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Identification of POMC processing products in single melanotrope cells by matrix-assisted laser desorption/ionization mass spectrometry

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Abstract The use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in identifying proopiomelanocortin (POMC) processing products in melanotrope cells of the pituitary intermediate lobe of *Xenopus laevis* was explored. Mass spectra were obtained with such a high sensitivity of detection that the peptides could be identified in a single melanotrope cell. In addition to known POMC processing products of the *Xenopus* melanotrope cell, t he presence of previously unidentified POMC-derived peptides was demonstrated. Together these POMC processing products accounted for the entire length of the POMC precursor. Furthermore, *Xenopus* possesses two genes for POMC and the sensitivity and accuracy of the MALDI-MS technique allowed identification of processing products of both the $POMC_A$ and $POMC_B$ gene. In addition, differences were obtained between the mass spectra of melanotrope cells from *Xenopus laevis* adapted to different conditions of background illumination. These results show that MALDI-MS is a valuable tool in the study of the expression of peptides in single (neuroendocrine) cells.

Key words: Matrix-assisted laser desorption/ionization mass spectrometr; Proopiomelanocortin; Melanotrope cell; Pituitary; *Xenopus laevis*

1. Introduction

In amphibians, the melanotrope cells of the intermediate lobe of the pituitary gland secrete a melanotropic peptide, α-melanocyte-stimulating-hormone (α-MSH), which causes pigment dispersion in dermal melanophores. Thus, the pituitary melanotropes play a pivotal role in the process of skin color adaptation. In lower vertebrates, as in mammals, α-MSH originates from a high molecular weight precursor protein, proopiomelanocortin (POMC). Based on thye cDNA nucleotide sequence, the complete amino acid sequence of POMC has been determined in various mammalian [1–6] and non-mammalian vertebrates [7–10]. THrough tissue-specific processing POMC can generate a number of bioactive peptides including adrenocorticotropic hormone (ACTH), α-, β- and γ-MSH, β-lipotropin (β-LPH) and β-endorphin [11].

The melanotrope cells of the amphibian *Xenopus laevis* are specialized in producing and secreting POMC-derived products. Although in some studies MSH peptides and endorphin end products [12–14] have been identified, little is known about the complete profile of POMC processing products within the melanotrope cell. The complexity of the POMC precursor makes identification of all processing end products using tranditional biochemical methods extremely difficult. The common practice of analysing neuropeptides by radioimmunoassay becomes arduous when applied to entire profiles. Moreover, this method can only be used for known peptide families. The introduction of high-mass mass spectrometric techniques and particle-induced desorption/ionization methods for analysing intact biopolymers [15–17] has resulted in the manufacturing of mass-specific analysers that (1) can detect peptides regardless of provenance and composition, (2) are fast and (3) can be used to characterize complete profiles in mixtures of different peptides [18,19]. One of these techniques is the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [20–22] which enabled the measurement of complete peptide profiles in single cells without prior peptide purification. For proteins of which the sequence is (largely) known, mass spectrometry permits a rapid, detailed confirmation of the sequence and the identification of possible protein modifications, e.g. acetylation, amidation, glucosylation and phosphorylation [23]. In the case of the *Xenopus* melanotrope cell, the majority of the peptides produced are derived from POMC [24]. The complete amino acid sequence of this precursor has been deduced from its nucleotide sequence [7]. *Xenopus* possesses two different POMC genes, named $POMC_A$ and $POMC_B$, which are similar (but not identical) in their amino acid sequences [25]. MALDI-MS would be an appropriate method to characterize the full peptide profile resulting from the processing of $POMC_A$ and $POMC_B$. Furthermore, because melanotrope cells of black-background adapted *Xenopus laevis* are very active in synthesizing and processing of POMC whereas melanotrope cells from white-adapted *Xenopus* serve as storage cells with an inactive biosynthetic apparatus [26,27], with MALDI-MS the hypothesis could be tested that cells from black-adapted *Xenopus* contain more (larger) POMC processing intermediates than cells of white-adapted *Xenopus*. The purpose of t his study was (1) to identify the different POMC ($POMC_A$ and $POMC_B$) processing end products and (2) to elucidate possible differences in the contents of melanotrope cells from animals adapted to different

