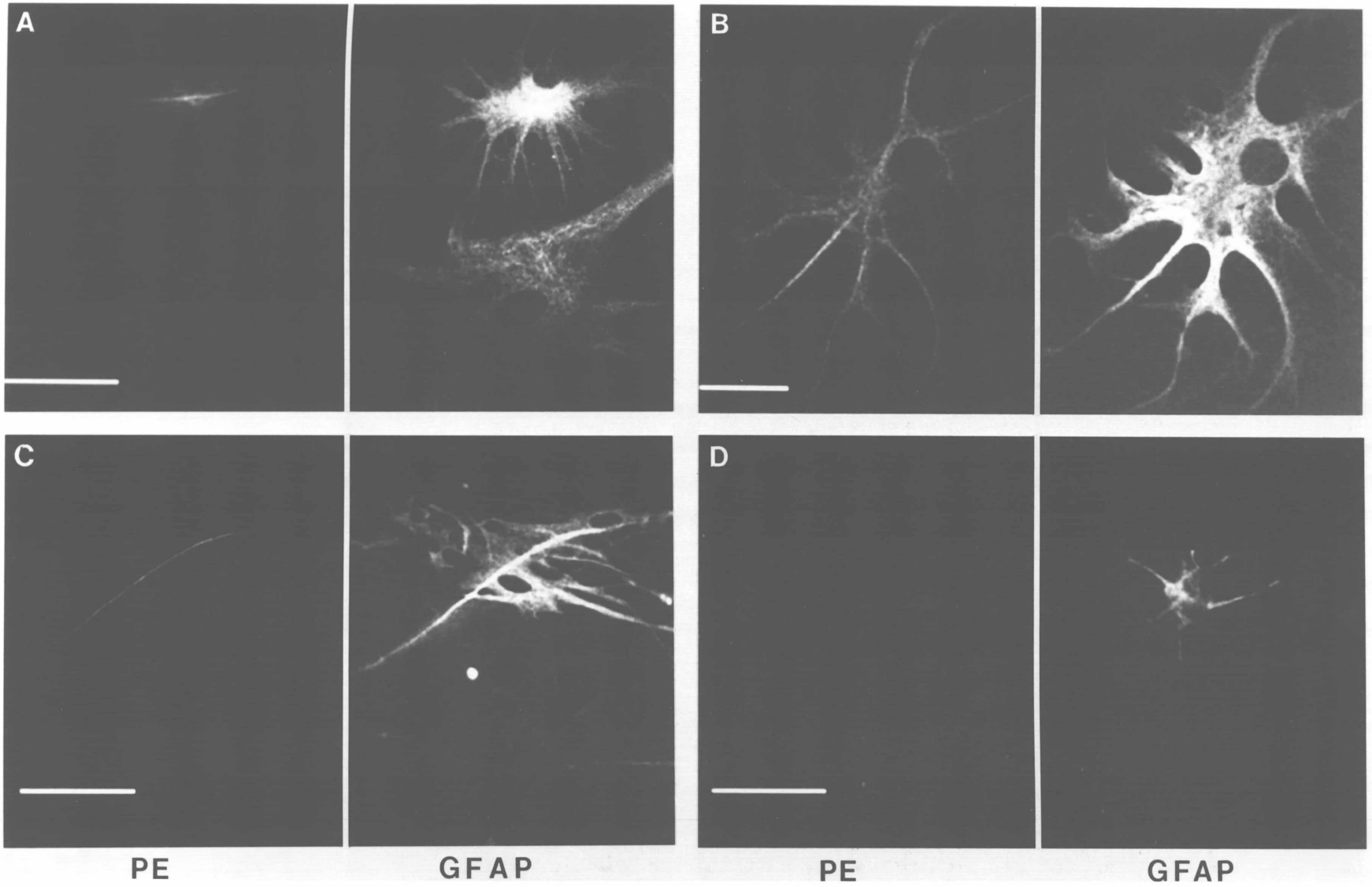
Astrocytes in dissociated cerebellar glial culture were dual labelled with PE-25 and anti-GFAP followed by FITC conjugated goat anti-mouse and Texas red conjugated anti-rabbit immunoglobulins. All are confocal micrographs: fluorescein optics - left hand panels; Texas red - right hand panels. These cells were at a higher density than those shown in Figure 6.6 which can influence the morphological appearance (see text).

(A) GFAP field shows examples of astrocytes with stellate-like (upper) and epithelioid-like (lower) morphology. Proenkephalin fluorescence is restricted to the cell with the stellate-like morphology.

Panels A, B, C all show a striking concentration of proenkephalin stain along a longitudinal axis.

(D) A process bearing cell, displaying strong GFAP fluorescence but absent (left hand panel) proenkephalin fluorescence.

Scale bars are 50 (A,C,D) and 25 (B) microns.


A
B
C
D
PE
GFAP
PE
GFAP

on astrocytes in sections of cerebellum (Goldman *et al.*, 1984; Curtis *et al.*; 1988). In view of this, I was unable to look for evidence of co-expression of GD_3 in sections.

All the panels in Figure 6.6 show strongly proenkephalin positive stellate astrocytes grown in subconfluent conditions. Cell counts performed when the cells are well separated like this reveal that the proportion of cells expressing proenkephalin, and the magnitude of expression, increase with time. After 2 days in culture, there are no clearly proenkephalin positive cells; after 6 days, between 10 and 20%, and after 8 days 30 to 40%, of all GD_3 positive process-bearing cells show strong proenkephalin immunostaining, with additional process-bearing cells showing a lesser degree of fluorescence, indicating graded expression. At all stages examined, there are some stellate astrocytes which do not express proenkephalin (as in Panel D, Figure 6.7).

