COMMENTARY

Identification of the adrenal protease that cleaves pro- γ -MSH: the dawning of a new era in adrenal physiology?

A B Bicknell

School of Animal and Microbial Sciences, The University of Reading, Whiteknights, PO Box 228, Reading, Berkshire RG6 6AJ, UK
(Requests for offprints should be addressed to A B Bicknell; Email: A.B.Bicknell@rdg.ac.uk)

Abstract

In respect to growth, the adrenal is a dynamic organ that requires constant stimuli from pituitary-derived POMC peptides to maintain its tonic state since either hypophysectomy or dexamethasone treatment results in rapid adrenal atrophy. It has been previously demonstrated that peptides derived from the N-terminus of the 16 kDa fragment of POMC not containing the γ -MSH sequence are potent adrenal mitogens both *in vitro* and *in vivo*.

However, since these shorter peptides are not found in the circulation, it has been suggested that they are generated by cleavage of the 16 kDa fragment by a specific protease expressed by the adrenal. This putative enzyme has recently been identified and this commentary describes the findings to date and highlights some of their possible implications.

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Introduction

The last few years of the 1970s were very significant years in the field of endocrinology. First, in 1977, Mains and colleagues (Mains *et al.* 1977) provided indirect evidence for the existence of pro-opiomelanocortin (POMC), the precursor of adrenocorticotrophin (ACTH), and 2 years later Nakanashi cloned the cDNA sequence encoding it (Nakanashi *et al.* 1979). A new era of multi-hormone polypeptide precursors was born and together with it a whole new series of implications for adrenal physiology. For the first time, it was unequivocally realised that ACTH was not the only hormone released from the pituitary during the stress response but co-secreted were two others, a 16 kDa N-terminal fragment that has become known as N-POMC(1–76) or pro- γ -MSH (pro- γ -melanocyte-stimulating hormone) and β -lipotrophin (β -LPH), which contains the β -endorphin sequence (Fig. 1).

Role of POMC peptides in adrenal growth

Evidence linking the maintenance of adrenal weight and the pituitary corticotroph sector (the cells that synthesise POMC) was well established since surgical removal of the pituitary (hypophysectomy) (Smith 1930) or suppression of corticotroph activity by dexamethasone administration

(Bransome 1968, Wright *et al.* 1974) results in rapid adrenal atrophy. The identity of the adrenal mitogen had been assumed to be ACTH since it was the only known product of the pituitary corticotroph and had been shown, albeit in hyperphysiological doses, to be mitogenic *in vivo* (Studzinski *et al.* 1963). In retrospect, it is possible that these early preparations of ACTH were contaminated with other pituitary peptides, but these results provided a simple explanation for the atrophy seen following hypophysectomy or prolonged steroid treatment.

The discovery of POMC raised the possibility that some of the other co-secreted peptides may play a part in adrenal maintenance. The first experimental evidence for this came from studies in rats where circulating ACTH was effectively neutralised by infusion of an anti-ACTH antibody. Although this resulted in a significant decrease in steroid levels, there was actually an increase in adrenal mitogenesis (Rao *et al.* 1978, Estivariz *et al.* 1982), suggesting that the decreased steroid levels promoted synthesis and release of POMC peptides. These results were further supported from experiments *in vitro* where it was shown that ACTH is antimitogenic to cultured rat adrenal cells (Hornsby 1984).

This evidence supported the notion that other POMC peptides distinct from ACTH may be involved with adrenal mitogenesis and prompted experiments to investigate if pro- γ -MSH was mitogenic towards the adrenal. It was quickly demonstrated that this glycopeptide

Figure 1 Processing of POMC in (A) the *pars distalis* and (B) the *pars intermedia*. All cleavages occur at dibasic residues shown as K (lysine) and R (arginine). POMC is N- (Asn⁶⁵) and O- (Thr⁴⁵) linked glycosylated (shown as N and O respectively). The O-linked glycan is of particular significance as cleavage of pro- γ -MSH to N-POMC(1-49) in the *pars intermedia* only occurs if it is absent. Recent work suggests that pro- γ -MSH secreted from the *pars distalis* is further cleaved at the adrenal by a serine protease (AsP) to generate N-POMC(1-52) that subsequently acts as an adrenal mitogen.