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Abbreviations: Ac, acetylated; AJP, acidic joining peptide; ACTH, adrenocorticotropic hormone; CLIP, corticotropin-like intermediate peptide; DHB, 2,5-dihydroxybenzoic acid; LPH, lipotropin; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MSH, melanocyte-stimulating hormone; POMC, proopiomelanocortin; TOF, time-of-flight.

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environmental light conditions. This was done by MALDI-MS, using single *Xenopus* melanotrope cells to prevent interference with other cell types present in the pituitary gland.

2. Materials and methods

2.1. Animals

Adult *Xenopus laevis* with a weight of approximately 35 g were obtained from our laboratory stock. Toads were kept under constant illumination on a black or a white background for at least 3 weeks at 22°C. They were fed trout pellets (Trouvit, Trouw, Putten, The Netherlands) once a week.

2.2. Preparation of single cells

The melanotrope cells of 8 animals were isolated from the pituitary neurointermediate lobe as described previously [28], with minor changes. In short, animals were anaesthetized for 15 min in a solution containing 0.1% MS222 (Sigma; St. Louis, MO, USA) and 0.15% $NaHCO_3$ (pH 6.8). After perfusing the animal with *Xenopus* Ringer's solution, containing 112 mM NqaCl, 2 mM KCl, 2 mM $CaCl_2$ and 15 mM HEPES (Calbiochem; La Jolla, CA, USA; pH 7.4) to remove blood cells, lobes were dissected and incubated for 45 min in Ringer's solution without $CaCl_2$ to which 0.25% (w/v) trypsin (Gibco, Renfrewshore, UK) had been added. Cells were subsequently dispersed in Ringer's solution by gentle trituration of the lobes with a siliconized Pasteur's pipette. The cells were readily identified on the basis of their characteristic round shape and used immediately for mass spectrometry.

2.3. Sample preparation

Before MALDI-MS only minimal sample preparation was necessary. The matrix used was 2,5-dihydroxobenzoic acid (DHB; Aldrich Chemie, Steinheim, Germany) dissolved at a concentration of 10 mg/ml in 0.1% trifluoroacetic acid (TFA; Merck, Darmstadt, Germany). After deposition of 1 μl of the matrix on a stainless steel target, the cells were placed in the matrix solution. The sample was dried in a cold air stream before loading into the mass spectrometer. Calibration was performed using a mixture of bovine insulin and horse cytochrome *c* (Sigma). The acid matrix used to retain the products directly from the melanotrope cells produced sufficient ions to eliminate purification artifacts.

2.4. Mass spectrometry

The mass spectrometer used was a VISION 2000 (Finnigan MAT, Bremen, Germany). This is a reflectron time-of-flight (TOF) laser desorption instrument equipped with a nitrogen laser at 337 nm. The laser beam was focused by a quartz lens system to a spot size of 70 μm in diameter. The ions generated were accelerated to a potential of 5 kV in the ion source and post-accelerated by a conversion dynode to a potential of 20 kV. The effective drift length of the instrument is 1.7 m. Ions were detected by a secondary electron multiplier and the signal was amplified and digitized by a high speed transient recorder linked to a 486 personal computer. Molecular weight data were collected and compared with computer-analysed POMC precursor fragments with known or predicted molecular weight, using the 'find mass' program (MacProMass, Beckman Research Institute of the City of Hope, Duarte, CA, USA). Mass measurements up to 5 kDa were reliable up to ± 3 Da. Therefore, a small search window can be used, resulting in fewer matches and consequently less ambiguity in identification.