Generally speaking, the proenkephalin immunostain is fibrous in nature and is stronger in some processes than in others (Panels A,B,C; Figure 6.6). At higher magnification, it is sometimes possible to discern low-level coalescence of proenkephalin-like material in the cytoplasm of the cell body (not shown), in addition to the diffuse stain of the processes. In addition, a coarse punctate stain can sometimes be seen over the nucleus, together with a bright rim of perinuclear staining (as in inset, Panel A, Figure 6.6; same cell as in the main picture in a different optical plane). Panels C and D in

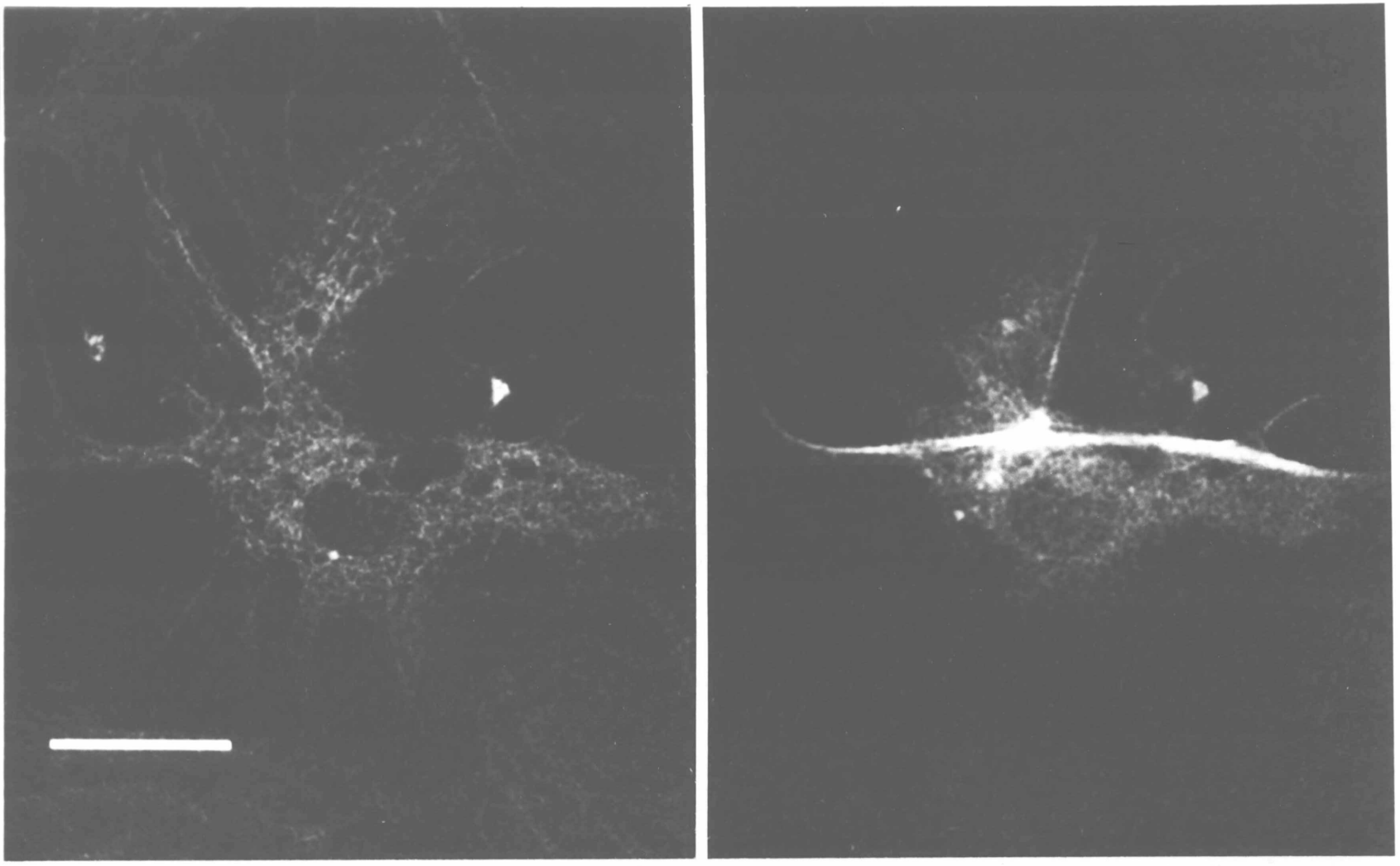
Figure 6.6 show a cell dual-labelled with PE Mab (C) and anti-Met-enkephalin (D), confirming absence of Met-enkephalin immunoreactivity.

Many of the proenkephalin-expressing cells display a striking axial concentration of the molecule (as in Panel B, Figure 6.6). This is also apparent in cells dual-labelled with PE Mab and anti-GFAP (Panels A,B,C; Figure 6.7). In these more epithelioid cells the restricted nature of the proenkephalin immunostain is particularly striking in comparison with the widespread distribution of GFAP throughout the cell. Overall, our impression from the quality and distribution of the stain is that proenkephalin appears to be co-localising with subsets of intermediate filaments. As this was a somewhat surprising finding, I also looked for microtubule association using a rat monoclonal anti-tubulin antibody (YL1/2) (Figure 6.8). Although microtubules are relatively sparse in mature fibrous astrocytes (see Peters, Palay and Webster, 1976), it was nonetheless apparent that microtubules were diffusely scattered throughout the cell and there did not appear to be any enrichment coincident with the proenkephalin stain. However, it remains possible that proenkephalin is associated with a subset of microtubules which may in turn be co-tracking with a subset of intermediate filaments.

Figure 6.8

Proenkephalin does not appear to be associated with microtubules.

Astrocytes in culture were fixed with methanol and acetone, then dual-labelled with anti-tubulin (YL1/2) and PE-25, as described in the text, and viewed with the confocal microscope. (Tubulin stain - fluorescein optics; PE stain - Texas red optics). The tubulin stain reveals that microtubules are diffusely scattered throughout the cell. In contrast, the proenkephalin molecule is concentrated heavily along a longitudinal axis, with no obvious enrichment of microtubules in this region.



