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Particular processing of pro-opiomelanocortin in *Xenopus laevis* intermediate pituitary

Sequencing of α- and β-melanocyte-stimulating hormones

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α- and β-melanocyte-stimulating hormones (α-MSH and β-MSH) have been isolated from *Xenopus laevis* neurointermediate pituitary and microsequenced. Intracellular α-MSH is not *N*-acetylated after proteolytic processing of pro-opiomelanocortin in contrast to mammalian α-MSHs. There is a high preservation of the melanotropic amino acid sequence common to all MSHs although in *Xenopus* β-MSH a histidine residue replaces the glutamic acid residue found in position 8 of mammalian β-MSHs.

Melanocyte-stimulating hormone; Proopiomelanocortin processing; Neurointermediate peptide; (*Xenopus laevis*)

1. INTRODUCTION

Mammalian (bovine, human and murine) pro-opiomelanocortins (POMC) have been fully characterized through cDNA [1,2] and gene sequencing [3–5]. Processing of this precursor (239 residues), however, differs in the anterior and intermediate lobes of the pituitary gland. Corticotropin (39 residues), an excised fragment of POMC, is mainly found in the first lobe whereas in the second further maturation of this peptide leads to the 13-residue α-melanocyte-stimulating hormone (α-MSH) and the 22-residue corticotropin-like intermediary pituitary peptide (CLIP) [6]. On the other hand β-lipotropin (91 residues), another fragment of POMC found in the anterior lobe, is split into β-MSH (18 residues) and β-endorphin (31 residues) in the intermediate pituitary. This particular tissue-specific processing involves the sequential actions of a paired basic residue-specific endopeptidase [7], a carboxypeptidase B-like enzyme [8] and a peptidyl glycine α-amidating monooxygenase [9,10]. Furthermore in mammals the 13-residue α-MSH is acetylated before secretion and this acetylation is necessary for displaying the full melanocytic activity [11]. In amphibians, processing of POMC in neurointermediate lobe has been studied through the pulse-chase technique [12,13] but identification of the end products by amino acid sequence has not yet been performed.

The nucleotide sequence of cloned cDNA for POMC from the amphibian *Xenopus laevis* has been determined and the amino acid sequence of the precursor has been deduced [14]. *Xenopus laevis* α-MSH and β-MSH have now been isolated from neurointermediate pituitary glands and subjected to microsequencing. Whereas cleavages of the precursor occur as expected at paired basic residues, α-MSH is not acetylated in contrast to its mammalian counterpart.

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2. MATERIALS AND METHODS

2.1. *Neurointermediate pituitaries*

Xenopus laevis (average body weight: 100 g), kindly provided by Professor J. Charlemagne from a breeding colony and kept

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for several weeks in the laboratory, were killed by decapitation and the two lobes of the pituitary gland (adenohypophysial and neurointermediate) were taken out and immediately used. Neurointermediate lobes (average weight: 1.2 mg) were homogenized with 0.1 M HCl (0.06 ml per gland) in a Potter-Elvehjem homogenizer for 4 min, then stirred for 4 h at 4°C. After centrifugation, the supernatant solution was recovered and subjected to high-pressure reverse-phase liquid chromatography.

2.2. *High-pressure reverse-phase liquid chromatography (HPLC)*

The supernatant was subjected to HPLC using a C-18 Nucleosil column (4.5 × 210 mm, particle size 5 μm) and an acetonitrile linear gradient (5–60%) containing 0.05% trifluoroacetic acid was applied for 55 min, followed by an isocratic elution with 60% acetonitrile/0.05% trifluoroacetic acid solvent during 5 min. A model 204 Waters chromatograph equipped with a model U6K manual injector, a model 680 solvent programmer, a model 441 UV absorbance detector and a model 730 data module, was used. Absorbance was monitored at 214 and 280 nm. 0.6-ml fractions were collected.

2.3. *Amino acid microsequencing*

Purified peptides (about 0.1–1 nmol) were subjected to microsequencing using an automated Edman degradation in an Applied Biosystems model 470 A gas-phase protein sequencer equipped with an on-line Applied Biosystems model 120 A analyzer under the conditions described by Hewick et al. [15]. Phenylthiohydantoin amino acids were identified and measured by high-pressure reverse-phase liquid chromatography in the on-line analyzer.