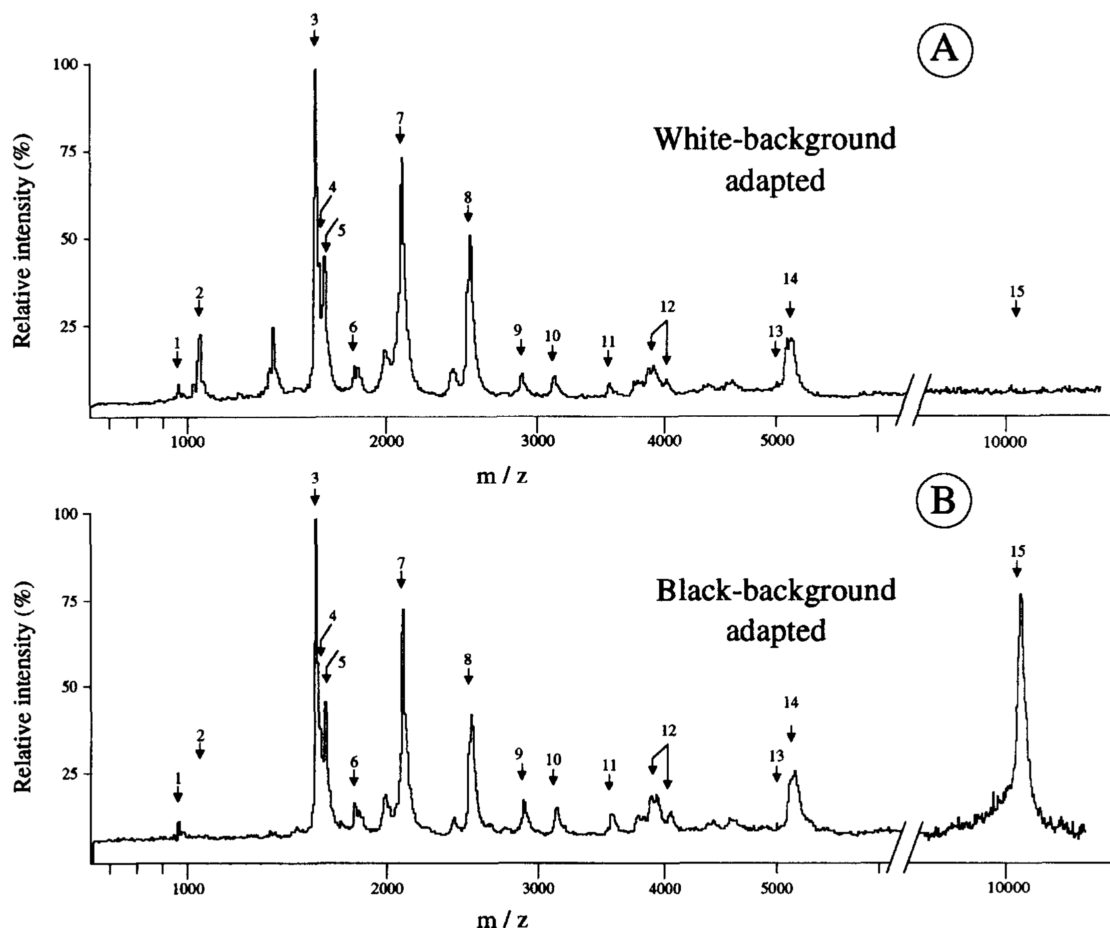

Fig. 1. Representative example of MALDI mass spectra from freshly isolated, single melanotrope cell of *Xenopus laevis*. Arrows indicate the position of identified POMC processing products (see Table 1). The spectra represent unprocessed data accumulated from 20 single-shot acquisitions without any filtering or backgruond substraction. (A) Spectrum from a melanotrope cell derived from a white-background adapted animal. (B) Spectrm from a melanotrope cell of a black-background adapted animal. Peak 4 was present as a small peak partially overlapping with peak 3 and peak 13 was not always detected in the spectrum. At higher resolution the presence of these products was clearly visualized.

