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Automated nanoflow liquid chromatography–tandem mass spectrometry for a differential display proteomic study on *Xenopus laevis* neuroendocrine cells

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Abstract

Many proteomic projects based on a comparison of protein profiles displayed on two-dimensional polyacrylamide gel electrophoresis rely on the identification of these proteins using peptide mass fingerprinting on a matrix-assisted laser desorption/ionization mass spectrometer after tryptic digestion. However, this approach is limited to an organism of which genomic information is largely available, i.e. when the total genome sequence is known. For other organisms, mass spectrometric sequence analysis is necessary for protein identification. We established a nano-LC–MS–MS system based on a quadrupole time-of-flight mass spectrometer, which allows automated sequence analysis of tryptic digestion mixtures from single gel spots. This system is applied in a differential-display proteomic study to identify differentially expressed proteins in the neuroendocrine cells of the neurointermediate pituitary of black- and white-background adapted *Xenopus laevis*.

Keywords: *Xenopus laevis*; Proteomics; Proteins

1. Introduction

The field of proteomics, the study of the protein complement of the genome of a certain cell type at a given moment [1], is booming after the recent disclosure of the genomic information of a number of organisms. Exploration of the proteome is mainly performed using differential display of the proteins isolated from cells grown under different conditions. Although several new strategies, implementing multidimensional chromatography [2] or isotopic labeling methods [3] coupled with mass spectrometry, have been established, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is still the most widely used method for the separation of complex protein mixtures obtained from lysates of cellular extracts. Differentially expressed proteins are detected by differences in intensity of staining and further identified by mass spectrometric means after in-gel digestion with endoproteinases, mainly trypsin.

The simplest method for protein identification is the so-called peptide mass fingerprinting (PMF) method [4], generally using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). When using mass spectrometric sequencing methods, either MALDI-

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TOF post-source decay analysis or electrospray ionization (ESI) tandem mass spectrometry (MS–MS), short sequence stretches (peptide sequence tags) can be obtained [5]. These data greatly improve the reliability of database searching. Some software tools (e.g. Sequest [6]) use the full set of MS and MS–MS data from a LC–MS–MS run to identify the protein. All of these methods have one main prerequisite: the sequence of the protein must be known. Therefore, these methods are excellent tools to study organisms of which the complete genome information is accessible. However, both prokaryotic and eukaryotic organisms that are excellent biological model systems exist for which this information is not available. Such a model system is represented by the neurointermediate pituitary lobe of the South-African clawed toad, *Xenopus laevis*. The proopiomelanocortin (POMC)-producing intermediate pituitary melanotrope cells are well tuned to study the secretory pathway in neuroendocrine cells. They play a pivotal role in the process of background adaptation [7]. Information concerning the color of the environment is perceived by the animals' visual system and is processed in the central nervous system resulting in a neural signal that reaches the melanotrope cells. By placing the animal on a black background, the melanotrope cells are triggered to release α-melanotrope hormone (α-MSH), which stimulates the dispersion of the black pigment melanin in the melanophores of the skin, causing a darkening of the animal. When the animal is placed on a white background, the secretion of α-MSH is inhibited, leading to an aggregation of melanin in the melanophores and a pale-colored skin. As α-MSH is cleaved from the prohormone POMC, an elevated release of α-MSH is accompanied by a higher rate of POMC gene transcription [8,9]. Consequently, melanotrope cells of black-background adapted *Xenopus* contain 30-times more POMC mRNA and display a much higher biosynthetic activity than those of white-background adapted animals. A strategy of black/white screening at the mRNA level has led to the identification of several gene transcripts [10] involved in neuroendocrine secretion in the neurointermediate lobe of *Xenopus*. However, a global picture of the changes at the protein level has not been demonstrated before. In the present study, we identified the proteins differentially expressed with respect to the adaptative behavior of the animal and, as such, contribute to a search for proteins which are associated with the specialized secretory function of neuroendocrine cells.