(it is both O and N linked glycosylated (Seidah & Chretien 1981)) had no trophic effect towards the adrenal in either hypophysectomised or dexamethasone-treated rats. However, the story was very different when in subsequent experiments shorter peptides lacking the γ -MSH sequence generated either by trypsin digestion or during purification from pituitary extracts were used. Using the incorporation of tritiated thymidine as a measure of hyperplasia, it was demonstrated that these peptides are

potent mitogens both *in vivo* and on isolated rat adrenal cells *in vitro* (Estivariz *et al.* 1982) whereas ACTH had no such properties. This finding posed an interesting question, namely that since these shorter peptides do not circulate in the blood how are they generated from pro- γ -MSH?

One model that has been used extensively to study the control of adrenal growth in the rat is that of the compensatory growth observed in the contralateral gland

Figure 2 Proposed model illustrating the interaction of POMC peptides with the adrenal cortex. ACTH and pro- γ -MSH are generated from the proteolytic processing of POMC in the pituitary (Fig. 1) and secreted into the circulation in response to stress. Pro- γ -MSH is consequently cleaved at the adrenal by AsP to generate the mitogenic fragment N-POMC(1–52) and (Des-Tyr¹, Des-Val²) γ_3 -MSH. N-POMC(1–52) binds to its receptor and results in increased cell proliferation while (Des-Tyr¹, Des-Val²) γ_3 -MSH binds to a distinct receptor (possibly antagonised by AgRP), resulting in the potentiation of the steroidogenic actions of ACTH mediated by the MC2 receptor.

following unilateral adrenalectomy. The control of this growth response has been clearly shown to be neurally mediated by activation of afferent and efferent nerve connections to and from the adrenal to the hypothalamus (Dallman *et al.* 1976, 1980). Several of these studies dismiss a hormonal role in the response on the basis that it will still occur in hypophysectomised animals. However, the data must be viewed with a degree of scepticism, as careful examination of the data would suggest that the so-called growth response is actually a slowing of the normal atrophy seen following hypophysectomy.

However the response also has a distinct hormonal input, and it was a study investigating this role that

provided the most convincing evidence for the mitogenic properties of the N-POMC peptides (Lowry *et al.* 1983). Using a series of different antisera to immunoneutralise specific POMC peptides it was demonstrated that the extreme N-terminal peptides were essential in eliciting the growth response, whereas immunoneutralisation of ACTH had no effect. However, the most intriguing result from this study was the finding that immunoneutralisation of the γ -MSH sequence completely inhibited the compensatory growth response. Bearing in mind the previous studies that had demonstrated that this peptide has no mitogenic properties and that its precursor pro- γ -MSH is the principal form present in the circulation, a hypothesis

was put forward. This proposed that pro- γ -MSH was specifically cleaved following secretion from the pituitary by a protease expressed by the adrenal to release the shorter, mitogenic N-POMC peptides, binding of the γ -MSH antibody inhibiting this cleavage (Lowry *et al.* 1983). Further evidence for this hypothesis came from the measurement of POMC peptides in the circulation of late gestational foetal sheep, a period characterised by rapid adrenal growth. In this study it was shown that levels of pro- γ -MSH progressively fall with a corresponding rise in shorter N-POMC peptides suggesting that cleavage of pro- γ -MSH results in the generation of the adrenal mitogen (Saphier *et al.* 1993).

More recently there has been much interest in POMC due to the discovery that MSH peptides play a crucial role in the control of feeding and this has prompted the generation of a POMC knock-out mouse (Yaswen *et al.* 1999). The phenotype of this animal, apart from being obese, includes the complete absence of any adrenal cortical tissue, perhaps not an entirely unexpected result but one that supports the role of POMC peptides in both adrenal growth and development.