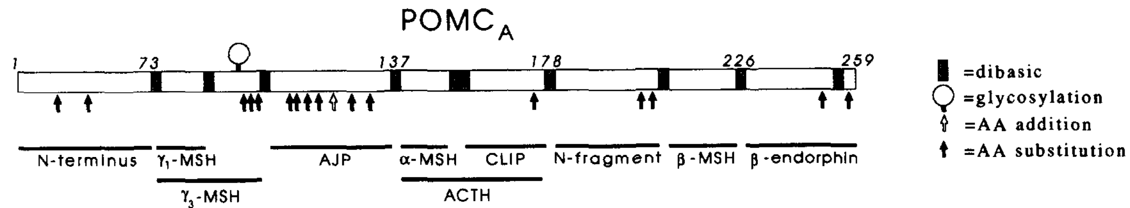

Fig. 2. Schematic representation of the $POMC_A$ precursor of the amphibian *Xenopus laevis*. The structure of the POMC gene was deduced from nucleotide sequence analysis [7]. Positions of amino acid differences between $POMC_A$ and $POMC_B$ are indicataed by filled arrows and the single amino acid addition in $POMC_B$ is indicated by an open arrow. The position of the glycosylation site is shown by the open circle. Sites where two or more basic amino acids occur are boxed. Processing products formed via processing at dibasic amino acids are indicated by horizontal lines. AA = amino acid.

3. Results and discussion

This study is based on the rationale that molecular weight information is sufficiently specific to tentatively identify all peptides that are either known to be present or can be predicted to occur in a given cell or tissue. This approach reduces the number of peptides that have to be analysed by more rigorous, but also more laborious and time-consuming, methods. Prediction is on the basis of 'classical' precursor processing at consecutive basic amino acids or by processing at 'consensus' sequence sites [29–31]. With single cells, especially with a homogeneous cell suspension such as the melanotrope cells of *Xenopus laevis*, that only express one major type of precursor molecule, mass accuracy becomes less important.

3.1. Complete mass profiles of melanotrope cells

The overview mass spectra of a single melanotrope cell from both white- and black-background adapted *Xenopus laevis* revealed 20 to 25 significant ions (Fig. 1). Clean and reproducible mass spectra were obtained up to a molecular mass of 10,000 Da. Mass accuracy for the peptides was in most cases better than ± 1 Da. The peaks with larger deviation may include unresolved components. The width of the peaks at higher mass slightly decreases accuracy.

The molecular weight data obtained in this study immediately pinpointed a number of the reported processing products of *Xenopus* POMC [12,13,32], ultimately accounting for the entire length of the POMC precursor (see Table 1, Fig. 2). Both desacetyl-α-MSH as well as α-MSH, β-MSH and lys-γ_1-MSH could readily be identified. However, the α-MSH peak was very small compared to the desacetyl-α-MSH peak. This abundance ofg the desacetyl-α-MSH peak over the α-MSH peak is in line with results obtained using radioimmunoassay for α-MSH [14,33,34]. As expected, only one ion was observed for each of the products, since these products show no difference in molecular weight whether derived from $POMC_A$ or $POMC_B$. The α,*N*-acetylated-β-endorphin{1–8} (POMC 229–236) was mainly present as sodium and potassium ions, resulting in shifts of 22.0 Da and 38.1 Da for the sodium and potassium ion, respectively. Thus, the measured molecular weight of this peptide differs from the calculated molecular weight showing shifts from 944.1 to 966.1 and 982.2, respectively. This result was confirmed by running synthetic α,*N*-acetyl-β-endorphin{1–8} (POMC 229–236) under the same conditions which resulted in the apperance of the 966.1 Da and 982.2 Da products, in the absence of the 944.1 Da H^+-ion. Only small peaks were observed for all acetylated endorphins (viz. α,*N*-acetyl-β-endorphin{1–8}, {1–27} and {1–31}). Because it is known that the *Xenopus* melanotrope cells contain high amounts of α,*N*-acetyl-β-endorphin{1–8} (POMC 229–236) [13], acetylation of these