Tubulin

PE

6.6 Discussion

I was surprised at the strength of the proenkephalin immunofluorescence given that previous workers have found little enkephalin immunostaining of adult rat cerebellum. The discrepancy between precursor forms and mature enkephalin peptides indicates markedly reduced proteolytic processing which suggests particular biological significance of enkephalin precursor peptides in this region of brain. The finding of apparently uncleaved proenkephalin within astrocytes is particularly intriguing. Its existence within astrocytes in all the brain regions I have examined so far suggests the possibility of a fundamental role in this cell type. Mature enkephalin peptides have previously been detected in germinative cells of 10 day old, but not adult, rat cerebellum (Zagon *et al.*, 1985). This would be in keeping with the detection of opiate binding activity only in the developing rat cerebellum (Tsang *et al.*, 1982). These previous studies suggest a role for small enkephalin peptides in cerebellar development. On the other hand, my results lead me to speculate that in the adult rat cerebellum, enkephalin precursor forms might have a non-opiate role which is quite distinct from that of the enkephalin peptides themselves.

6.6.1 Enkephalin precursor forms within cerebellar neurons

The observation of proenkephalin immunofluorescence in Golgi cells was not a totally unexpected finding. Met-

enkephalin-Arg^6-Gly^7-Leu^8 (Met-enk-RGL) immunofluorescence in Golgi cells has previously been reported in association with γ-aminobutyric acid (GABA) (Ibuki *et al.*, 1988). COOH-terminally directed antibodies to Met-enk-RGL often cross-react with high molecular weight proenkephalin-derived peptides which terminate with this sequence. Therefore, this result is consistent with my own finding of PE-1 immunofluorescence which together suggest the presence of high molecular weight cleavage products, indicating limited proteolytic processing in Golgi cells.

The somata of a subpopulation of Purkinje cells also show strong proenkephalin immunofluorescence. Proenkephalin gene expression in these cells is confirmed by the in situ hybridisation experiment (Figure 6.5). It is interesting that there is also fluorescence in macroneurons within deep cerebellar nuclei upon which Purkinje cell axons terminate. This might suggest participation of proenkephalin and its high molecular weight cleavage products in the efferent arm of the cerebellar neuronal circuitry.

6.6.2 Proenkephalin in a subpopulation of astrocytes

In cerebellar sections, subpopulations of both grey and white matter astrocytes show strong proenkephalin immunofluorescence. From an examination of earlier postnatal stages, this appears to be a relatively late developmental phenomenon, since astrocytes which express proenkephalin do not appear until the second postnatal

week. The staining then progressively increases and is maintained through at least to young adulthood. The proportion of glia which express proenkephalin also increases with time. By 6 weeks, the majority of astrocytes within central white matter do express proenkephalin, although in folial white matter and in grey matter a smaller proportion of the total astrocyte population show expression. The staining is unequivocal with six of the PE Mabs, and can be reproduced in dissociated glial cultures established from 8 day old rat cerebella. Proenkephalin mRNA has been reported in cultures of astrocytes from different brain regions, including cerebellum (Vilijn *et al.*, 1988; Shinoda *et al.*, 1989), and also in the rat C6 glioma cell line (Yoshikawa and Sabol, 1986a). Our findings of proenkephalin in astrocytes both in cryostat sections and in culture, confirms that these previous reports of proenkephalin mRNA in astrocytes are not due to a tissue culture artefact. One apparent difference however, is that I find that proenkephalin expression by astrocytes is a relatively late developmental phenomenon and increases through to adulthood. Nevertheless, although these previous studies analysed cultures prepared from embryonic and early postnatal rats, in both cases analysis of mRNA was not carried out until after 3 to 4 weeks in culture. Since astrocytes have been shown to be capable of developing *in vitro* on the same schedule as *in vivo* (Williams *et al.*, 1985), the effective developmental stage of these cultures

will have been much later.

In dissociated glial cultures from cerebella of postnatal day 8 animals, I found striking proenkephalin staining of a subpopulation of process-bearing astrocytes from 3 days in culture which became maximal between 9 and 12 days. These time points can be regarded as roughly equivalent to postnatal day 11, 17 and 21 respectively. Thus, on a preliminary assessment, the temporal expression of proenkephalin in culture would seem to parallel that in the developing brain where proenkephalin-expressing astrocytes first appear some time between postnatal days 5 and 10. Proenkephalin positive cells in subconfluent culture exhibit a stellate phenotype, all express GFAP and more than 95% co-express the surface ganglioside GD_3 as detected with the Mab LB_1. In these respects, these cells resemble type-2 astrocytes first described by Raff and co-workers (1983a) in rat optic nerve cultures. However, astrocyte classification is controversial as reliable, universally accepted surface antigen markers for the respective lineages are not available.

6.6.3 **Implications**

There has been accumulating evidence of unique properties of type-2-like as compared with type-1-like astrocytes. Indeed, some of their properties are more classically "neuronal". For example, they can bind tetanus toxin (Raff *et al.*; 1983a), and they exhibit "neuron-like" GABA transport (Johnstone *et al.*, 1986). Furthermore,

they display a complex ion channel phenotype (Barres *et al.*, 1988), and there has been a recent demonstration of glutamate activated ion channels in type-2 cerebellar astrocytes in culture (Usowicz *et al.*, 1989). Neuropeptide gene expression therefore provides a further dimension to the remarkable hybrid mixture of neuronal and glial properties displayed in this cell type.