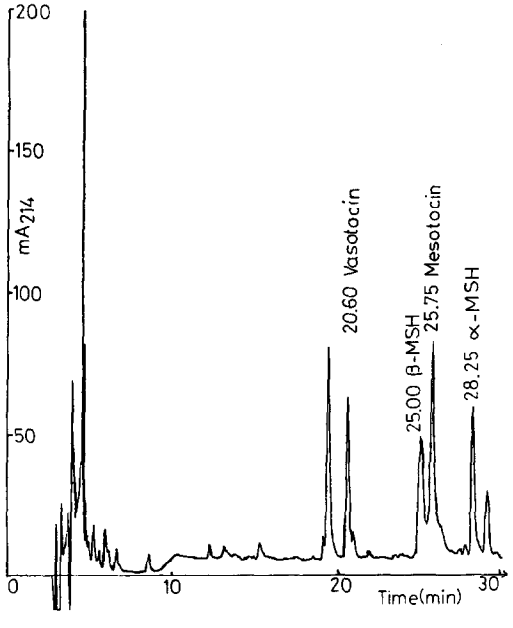

Fig.1. Separation of neurointermediate pituitary peptides of *Xenopus laevis* by HPLC. Retention times in minutes are indicated.

3. RESULTS AND DISCUSSION

Three neurointermediate lobes have been extracted and the supernatant solution has been subjected to HPLC under the conditions described above. Several peptides have been detected (fig.1). Vasotocin and mesotocin have been identified by their retention times (RT) compared with those of synthetic peptides, their amino acid compositions and sequences, and their biological activities [16]. Materials corresponding to the peaks with RT 25.00 and 28.25 min, respectively, have been concentrated and submitted to microsequencing. Results are given in table 1.

α-MSH can be recognized in the peak with RT 28.25 min by its amino acid sequence up to residue 10. This sequence is identical to the one deduced from the cDNA [14] assuming a processing cleavage at the pair of basic residues upstream alanine. Determination of the α-MSH N-terminal sequence on the one hand proves the processing

Table 1

Microsequence analysis of *Xenopus laevis* α-MSH and β-MSH (material isolated from 3 neurointermediate lobes)

α-MSH (RT = 28.25)				β-MSH (RT = 25.00)			
Cycle	Residue	Yield (pmol)	Deduced from DNA	Cycle	Residue	Yield (pmol)	Deduced from DNA
1	Ala	36.5	Ala	1	unid.		Asn
2	Tyr	28.5	Tyr	2	Gly	22.5	Gly
3	Ser	3.5	Ser	3	Asn	42.0	Asn
4	Met	16.5	Met	4	Tyr	30.5	Tyr
5	Glu	20.5	Glu	5	Arg	18.5	Arg
6	His	21.0	His	6	Met	26.0	Met
7	Phe	14.5	Phe	7	His	19.0	His
8	Arg	10.0	Arg	8	His	16.0	His
9	Trp	8.5	Trp	9	Phe	27.5	Phe
10	Gly	4.0	Gly	10	Arg	12.5	Arg
11	–	–	Lys	11	Trp	18.0	Trp
12	–	–	Pro	12	Gly	12.0	Gly
13	–	–	Val	13	Ser	2.5	Ser
14	–	–	Gly	14	Pro	15.0	Pro
15	–	–	Arg	15	Pro	9.5	Pro
16	–	–	Lys	16	Lys	<2.0	Lys
				17	–	–	Asp
				18	–	–	Lys
				19	–	–	Arg

unid., unidentified

cleavage, on the other shows that the N-terminal end is not acetylated in contrast to *N*-acetylated mammalian α-MSHs [11]. The retention time of synthetic *N*-acetylated α-MSH (Ciba) is clearly different (30.25 min).

In a study on the processing of the frog *Rana ridibunda* POMC, it has been suggested, from HPLC behaviour, that intracellular α-MSH is not acetylated [13] and this feature might therefore be general in amphibians. It is likely that a second processing cleavage occurs in *Xenopus* POMC at the level of the pair Arg-Lys downstream and, through carboxypeptidase B and α-amidase, valine is amidated as it is in mammalian α-MSH.

β-MSH can be recognized in the peak with RT 25.00 min by the amino acid sequence up to residue 16. From the cDNA sequence and assuming a processing cleavage at the Lys-Lys pair upstream Asn, the amino acid sequence of *Xenopus* β-MSH has been deduced [14]. In our experiment, for an unknown reason, the first residue of the purified β-MSH could not be identified but the 15 following amino acids are identical to those deduced (table 1).

It can also be admitted that a second processing cleavage occurs at the level of Lys-Arg downstream Asp-18, as in the mammalian intermediate lobe, with separation of β-MSH from β-endorphin [17]. C-terminal basic residues are subsequently removed by a carboxypeptidase B-like enzyme.