Table 1
Summary of mass data of identified POMC processing products obtained from *Xenopus* melanotrope cells

Peptide	Peak Nr.	$POMC_A(POMC_B)$	$[M + H^+]_c$	MALDI-MS	▲
N-terminus	2	26 34	1070.2	1069.4	− 0.8
N-terminus	14	26–73	5139.7 (5183.9)	5139.1 (5182.7)	− 0.6 (− 1.2)
N-terminus	15	26–101	ST	> 10,000	
Lys-γ_1-MSH	5	75–86NH_2	1657.0	1656.0	− 1.0
Lys-γ_3-MSH	13	75–101	ST	5006.8 (5050.2)	− 1.8 (− 0.6)
AJP	12	104–137 (104–138)	3885.1 (4042.2)	3882.7 (4041.5)	− 0.1 (− 0.7)
Des-Ac-α-MSH	3	140–152NH_2 (141–153NH_2)	1607.9	1607.0	− 0.9
α-MSH	4	140–152NH_2 (141–153NH_2)	1649.9	1648.9	− 1.0
CLIP-fragment	6	157–172 (158–173)	1818.0	1817.2	− 0.8
CLIP	8	157–178 (158–179)	2538.8 (2522.8)	2537.9 (2522.4)	− 0.9 (− 0.4)
N-fragment	9	181–205 (182–206)	2895.1 (2965.1)	2897.2 (NI)	+ 2.1 (NI)
β-MSH	7	210–226 (211–227)	2100.3	2099.7	− 0.6
Ac-β-Ep{1–8}	1	229–236 (230–237)	944.1	966.4 (ST)	+ 0.3 (ST)
Ac-β-Ep{1–27}	10	229–255 (230–256)	3125.6 (3139.6)	3123.6 (3139.1)	− 2.0 (− 0.5)
Ac-β-Ep{1–31}	11	229–259 (230–260)	3567.2 (3566.2)	3566.8 (3564.8)	− 0.4 (− 1.4)

Peak numbers are related to the peaks as shown in Fig. 1. Amino acids 1–25 of the POMC precursor comprises the signal peptide which is generally not found. The complete $POMC_A$ precursor comprises 259 amino acids (260 for $POMC_B$; [25]). The position of the peptides within the precursor and the calculated average molecular mass of $POMC_A$ (and $POMC_B$) processing products have been indicated, as well as the molecular weight products found by MALDI-MS. The difference between calculated average mass and measured molecular mass of the protonated ions is indicated by ▲. AJP = acidic joining peptide, Ac = acetyl, NI = not identified, ST = see text.