The apparent predominance of uncleaved proenkephalin within cerebellar astrocytes would be in keeping with the previous finding of largely unprocessed proenkephalin in the C6 glioma cell line (Yoshikawa and Sabol, 1986b), which may therefore represent a clonal line of the subpopulation of proenkephalin-expressing astrocytes. It is also in agreement with the findings of Shinoda and co-workers (1989) who detected free Met-enkephalin in cortical and striatal astrocytes, and not in cerebellar astrocytes. Interestingly, free Met-enkephalin levels were much higher in striatal than in cortical astrocytes, despite comparable mRNA levels. Taken together with my findings, these data suggest that astrocytes from different brain regions may exhibit differential proteolytic processing, in an analogous way to neurons. With respect to my own data, I find it quite remarkable that such an unstable molecule, which is readily cleaved by a variety of proteases, should be present in such apparent abundance within astrocytes. This leads one to wonder if there may be a mechanism operating to prevent proteolytic cleavage of proenkephalin. One possibility is

that the molecule is folding in a very different way in these cells, restricting the accessibility of cleavage sites. However, the molecule already has a significant degree of tertiary complexity even in cells which process the molecule, as evidenced by the inability of enkephalin antibodies to cross-react with the intact prohormone. Such conformation could be a means of generating ordered rather than random cleavages. Thus, folding of the protein may be an insufficient explanation for protection of the molecule to this degree. Interestingly, a factor which has potent serine protease inhibitory activity has been purified from medium conditioned by C6 glioma cells (Guenther *et al.*, 1985) and which has been shown to belong to the family of protease inhibitors, the protease nexins (Gloor *et al.*, 1986). This glia-derived nexin promotes neurite outgrowth and regulates migration of neuronal cells. It is tempting to speculate that co-expression, and perhaps co-packaging, with this serine protease inhibitor could protect proenkephalin from attack by cellular proteases.

The distribution and diffuse, fibrous nature of the proenkephalin stain within the cultured astrocytes strongly suggests co-localisation with a subset of intermediate filaments. I was somewhat surprised at this finding since there is no precedent for association of such a molecule with intermediate filaments. However, since this is cell type not known to possess storage granules, (for a review of the ultrastructure of "fibrous" astrocytes see Peters, Palay and Webster, 1976), there are

no immediately obvious candidate organelles for the packaging and transport of such a molecule. Thus, the absence of punctate staining is not too surprising. On the other hand, association with filamentous subsets of cytoskeletal elements could be a means of generating the sometimes striking axial concentration of the molecule. The purpose of this longitudinal localisation is at this stage very difficult to predict. Perhaps one might speculate astrocytic outgrowth and thereby orientation of the cell, or maybe even in directing the migration of neurons along the glial cell, if the molecule were presented at the cell surface.

Although neuropeptide genes were among the first to be cloned, the relevance of the encoded propeptide precursor molecules has remained unclear, leading to an assumption that biological activity requires liberation of the mature peptides. My finding of proenkephalin in cerebellar neurons and astrocytes suggests a non-opiate role for the enkephalin precursor, distinct from that of its processed products. The elucidation of the role of the precursor within astrocytes, together with an ultrastructural analysis of its subcellular distribution, will be particularly challenging.

CHAPTER 7

DISCUSSION

I have succeeded in using a recombinant DNA approach to generate a series of monoclonal antibodies to the mammalian enkephalin precursor, proenkephalin A. Having applied this method to one of the most complex and unstable of the neuropeptide precursors, the technique should now be widely applicable to other propeptide molecules. The availablity of antibodies to such molecules should greatly facilitate the understanding of complex biosynthetic pathways involved in the generation of multiple potentially bioactive products of a single gene.

7.1 Fusion proteins

Since proenkephalin is a highly unstable molecule and therefore difficult to purify, the approach I took was to generate hybrid genes consisting of proenkephalin cDNA fused to the *lac*Z gene which were expressed within *E. coli* as fusion polypeptides. After purification, these chimaeric molecules could be used directly as immunogens. Coupling of the "target" sequence to a bacterial gene product serves two purposes. Firstly, it appears to protect the eukaryotic component from proteolytic degradation within the bacterium. Secondly, the hybrid polypeptide effectively provides a ready-made "hapten-carrier" complex for immunisation. Within the fusion polypeptide, the large "carrier moeity" (in this case β-galactosidase) may provide "T-cell epitopes" which could theoretically stimulate the immune response to B-cell

epitopes of proenkephalin by binding to T-helper cells (Lamb *et al.*, 1987). (The study by Lamb and co-workers demonstrated stimulation of cloned T-cells by expressed cDNA fragments of a tuberculosis antigen. This would suggest a mechanism independent of major histocompatibility complex molecules.)

A theoretical difficulty of covalent conjugation to a highly immunogenic bacterial protein is that the desired response may be effectively overshadowed, at least in the initial stages of immunisation. My own results demonstrated a striking immunodominance of β-galactosidase over proenkephalin. I was alerted to this at an early stage as my initial screening method involved comparing fused and unfused β-galactosidase in an ELISA system. This approach was disappointing, however, as residual splenocytes which had not been fused to myeloma cells secreted sufficient anti-β-galactosidase to mask any anti-proenkephalin activity secreted by hybridoma clones. The degree of immunodominance was a problem again at a later stage when I screened clones from immunisation with the full-length proenkephalin sequence against a CAT construct which happened to contain 17 amino acids of β-galactosidase which had been carried over during the subcloning procedure. Equivalent constructs had however been successfully used by Dr. David Lane's laboratory at the ICRF to generate antibodies to large T (personal communication). It was my intention to perform a secondary screen to exclude any antibodies binding to this short β-

galactosidase sequence. However, as it turned out, all 10 of the positive clones were directed to this 17 amino acid "contaminating sequence" (in the middle of the fusion protein) and **none** were directed to the 267 amino acid preproenkephalin sequence. An immune response to the proenkephalin sequence was eventually obtained at a much later stage, presumably due to induction of tolerance to the bacterial moeity. In fact, it took nearly 18 months to elicit an immune response to the full-length proenkephalin sequence. This was despite optimal immunisation conditions, such as long intervals between booster injections to allow antibody titre to decline. It is my assumption that the proenkephalin portion of the protein was presented in a "familiar" way to the animal. It could be relevant that the animals were near the end of their life-span before they mounted an immune response. This could have been because they were less tolerant of "self" which is a common feature of old age.