For *X. laevis*, the amount of genomic information available in routinely accessible databases is rather limited and is mainly restricted to expressed sequence tag (EST) databases containing only partial sequence information. Therefore, protein identification on 2D gels from *Xenopus laevis* cells relies on de novo protein sequence analysis. We improved a previously published automated nano-LC–MS–MS method for the identification of the differentially expressed proteins [11]. We show here the outlines of this system, and present results of protein identifications.

2. Materials and methods

2.1. Chemicals

Acetonitrile was purchased from Biosolve (Valkenswaard, Netherlands). The water used was Milli-Q grade (Millipore, Bedford, MA, USA). Formic acid was from Panreac (Barcelona, Spain). Argon was from Air Liquide (Paris, France). Tris base and dithiotreitol (DTT) were from Life Technologies (Paisley, UK). Phosphate-buffered Saline (PBS), trichloroacetic acid (TCA), diethylether, ethanol, Tris–HCl buffer, and methanol were from Merck (Darmstadt, Germany). Deoxycholic acid (DOC), thiourea, phosphoric acid, ammonium sulphate, iodoacetamide and ammonium hydrogen carbonate were from Sigma–Aldrich (St. Louis, MO, USA). Urea and glycerol were from Fluka (Buchs, Switzerland). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and sodium dodecylsulphate (SDS) were from ICN (Costa Mesa, CA, USA).

2.2. Animals and tissue material

Adult *Xenopus laevis* were bred and reared in the laboratory. In all experiments neurointermediate lobes from fully black-background and fully white-background adapted animals were used and always extirpated in the morning.

2.3. Sample preparation and protein extraction

The dissected neurointermediate lobes were collected dry in a glass/glass potter. Homogenization took place in 250 μl per gland of a solution containing 40 m*M* Tris base (pH 9.5) and 10 m*M* Pefablock (Roche, Basel, Switzerland). The proteins were precipitated by a TCA–DOC procedure mainly based on the method described by Molloy et al. [12]. The homogenized sample was filled up to 900 μl with ice-cooled 10 m*M* PBS (pH 7.4), mixed first (by vortex) with 100 μl ice-cooled 3% DOC and then with 110 μl 100% TCA. This sample was left for 15 min on ice and then centrifuged at 21 000 *g* at 4 °C. The precipated material was washed in 1 ml diethylether–ethanol (1:1), thoroughly mixed (by vortex) and centrifuged again at 21 000 *g* for 20 min. After 3 further washing steps, the recuperated precipitate was air-dried for about 2 h. This dry material was then dissolved in 40 μl lysis buffer containing 7 *M* urea, 2 *M* thiourea, 4% 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate (CHAPS), 1% DTT and 0.8% IPGphor buffer (pH 3–10L; Amersham-Bioscience) and sonified 3 times for 5 s on ice-cooled water.

2.4. Two-dimensional electrophoresis

The 2-D electrophoretic analysis was carried out on the immobilized pH gradient (IPG) system and the SDS–PAGE system with ready-made gradient gels developed by Amersham-Biosciences (Uppsala, Sweden). The procedure was mostly based on the technical protocols described by Görg et al. [13]. For electrophoresis in the first dimension, a premade IPG drystrip (11 cm) was placed in the porcelain electrophoretic holder containing the aforementioned 40-μl sample and 160-μl rehydration buffer [8 *M* urea, 0.5% CHAPS, 0.5% IPGphor (pH 3–10L), 0.28% DTT and 6.4 μl bromophenol blue (Bio-Rad, Hercules, CA, USA) solution (1 mg/ml]. The holder was then filled with 300 μl IPGphor cover fluid and put under an electric field of 50 μA per strip at 20 °C. Further rehydration took place at 30 V for 12 h (360 V h) The isoelectric separation was carried out in 3 steps going from 500 V for 1 h (500 V h), 1000 V for 1 h (1000 V h) and 8000 V (16 000 V h) for 2 h. Each electrophorized strip was then prepared for the second electrophoretic run by incubation under shaking for 15 min in equilibration buffer (6 *M* urea, 30% (w/v) glycerol and 2% (w/v) SDS dissolved in 0.05 *M* Tris–HCl buffer, pH 8.8), containing 2 μl 1% bromophenol blue and 20 mg DTT per ml equilibration buffer, and 15 min in equilibration buffer containing 40 mg iodoacetamide per ml. After a washing step of a few seconds in distilled water, the strip was dried on filter paper and placed on the premade SDS–PAGE gel (ExcelGel SDS gradient 8–18%, 0.5 mm thick). The horizontal electrophoresis in the Multiphor II system took place in two steps under the following conditions: 35 min at 600 V, 20 mA, 30 W and, after removing of the strip, at 110 min at 600 V, 50 mA, 30 W.