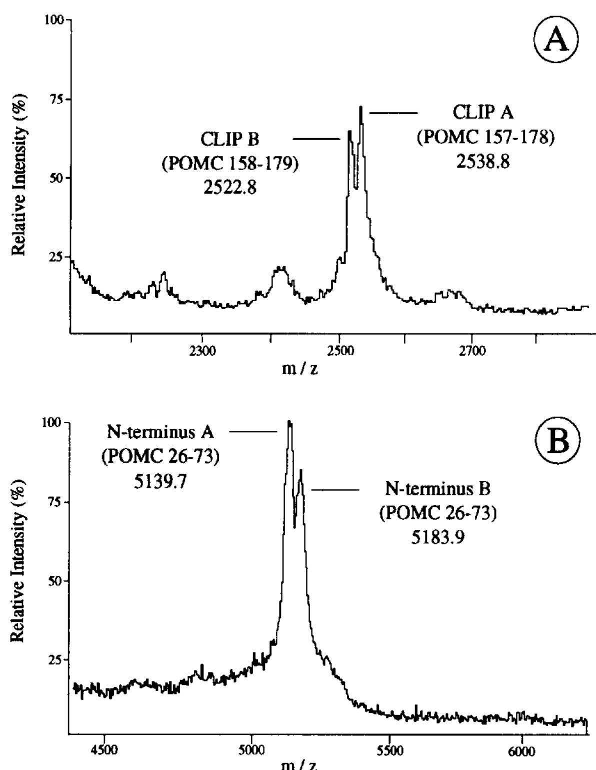

Fig. 3. MALDI mass spectrum from a single melanotrope cell confirming the presence of both $POMC_A$ and $POMC_B$ gene products. (A) Corticotropin-like intermediate peptide from $POMC_A$ ($[M + H^+]_c$ = 2538.8) and from $POMC_B$ ($[M + H^+]_c$ = 2522.8). (B) The N-terminal peptide (POMC 26–73) derived from $POMC_A$ ($[M + H^+]_c$ = 5139.7) and $POMC_B$ ($[M + H^+]_c$ = 5183.9). The spectra represent unprocessed data accumulated from 10 single-shot acquisitions without any filtering or background substraction.

endorphins may interfere with the matrix or with the ionization/detection possibility of the mass spectrometer. Possibly due to the complexity of the peptide contents of the melanotrope cell the mass spectrometric signal of these peptides is suppressed and thus prohibits detection of all analytes. The mechanism of this suppression is still unknown [35]. Although the absence of a signal does not prove that a peptide is not there, the absence of non-acetylated endorphins in the melanotrope cells confirms previous findings [14,36]. These results confirm the observations concerning the spatial difference in acetylation of α-MSH and endorphins, while the endorphins are rapidly acetylated after synthesis (so, no non-acetylated endorphins can be found within the cell), acetylation of desacetyl-α-MSH occurs at the moment of secretion, which explains the small amount of α-MSH present within the cell [32–34,36].

3.2. *Different $POMC_A$ and $POMC_B$ processing end products*

Several peptide products, unique to either the $POMC_A$ or the $POMC_B$ gene, were found. Only small differences in the height of the peaks of $POMC_A$ and $POMC_B$ end products were visible, indicating similar amounts of $POMC_A$ and $POMC_B$ end products and a similar processing. This is in agreement with the biosynthesis and the level of expression of the two POMC genes [37]. The corticotropin-like intermediate peptides (CLIPs) derived from $POMC_A$ and $POMC_B$ have a difference in molecular mass of only 18 Da, but both peptides were clearly present (Fig. 3A). Interestingly, no evidence for posttranslational modification of this product in *Xenipus laevis* melanotropes could be obtained, although it has been shown that both rat and human CLIP can be phosphorylated as well as glycosylated [38,39]. Two different peaks were also observed to match the N-terminal 26–73 peptide of the two POMC genes (Fig. 3B). These peptides were the most favourable matches for the ions seen, with an average molecular weight of 5139.1 and 5182.7. Recent biosynthesis studies have shown that this N-terminal peptide is a secretory product rapidly formed from a larger γ-MSH-containing POMC product [40].

3.3. *ψ-MSH peptides in* Xenopus *melanotropes*

The POMC precursor protein of *Xenopus laevis* contains only one glycosylation site, which is present in the γ-MSH region (Fig. 2) [[25]. Nevertheless, cleavage at this site results in several peptide products containing the glycogroup (Lys-γ_3-MSH and the N-terminal peptides harbouring this γ-MSH). Since glycopeptides are not always seen in mass spectrometr [18] and since the carbohydrate structures of the intermediate glycopeptides are not known, the identification of these peptides is not straight forward. Nevertheless, both $POMC_A$ Lys-γ_3-MSH and $POMC_B$ Lys-γ_3-MSH have been isolated and their molecular weights identified by electrospray mass spectrometr [40]. Although only small peaks were found in the melanotrope cells, the molecular weights for the Lys-γ_3-MSH peptides have now been confirmed by MALDI-MS. Consistent with earlier reports [41–43] we observed Lys-γ-MSH and not γ-MSH. Unless flanked by a proline, all basic amino acids are generally removed after cleavage of the neuropeptide precursors at basic sites [31,44]. It remains an unanswered question as to why the cleavage procedure for Lys-γ-MSH is so different from that occurring at other basic sites.