The poor immunogenicity of proenkephalin made it imperative to devise a screening approach where anti-β-galactosidase activity could be disregarded. To achieve this, I generated hybrid polypeptides consisting of a proenkephalin sequence identical to that used in the original immunogen, but fused to a different bacterial gene product. As discussed above, it later became apparent that it was imperative to screen against constructs devoid of any β-galactosidase sequence. To screen antibodies raised to the central proenkephalin segment, I made a

fusion peptide containing 31 amino acids of the phage lambda *cII* gene product. As this protein was considerably smaller than the corresponding β-galactosidase fusion protein, and present in much lower abundance, gel purification would have been difficult to achieve. Initially, I chose an ELISA method of screening, comparing total lysates from bacteria expressing the fusion proteim with lysates containing the unfused *cII* gene product. This was unsuccessful, however, as the overall sensitivity of the system was compromised by the high total levels of bacterial protein. Instead, I screened against *cII*-proenkephalin fusion protein blotted onto nitrocellulose after SDS-PAGE fractionation of total bacterial lysates, as described in Chapter 3. At the outset, I was concerned that using an immunoblotting screening method might prejudice against the selection of antibodies to conformational epitopes. However, it is now thought by some that protein renaturation can occur to a degree during the blotting procedure. Indeed, preliminary data suggests that at least some of my antibodies are to 3-D epitopes. What is perhaps more surprising is that any immune response to apparently complex epitopes has been achieved, given that no attempt was made to renature the fusion protein following gel purification. However, it has been the experience of others that in many cases this is not necessary as the protein re-folds following injection into the animal. For example, monoclonal antibodies raised to SV40 T antigen fusion proteins injected in a denatured

form are able to immunoprecipitate T antigen, indicating an idiotypic response to "native" epitopes (Mole and Lane, 1985). In my own case, I have had some difficulty in immunoprecipitating proenkephalin from the PC12::hPE transformants which is probably related to the instability of the proenkephalin molecule.

7.2 Application of the Mabs in the investigation of the cell type specific processing of proenkephalin

I now have a comprehensive range of antibodies which display differential recognition of intact proenkephalin and subsets of high molecular weight cleavage products. None of the antibodies recognise mature enkephalin peptides, terminal products of proteolytic processing, for which there are a variety of commercially available antisera. They will therefore provide powerful reagents with which to study tissue specific and cell type specific patterns of proteolysis. Furthermore, since they work well in immunofluorescence, they will enable intracellular immunolocalisation studies to be carried out. These will provide information on proteolytic processing in relation to compartmentalistion within the cell.

Variations in proteolytic processing in different cell types could occur for several reasons. There may be differential expression of specific endoproteases; compartmentalisation in distinct populations of secretory vesicles with different intragranular environments could lead to selective cleavage events; or, differences in

post-translational modifications could lead to variation in the accessibility of cleavage sites due to differences in folding. As discussed in Chapter 1, there is now good evidence that primary cleavage events of complex peptide precursors such as the pro-ELH, can be initiated within the trans-Golgi, with subsequent cleavages occurring in secretory vesicles. If cleavage is initiated within the Golgi, there is then the potential for different primary cleavage products to be sorted into different subsets of vesicles (Fisher *et al.*, 1988). This gave important insight into possible cellular mechanisms for the generation of diverse patterns of proteolysis even within a single cell type. Furthermore, segregation into different vesicle populations could theoretically allow differential release of particular subsets of cleavage products.

Since the antiproenkephalin Mabs exhibit differential recognition of the precursor and subsets of high molecular weight cleavage products, they will allow the study of initial cleavage events of the precursor. The Mabs PE-1 and PE-2 are able to distinguish between subsets of high molecular weight cleavage products which have been proteolytically shortened either from the NH_2 or COOH-terminus of the molecule. Interestingly, my data suggest that such forms can co-exist within a single cell type, the adrenal chromaffin cell (Chapter 3 and Spruce *et al.*, 1988). I propose to go on now to examine the subcellular localisation of proenkephalin and its cleavage products

within primary cultures of adrenal chromaffin cells. If particular secretory vesicles demonstrate specific PE-1 or PE-2 immunoreactivity, this suggests that distinct vesicle populations are responsible for cleavages initiated from opposite termini. This may arise either from differential expression of specific processing enzymes, or from differing intragranular environments which allow only selective proteolysis. Another interesting question to address would be whether the precursor is cleaved at the level of the trans-Golgi. Certainly, in chromaffin granule isolates there is only a very small amount of the 35-kDa form which I speculate may represent the intact precursor. This could be explained if the majority of molecules had undergone their first cleavage within the Golgi apparatus. If this is the case, precursor immunoreactivity would be expected to be largely confined to the Golgi apparatus and absent from mature secretory vesicles.

Final steps in the processing pathway involved in the liberation of free enkephalin peptides will also be worthy of examination at the subcellular level. To investigate this, I will compare immunoreactivity with antibodies such as PE-1 and PE-2 that recognise intermediates in the processing pathway, with antibodies to mature enkephalin peptides which do not cross-react with extended forms. It is perhaps over-simplistic to assume that all proenkephalin-derived peptides will be localised in the dense core vesicle component of the regulated secretory pathway. It is now appreciated that neuroendocrine cells

also possess small electrolucent vesicles (SELs) which lack a dense core and which also participate in regulated secretion. These SELs are the equivalent of small synaptic vesicles found at nerve terminals. Such organelles undergo more rapid exocytosis after receipt of an appropriate stimulus than their dense core counterparts and are thought to be involved in neurotransmitter release. There is electrophysiological evidence that enkephalins can initiate action potentials (Henderson, 1983), thereby acting as classical neurotransmitters, in addition to acting as neuromodulators and neurohormones. If this is the case, one might anticipate that at least a proportion of the small enkephalin peptides reside in SELs, to be available for rapid release, in addition to being contained within dense core vesicles.