A comparison of amino acid sequences of mammalian and *Xenopus laevis* melanotropic peptides is shown in fig.2. There is a great conservation for α-MSHs since they differ only by the first residue (Ser or Ala). In the case of β-MSHs, the number of variations is higher: positions 2, 4, 6 and 8 are different (fig.2). The melanotropic sequence common to mammalian α-MSHs and β-MSHs [11] is preserved in amphibians. The replacement, however, of a glutamic acid residue by a histidine in the fourth position of this sequence in *Xenopus* β-MSH is worthy to note.

Substrate conformation-directed proteolytic processing has been suggested for vasopressin – neurophysin – copeptin precursor involving a two-step maturation [18,19] and such a mechanism has been proposed for the cleavage of β-lipotropin into

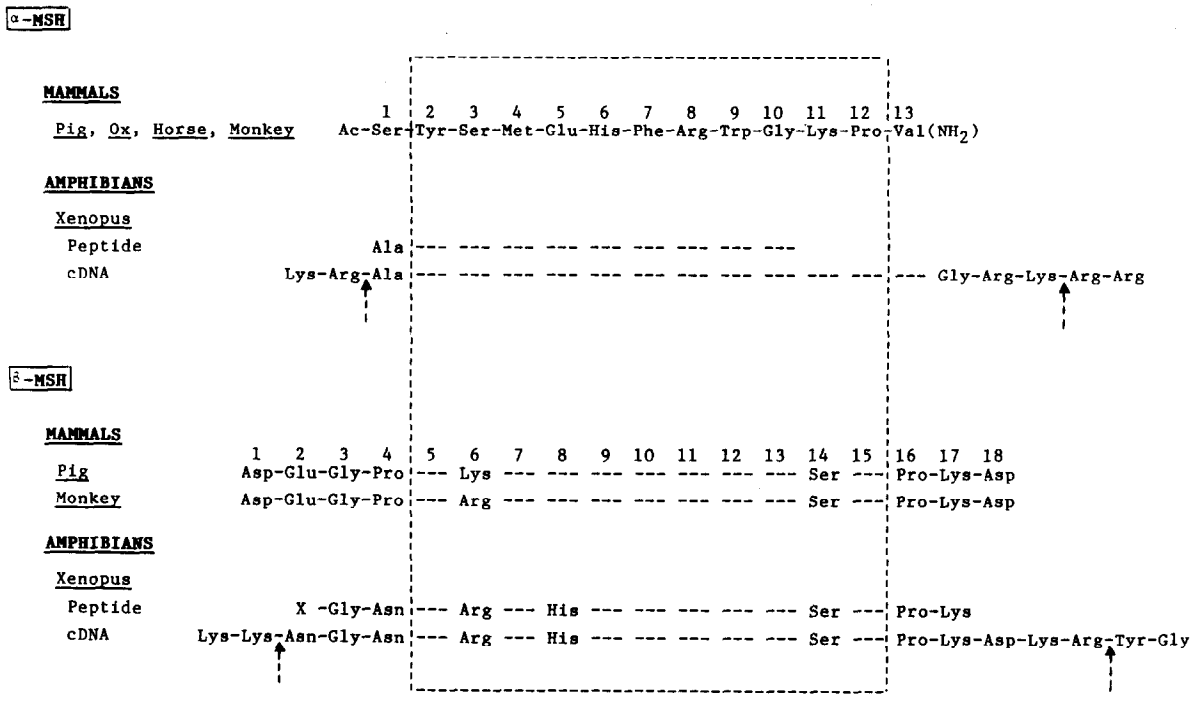

Fig.2. Comparison of *Xenopus laevis* α-MSH and β-MSH with mammalian counterparts. Amino acids identical to those of mammalian α-MSH are indicated by dashes. Homologous sequences in the two families, α-MSHs and β-MSHs, are boxed.

β-endorphin [20]. Pulse-chase studies using radioactive amino acids and sugars performed on mouse pituitary tumor cells (AtT-20) suggest that 20 kDa POMC is a branch-point in the processing pathways [21]. It can be converted either to 4.5 kDa corticotropin by proteolytic processing or to 32 kDa POMC by the further addition of carbohydrate, the 32 kDa form giving in turn a 13 kDa glycosylated corticotropin. Because proteolytic processing of POMC differs in anterior and intermediate pituitary lobes although the same type of bibasic cleavage sites is involved, it seems of interest to compare the enzyme content of the secretory granules of both tissues. Furthermore, because a secreted acetylated form of α-MSH has been detected in amphibians [12,13], the acetylating enzyme is probably not present in the intermediate pituitary granules but perhaps in the plasma membrane, acting during exocytosis.

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