3.4. *Unexpected POMC peptides*

The usefulness of the 'find mass' program for the recognition of unexpected peptides was demonstrated for C-terminally shortened forms of CLIP, of the so called N-fragment (POMC 181–207), and of the N-terminal peptide (POMC 26–73). On the basis of the computer analysis the C-terminally truncated CLIP (POMC 157–172) is the peptide with amolecular weight of 1817.2 (peak 6). Furthermore, C-terminally shortened products of the N-terminal and the N-fragment were found to fit ions (peak 2 and 9) in the mass spectrum. CLIP, the N-terminal peptide and the N-fragment are both acidic and contain high molecular percentages of proline, glutamic acid, aspartic acid and threonine. This fact has been linked to fast proteolytic degradation in vivo [45] and explains the presence of the truncated forms of the N-fragment, the N-terminal and CLIP found in this study.

3.5. *Unidentified products*

Some peaks could not be identified as POMC processing products by MALDI-MS (MW 1388.9, 2002.2, 2422.3). Possibly, these peaks represent posttranslationally modified POMC end products (e.g. by amidation, acetylation, phosphorylation or glycosylation). Alternately, they may be non-POMC peptides produced by the *Xenopus* melanotrope cell. The post source decay technique, which is currently in a developmental stage, will probably allow the extraction of sequence information from these products [46]. Other POMC peptides deduced

by computer analysis matching ions present in the spectrum were judged by us to be poor candidates either because they (1) contain dibasic cleavage sites not expected to survive in the intermediate pituitary or (2) their processing would require the unlikely cleavage of a dibasic cleavage site next to a proline.

3.6. Background adaptation

Although the MALDI-MS profiles of cells derived from black-background adapted animals (Fig. 1A) and those of white-background adapted animals (Fig. 1B) showed little qualitative difference, the relative intensity of several peaks was quite different. Major differences were found for instance in the higher molecular weight region, where the 10 kDa product (peak 15) was more pronounced in cells derived from black animals compared to the peak in cells of white-adapted toads. This ion might be identified as a POMC processing product. The N-terminal processing product of POMC (26–101) contains the Lys-γ_3-MSH and has a molecular weight of 10 kDa. Previous findings have reported the existence of large intermediates in the processing of POMC to its end products in melanotrope cells from black-background adapted *Xenopus* [24] and such a N-terminal product has been identified as a major processing product on SDS gel electrophoresis [41]. It seems likely that the 10 kDa ion is this product. Some other products in the mass profile with a lower molecular weight (peak 2 and an unidentified molecular ion at *m/z* 1388.9) were more pronounced in melanotrope cells of white-adapted *Xenopus* than in cells of black-adapted animals. The physiological significance of this difference in processing end products is not known. However, these findings are in agreement with the idea that melanotropes of black-background adapted animals are very active in the biosynthesis and processing of POMC and thus will contain larger intermediates, while melanotropes of white-backgruond adapted animals serve as storage cells, containing completely processed POMC end products [14,27,35].

3.7. Conclusions

Single pituitary melanotrope cells can be efficiently charted by MALDI-MS and reveal a complete spectrum of the POMC-derived peptides. Although the samples are initially in a physiological environment, neither sample pretreatment nor separation steps are necessary. This offers a fast screening which is minimizing artifacts caused by extraction or separation procedures of conventional methodologies, and offers the possibility to directly mass analyse proteins present in naturally occurring biological fluids. All previously identified and several new POMC end products in melanotrope cells of *Xenopus laevis* were identified by the MALDI-MS method. In addition, the differences in processing end products of $POMC_A$ and $POMC_B$ could be resolved. While little can be said of the absolute concentration of the peptides in the profiles, clear differences between the relative intensities of the peaks show that MALDI-MS can be used to demonstrate different peptide contents in melanotrope cells from animals under different physiological conditions.

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