7.2.1 **The investigation of intracellular trafficking by transfection of proenkephalin cDNA into neuroendocrine cell lines**

In order to extend my investigations into the processing of proenkephalin in relation to subcellular events, I propose to generate a number of neuroendocrine cell lines stably transformed with the proenkephalin gene. So far, I have generated stable transformants of PC12 cells which express high levels of the uncleaved enkephalin precursor. I wish now to generate a stable line which expresses high levels of processed products. The power of this approach lies in the fact that one can

directly investigate proteolytic processing in relation to intracellular trafficking by the introduction of mutated proenkephalin molecules with potentially altered "sorting domains" (for further discussion see section 7.2.2). Furthermore, introduction of the molecule into "heterologous cell lines" - cells which do not express the endogenous enkephalin gene - can also be used to study the fidelity of prohormone converting enzyme activity.

Available data so far suggests that some cell lines, such as AtT20 anterior pituitary cells, are capable of processing a wide range of "foreign" prohormones, in addition to their endogenous gene products. Other cell types appear markedly restricted in their processing capability. It is not surprising that cell lines which do not possess a regulated secretory pathway, such as NIH 3T3 cells, do not process transfected molecules. It is more surprising that some cell lines which do possess storage granules are also unable to process prohormone molecules, such as the GH_4C1 line (Thomas *et al.*, 1986). Thus, the presence of converting enzyme activity is not necessarily correlated with the possession of secretory granules.

Since some cell lines have a broad substrate range of processing enzyme activity, this would suggest that converting enzymes may not be specific for a particular prohormone. Furthermore, *in vitro* studies of purified processing enzyme activity have suggested a similar lack of specificity. However, it is then difficult to explain the diversity of proteolytic cleavage profiles seen *in*

vivo even in the same cell type, such as in enkephalinergic neurons in different brain regions. One possible explanation for the apparent lack of specificity of converting enzymes could be that a number of similar but discrete enzymic activities have been co-purified. It would seem not unreasonable if an analogous situation existed to that of the kallikrein enzymes which are involved in growth factor maturation. Cloning of the genes established that there is a kallikrein enzyme gene family which encodes enzymes with highly homologous regions responsible for enzymic activity but non-homologous regions lining the substrate binding pocket of the enzyme leading to substrate specificity (Mason *et al.*, 1983). This question will hopefully be solved when genes encoding prohormone converting enzymes are finally cloned, which a number of laboratories are working towards.

An alternative explanation for the diversity of proteolysis is that different local environments within the cell, for example differing pH and ionic concentrations, allow differential cleavages; or, alteration in post-translational modifications resulting in changed tertiary conformation around cleavage sites, could change enzyme affinity at these sites and thereby dictate altered cleavages. It is my opinion that a combination of all of these factors may well be necessary to generate the complexity of post-translational events. I am particularly interested in investigating processing events in relation to compartmentalisation within the

cell.

So far, I have generated stable transformant lines of PC12 cells which express high levels of unprocessed human proenkephalin, despite apparently correct targeting to secretory granules. This was somewhat surprising, given that PC12 cells process the endogenous proenkephalin, albeit present at much lower levels than the exogenous molecule. If processing enzymic activity is present at a comparably low level, this suggests coordinate regulation of a prohormone gene and its processing enzymes. I am presently exploring ways of attempting to induce the endogenous converting enzymes. So far, I have tried a variety of differentiation promoters, without success. I had been hopeful that sodium butyrate, an agent which apparently induces differentiation along the chromaffin cell pathway, might be effective, since it had previously been shown to stimulate processing of endogenous proenkephalin within PC12 cells. Reserpine was also ineffective, despite its ability to stimulate proenkephalin processing within primary chromaffin cell cultures. I want to proceed now to try chronic treatment with cyclic AMP or phorbol ester, both of which induce the enkephalin gene and may therefore stimulate its corresponding converting enzymes also. Another approach would be to "super-transfect" the PC12 / proenkephalin transformants with cloned genes of putative endoproteases. So far, only one is available - the gene encoding the yeast kex-2 protease (Julius *et al.*, 1984). Mammalian

endoprotease genes will hopefully be cloned soon.

A possible explanation for the poor processing capability of the PC12 transformants could be that the intragranular environment is not conducive to prohormone cleavage. For example, one unusual feature of granules within PC12 cells is that they lack chromogranin A, despite possessing chromogranin B. In Chapter 5, I speculated that possible interaction of chromogranin with proenkephalin might promote processing. It would be interesting to "super-transfect" chromogranin A cDNA into the transformed line to attempt to stimulate processing.

I have recently obtained preliminary evidence, in collaboration with Dr. Susan Jackson, that the PC12 transformants fail to release proenkephalin upon stimulation with a variety of secretagogues including 60mM potassium, carbachol, nicotine, cyclic AMP, forskolin and phorbol ester. This was determined by measurement of Met-enkephalin in the media before and after trypsinisation. Trypsinisation releases enkephalins from extended forms and provides an indirect means of measuring precursor forms. Surprisingly, only free Met-enkephalin was released (at levels comparable to those released from non-transformed cells and therefore compatible with endogenous viz. rat enkephalin), and not high molecular weight enkephalin (corresponding to exogenous viz. human, proenkephalin). These studies are currently being repeated to confirm this potentially interesting finding. One possibility could be that the molecule has been held

up in immature clathrin-coated vesicles (Orci *et al.*, 1987) which are not available for release. I propose to examine this possibility by immunolocalisation of the proenkephalin molecule after treatment of the transformed cells with nerve growth factor, which promotes neurite outgrowth. Immature granules under these circumstances would be localised proximally rather than out at the tips of the processes. Another reason for the failure of release could be that the intact precursor is membrane-bound, therefore preventing secretion of the molecule upon exocytosis. This will only be clarified by detailed analysis of the subcellular distribution of the molecule, both by biochemical fractionation and immunoEM examination, which I propose to carry out in due course.