Identification of adrenal secretory protease

Throughout the whole of this time, the pro- γ -MSH cleavage hypothesis remained unchallenged before we set out to substantiate it using modern molecular techniques, firstly to demonstrate that proteolysis was required for adrenal growth and then to set about identifying the putative protease involved (Bicknell *et al.* 2001). The first point was relatively simple to address – based on the assumption that the candidate protease was likely to be secreted or retained on the adrenocortical cell surface, we demonstrated that injection of the serine protease inhibitor aprotinin into groups of rats prior to unilateral adrenalectomy significantly decreased the increase in weight compared with saline-treated controls. It was also found that the same inhibitor significantly decreased the growth rate of the mouse Y1 adrenocortical tumour cell line. As both of these results suggested that proteolysis was necessary for adrenal growth both *in vivo* and in an adrenal-derived cell line, we attempted to identify the protease involved. We decided that the best way to achieve this was to use a molecular approach and used the conserved sequence surrounding the catalytic His/Asp/Ser residues of the trypsin family to design degenerate oligonucleotides. These were then used in PCR reactions with cDNA templates derived from normal and compensatory growing adrenals. Using this approach we identified three serine proteases: the first of these was identified as tissue plasminogen activator (tPA), which had previously been identified in the adrenal medulla; the second as adipsin, a protease known to be involved with adipocyte differentiation; and finally a novel sequence which we investi-

gated further. Cloning of the full-length sequence revealed it to encode a 28 kDa protein containing the classical His/Asp/Ser catalytic triad while computer analysis of the sequence predicted it to contain a secretory signal sequence and because of this feature the enzyme was named AsP (for adrenal secretory protease). *In situ* hybridisation localised expression to the zona glomerulosa/fasciculata – the region in which cortical cells undergo mitosis before migrating inwards towards the medulla. Although we did not perform an exhaustive survey, AsP expression appeared to be limited to the adrenal as well as the Y1 cell line.

The fact that Y1 cells expressed AsP suggested that they could be used as a model to investigate the role of AsP in adrenal growth. By transfecting Y1 cells with plasmid constructs expressing antisense AsP RNA we reduced the levels of endogenously expressed AsP. The observation that these cell lines grew at a reduced rate when compared with control cell lines expressing sense AsP RNA suggested that Y1 cells need the enzyme for cell growth.

We then turned our attention to the question as to whether AsP could cleave pro- γ -MSH. Two pieces of indirect evidence suggested that it could; the first of these was the observation that only the sense cells could respond to exogenous pro- γ -MSH (purified from bovine pituitaries), suggesting that the antisense cells had an impaired ability to activate the pro- γ -MSH. Secondly, we also found that if the cells were grown in media stripped of γ -MSH containing peptides the growth rate of the sense cells was reduced to that of the antisense cells. This result suggested the growth advantage of the sense cell was derived from the fact that they could activate pro- γ -MSH in the media.

Encouraged by these results, we looked for more direct evidence that AsP could cleave pro- γ -MSH. Homology modelling using the known crystal structures of trypsin and chymotrypsin was used to generate a putative 3D structure of AsP. This model suggested a high density of arginine residues on the posterior face to the active site that could possibly anchor the enzyme on the cell surface by ionic interaction. Using immunocytochemistry with an antibody against AsP we showed that the enzyme is retained on the surface following secretion. The next logical step was to test for activity. Since the experimental evidence had suggested that pro- γ -MSH required removal of the γ -MSH sequence to express its mitogenic activity, it seemed likely, in common with the other processing sites in POMC, that cleavage occurred at the dibasic arginine/lysine site at position 48/49. We consequently tested for activity using an internally quenched fluorescent peptide substrate that fluoresces upon cleavage, the sequence of which was identical to that of pro- γ -MSH surrounding the 48/49 dibasic cleavage site. With this substrate we demonstrated enzyme activity upon the surface of Y1 cells transfected with the sense constructs with correspondingly less activity on the antisense cells, suggesting that AsP

could cleave the peptide. We next determined the cleavage site within the peptide by digesting the peptide with immuno-purified AsP and subsequently analysing the cleavage products by mass spectrometry. The results of this, surprisingly, showed that cleavage occurred not at the dibasic site, but two residues towards the C-terminal between the valine and methionine at positions 52/53. Although these results strongly suggest the identity of the adrenal mitogen to be N-POMC(1–52), this cleavage site remains to be fully substantiated from analysis of products generated from AsP digestion of full-length pro- γ -MSH.