7.2.2 **Immunological approaches to the investigation of protein trafficking**

In Chapter 5 the possible biological relevance of the shared antigenic determinant between proenkephalin and chromogranin A was discussed. There are examples in the literature of monoclonal antibody cross-reactions which have revealed meaningful homologies. For example, a Mab against the oncogene protein SV40 large T antigen specifically cross-reacts with a host cell protein, p68, which is involved in cell growth (Lane and Hoeffler, 1980). Possibly the most exciting biological reason for the shared epitope in chromogranin and proenkephalin could be that this represents a "sorting domain" responsible for

targeting these molecules to the regulated secretory pathway. One possible way of establishing whether the PE-15 epitope represents such a trafficking domain might be to microinject mRNA from PE-15 hybridoma cells into a suitable secretory cell line. A recent study from Dr. Huttner's laboratory described microinjection of mRNA encoding an anti-secretogranin I Mab into the PC12 cell line which contains abundant secretogranin I (chromogranin B) within its dense core granules (Rosa *et al.*, 1989). The translated immunoglobulin molecule would normally be secreted in a constitutive fashion, but in this case it became complexed with the endogenous secretogranin molecule and was diverted into the regulated pathway. Thus, if PE-15 hybridoma mRNA was microinjected into, for example, the PC12 line stably transformed with human proenkephalin, the translated immunoglobulin molecule might complex with proenkephalin and be transported via the regulated secretory pathway. However, if it were to neutralise a functionally important domain, in particular a sorting domain, then correct intracellular transport of the proenkephalin molecule could be prevented. Since this cell line is unusual in having negligible amounts of chromogranin A, the potential cross-reaction of PE-15 with CRGA, which may otherwise have confused the issue, can be disregarded.

Such an immunological approach to the investigation of protein trafficking and sorting had been pioneered a few years earlier. Burke and Warren in 1984 showed that

microinjection of mRNA encoding an anti-Golgi antibody inhibited intracellular transport of a viral membrane protein. However, the potential of such an approach to interfere with sorting information necessary for regulated as opposed to constitutive secretion had yet to be established. The alternative approach to this problem has centred on genetically engineered secretory molecules. For example, DNA molecules encoding chimaeric proteins possessing domains of regulated molecules fused to constitutive molecules have been transfected into cell lines to investigate effects on sorting. For example, it was found that a chimaeric protein comprised of growth hormone (a regulated secretory protein) fused to the G protein of vesicular stomatitis virus (a constitutively secreted protein) was packaged into regulated secretory granules of AtT20 cells (Moore and Kelly, 1986). Thus, it was suggested that "regulated information" was dominant over "constitutive information". More recently, the "pro" region of prosomatostatin fused to α-globin diverted this molecule into the regulated pathway (Stoller and Shields, 1989). Thus, "targeting information" was for the first time localised to a particular region of a molecule. Mutated secretory molecules have also been created, in the hope of inactivating sorting information. For example, DNA encoding truncated trypsinogen molecules was introduced into PC12 cells (Burgess *et al.*, 1987). However, the mutated molecules remained in the regulated pathway and this approach has not yet shown perturbation of transport

of such molecules. An inherent disadvantage in creating genetically altered secretory molecules is that tertiary folding may be altered which may in itself disrupt transport in a non-specific way. Thus, the immunological approach has a potential advantage since it does not require alteration of the structure of the secretory molecule. However, antibody binding might indirectly interfere with recognition of sorting information or with aggregation due to stearic hindrance.

Macromolecular interactions that could be important in intracellular trafficking may also be revealed by immunoprecipitation experiments. There are examples in the literature of monoclonal antibodies which have co-precipitated molecules bound to each other *in vivo*. For example, Mabs to the adenovirus E1A proteins were found to co-precipitate the 105kDa retinoblastoma gene product from adenovirus infected cells, due to complexing of E1A proteins with the 105kDa host cellular protein within these cells. Thus, Mabs have the potential to identify intermolecular interactions even in the absence of shared antigenic determinants. In Chapter 5, I speculated that the diversion of proenkephalin into dense core aggregates could be mediated by binding to chromogranin. One way of determining this would be to see if any of the anti-PE Mabs co-immunoprecipitate chromogranin A from cells co-expressing proenkephalin and chromogranin, such as adrenal chromaffin cells. This approach could also potentially reveal binding to other putative intracellular binding

proteins, such as the "sortases".

7.2.3 **Astrocytes: a novel type of neurosecretory cell?**

The expression of proenkephalin by glia was a surprising finding, since these cells have not previously been considered to be part of the neuroendocrine network. Ultrastructural analysis of the intracellular localisation of proenkephalin within astrocytes should pose interesting cell biological questions. The localisation of the molecule apparently in association with subsets of intermediate filaments may reveal novel pathways in intracellular trafficking. In "classical" neurosecretory cells, peptides are packaged into secretory vesicles which are transported to the cell periphery along microtubule networks. To date, there has been no convincing evidence for a transport function of intermediate filaments. Although I have yet to show that the proenkephalin molecule is released from astrocytes, the data from Shinoda and co-workers (1989) indicated that Met-enkephalin was released from cortical and striatal astrocytes in culture. However, there was only a very low level of basal ("constitutive") release which was essentially unresponsive to a secretagogue (forskolin). If the intact proenkephalin molecule is released, it will be interesting to determine if this occurs in a regulated manner. In typical neurendocrine cells, it has been observed that unprocessed prohormone molecules exit from the cell in a constitutive fashion (Moore and Kelly,

1983). However, rather than this being a chosen route of exit, it seems more likely that the phenomenon is due to defaulting molecules bypassing the secretory granule enzymes. If unprocessed proenkephalin is secreted in a regulated fashion, this would support the idea that the processed state of the molecule *per se* does not dictate its manner of exit. It remains possible of course that unprocessed proenkephalin has a purely intracellular role in astrocytes.