Taken together, the results from these experiments provide substantial evidence supporting the pro- γ -MSH cleavage hypothesis and have some interesting implications.

Cleavage of pro- γ -MSH to generate N-POMC(1–52) may provide a clue as to explaining the role of the pars intermedia in adrenal physiology. POMC expressed in the pars intermedia (Fig. 1) is more extensively processed than in the pars distalis and results in the generation of N-POMC(1–49) from cleavage of pro- γ -MSH. However, the cleavage of pro- γ -MSH only occurs in the pars intermedia if the peptide lack the O-linked glycosylation (H P J Bennett, personal communication), and consequently the resulting N-POMC(1–49), unlike pro- γ -MSH secreted from the pars distalis, lacks these O-linked sugar moieties (Fig. 1). It would seem logical that this peptide could be an adrenal mitogen but it has been demonstrated that the pars intermedia plays no significant role in adrenal growth (Lowry *et al.* 1983), which corroborates recent work on foetal sheep adrenals that also suggests that N-POMC(1–49) is mitogenically inactive (Ross *et al.* 2000). This has interesting implications surrounding the ligand specificity of the N-POMC receptor. The observation that N-POMC(1–28) is mitogenic (Fassnacht *et al.* 2000) and the fact that incorrect alignment of the two N-terminal disulphide bridges leads to loss of activity (Denef *et al.* 2001) suggest that the mitogenic properties of the N-POMC peptides reside in their N-termini. It is then perhaps slightly surprising that N-POMC(1–49) is inactive, raising the possibility that it has a structure that stops it from binding/activating the N-POMC receptor. An alternative hypothesis may be that the O-linked glycosylation present on pro- γ -MSH derived from the pars distalis is functionally necessary for high-affinity binding of the peptide to the receptor. Whatever the reason, the isolation and characterisation of the receptor should provide the answers to this interesting enigma.

A second implication from these results is the role of the C-terminal γ -MSH peptide generated from cleavage of pro- γ -MSH. Various studies have demonstrated that pro- γ -MSH can potentiate ACTH-induced steroidogenesis by up to sixfold (Al Dujaili *et al.* 1982) and this activity resides in the C-terminal γ -MSH portion of the peptide (Seger &

Bennett 1986). A possible receptor that could mediate this response is the MC3 receptor, which has a high affinity for γ -MSH and is expressed in the adrenal gland of certain species (Takeuchi & Takahashi 1999 and unpublished observations). However, the γ_3 -MSH generated from the cleavage of pro- γ -MSH by AsP would result in the absence of the first two residues that may alter its affinity for the MC3R. This coupled together with the fact that γ_3 -MSH generated from pro- γ -MSH is N-linked glycosylated (which has been shown to prevent binding of pro- γ -MSH to the MC3R (Bert *et al.* 1999)) raises the possibility that a further adrenal melanocortin receptor may exist. This poses the additional question as to the role of the melanocortin receptor antagonist, Agouti related protein (AgRP), which is expressed across the whole adrenal cortex (Bicknell *et al.* 2000) and could play a role in the local control of steroid synthesis by antagonising such a receptor (Fig. 2).

The cleavage of pro- γ -MSH by AsP leads to the interesting scenario that the adrenal has a degree of control over its own tonic state, independent from hypothalamic stimulation of the pituitary gland. By adjusting the expression levels of either AsP or the receptor the adrenal can modulate its size independent from the steroidogenic influences of ACTH. This gives rise to interesting questions with regard to the control of AsP expression and places either enzyme or receptor as a potential candidate for the cause of adrenal tumours, a notion perhaps supported by the dependence of the Y1 tumour cell line on the expression of AsP.

It has been 18 years since the pro- γ -MSH cleavage hypothesis was proposed. The cloning and characterisation of AsP finally provides the experimental evidence to support it, but with it a whole new series of questions have been posed. Perhaps another exciting era of adrenal physiology has just begun.

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