I was very surprised at the apparent co-localisation of proenkephalin with intermediate filaments and considered for some time, the possibility of an artefactual cross-reaction. Against this is the fact that only a subset of filaments within a subpopulation of astrocytes show immunoreactivity. Furthermore, five Mabs show an identical staining pattern. Whilst I cannot at this stage prove that they are directed to different epitopes, they do have different subclasses (IgG1 and IgG2a). All five antibodies give a strong punctate staining pattern in neurons but a "fibrous" stain in astrocytes. Perhaps the intracellular distribution is less surprising when one considers that, in classical ultrastructural studies, "fibrous" astrocytes have not been demonstrated to possess storage granules although they do possess heterogeneous electron dense bodies which are thought to be lysosomal. There are therefore no organelles which one would readily associate with the packaging and transport of a propeptide molecule. There

has been an interesting recent description of a 48-kDa intermediate filament associated protein in reactive-type astrocytes (Abd-el-Basset *et al.*, 1989). Although the immunoreactivity is apparently continuous along the filaments at the light microscope level, at the ultrastructural level the molecule is concentrated in pockets at frequent intervals along the filaments, in contrast to the uniform distribution of the integral filament protein, GFAP. It is not clear from the paper whether these foci are bounded by a membrane. However, these observations could provide insight into the unusual distribution of the proenkephalin molecule. I propose to perform an ultrastructural analysis in collaboration with Dr. John Lucocq in the Department of Biochemistry at Dundee. In the longer term it would be interesting to microinject mRNA encoding the PE Mabs into glia, to track the molecule on its subcellular route.

The findings of Shinoda and co-workers (1989) suggest that cortical and striatal, but not cerebellar, astrocytes are able to process the proenkephalin molecule to completion since they have detected free Met-enkephalin. This is difficult to reconcile with the fact that these cells may not possess classical storage vesicles. A possible explanation is that a proportion of the proenkephalin molecule has been targeted to lysosomes where it could undergo non-specific proteolysis to liberate free Met-enkephalin. In this situation, one would expect an absence of the characteristic processing

intermediates. Indeed, a description in Shinoda's paper of a chromatographic profile of cortical extracts indicates a single high molecular weight peak of trypsin-releasable Met-enkephalin (unprocessed precursor) with no "lower molecular weight products" (processing intermediates). This could be compatible with diversion of a proportion of the molecule to lysosomes. Another possibility is that different populations of astrocytes possess differential processing capability. My experiments indicate that unprocessed proenkephalin may be restricted to type-2-like astrocytes. However, the experiments of Vilijn and colleagues (1988) indicated that type-1-like astrocytes were synthesising proenkephalin mRNA as they used a method of culture which would have been expected to enrich for this population of cells. Taken together with the other available data, this may suggest that type-1-like astrocytes proteolytically process the molecule whereas type-2-like cells do not. Indeed, type-1 astrocytes would be expected to possess a specialised secretory system since they release growth factors including platelet derived growth factor (Richardson *et al.*, 1988) and ciliary neurotrophic factor-like activity (Lillien *et al.*, 1988) which are involved in the maturation of oligodendrocytes and type-2 astrocytes respectively.

The apparent abundance of unprocessed proenkephalin within cerebellar astrocytes is also a very surprising finding, given that the molecule is ordinarily unstable.

As discussed previously, altered folding of the molecule would seem unlikely to be the sole explanation. It is tempting to speculate that the lack of processing may be related to the co-expression of glia-derived nexin, a potent protease inhibitor (Gloor *et al.*, 1986). There are some interesting parallels between the expression of glia-derived nexin and that of proenkephalin. Both appear in the second post-natal week and are present only in subpopulations of GFAP positive cells. Both exist in the C6 glioma cell line which may represent a clonal line of the subpopulation of proenkephalin-expressing astrocytes. Interestingly, Schwann cells, peripheral glial cells, are rich in glia-derived nexin (D. Monard, personal communication) in which Dr. Ann Mudge (University College, London) has recently found proenkephalin immunoreactivity, using my antibodies. In C6 cells and primary astrocyte cultures, GDN immunoreactivity is strongest in the perinuclear region which may indicate concentration in the Golgi region. Since Golgi membrane-associated opioid precursor converting enzyme activity has been shown to be of a serine protease type (Demmer and Brand, 1986), it could be relevant that GDN is a specific serine protease inhibitor. It is not inconceivable that, if primary cleavage of the precursor were inhibited within the Golgi, subsequent processing events in the cascade could not take place. However, at this stage, these are no more than tantalising speculations. In the first instance, I propose to look carefully for co-expression of GDN and

proenkephalin, using an anti-GDN antibody kindly given to me by D. Monard. If an identical population of cells co-express the two molecules, it will be interesting to investigate further.

Since somatostatin peptides have also been detected in cerebellar astrocytes (Shinoda *et al.*, 1989), it will be interesting to compare the intracellular distribution of proenkephalin with somatostatin in these cells. In classical neurosecretory cells, both propeptides are targeted to dense core secretory vesicles. A different distribution of the molecules within astrocytes will raise the interesting possibility that sorting information can be interpreted differently in different cell types.

7.3 Concluding remarks

The existence of proenkephalin gene products within many different cell types suggests either wide-ranging functions or a fundamental role in cellular processes. Of particular interest, a number of studies have pointed to a developmental role for the gene. Preproenkephalin mRNA is expressed at high levels in mesodermal lineages during mouse development (Keshet *et al.*, 1989) and is also present in the developing rat heart (Springhorn and Claycomb, 1989). Despite mRNA levels in heart which are even higher than in brain, there is no free enkephalin peptide, which suggests lack of processing of the precursor (Howells *et al.*, 1986). It has been suggested that small enkephalin peptides may play a role in neuronal

development (Zagon *et al.*, 1985). My observations in the developing cerebellum indicate that the enkephalin precursor is expressed at a high level in early postnatal cerebellar neurons (unpublished) and is relatively down-regulated at later stages when it is confined to a small subpopulation of neurons which are nonetheless apparently rich in the molecule (Spruce *et al.*, 1990). The expression by glial cells appears later, in the second postnatal week, and increases through to adulthood (Spruce *et al.*, 1990). In this respect, the pattern of glial expression resembles that in the heart, which together suggests that the molecule could have a role in differentiation of the cell. It will clearly be important now to explore the role of proenkephalin in development which will be my aim in the longer term.

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