Each electrophorized SDS–PAGE gel was fixed overnight in 2% phosphoric acid, 50% ethanol and 48% distilled water. The gel was then washed 3 times for 20 min in distilled water and stained for 48 h in a 0.2% Coomassie Brillant Blue G 250 (Serva, Heidelberg, Germany) solution containing 34% methanol, 17% ammonium sulphate and 3% phosphoric acid. Destaining was followed by incubation in 10% methanol.

2.5. In situ digestion

Differently expressed spots were cut from the gel and individually stored in Eppendorf tubes (Hamburg, Germany). The gel pieces were prepared for digestion following a protocol slightly modified from Rosenfeld et al. [14]. They were washed twice with 150 μl of 200 m*M* ammonium hydrogen carbonate in acetonitrile–water (50:50) (20 min at 30 °C), and allowed to dry in the air. The tubes were then chilled on ice and 10 μl of digestion buffer (50 m*M* ammonium hydrogencarbonate) containing 160 ng modified trypsin (Promega, Madison, WI, USA) were added. The samples were kept on ice for 45 min to allow the enzyme to enter the gel. A 50-μl volume of digestion buffer was added and the samples were incubated overnight at 37 °C. The supernatant was recovered, and the remaining peptides were extracted from the gels by washing twice with 60% acetonitrile/0.1% formic acid in water. The extracts were combined together and the sample then dried in a Speedvac concentrator (Thermo Savant, Holbrook, NY, USA). The samples were redissolved in 10 μl

5% acetonitrile/1% formic acid prior to mass spectrometric analysis.

2.6. Nano-LC–MS

We used a commercial nano-HPLC system, i.e. an Ultimate Micro LC system combined with a FAMOS autosampler system (LC-Packings, Amsterdam, Netherlands). The pump is a classical reciprocal pump system used at 150 μl/min. It has a built-in flow splitting system to reduce the flow-rate to 100 nl/min. Essentials of the set-up are outlined in Fig. 1. The samples, generally 4 μl of the peptide extract, were loaded onto the column (PEPMAP, 15 cm×75 μm I.D., LC-Packings) using an on-line preconcentration step on a micro precolumn (2 mm×800 μm I.D.,) cartridge. This was proven to be an essential step for both reducing sample loading times and desalting. The washing step was performed using 0.1% formic acid in water delivered at 10 μl/min by a 130A syringe pump (Applied Biosystems, Foster City, CA, USA). After 10 min, valve A was switched to connect the precolumn to the separating column and the gradient was started. A linear gradient from 5% acetonitrile/0.1% formic acid in water to 80% acetonitrile/0.1% formic acid in water, over a period of 50 min, was performed. The column was connected to the UV detector equipped with a U-shaped cell and then linked directly via a 25-μm I.D. fused-silica capillary to a nano-LC electrospray device developed in the laboratory, using PTFE sleeves. This device holds a New Objective PicoTip needle (Woburn, MA, USA), which is a Au–Pd-coated nano electrospray needle with a 10-μm tip. The instrument was set to perform a MS survey scan of 2 s with m/z range of 400–1500. Any peak with a threshold of 100 counts/s was automatically detected and selected by the quadrupole for fragmentation. The collision gas was Ar (1 bar) and the collision energy was kept at 32 V. The scan time was 1 s, with m/z from 50 to 2000. The instrument was used in the positive ion mode and calibrated prior to analysis using horse myoglobin.

2.7. Data interpretation and similarity searching

The MS–MS spectra were interpreted manually, supported by software from the manufacturer (Micromass) that is implemented in the MassLynx software controlling the quadrupole Time-of-Flight (Q-TOF) system. It consists of the MaxEnt Sequence tool which was used to convert multiple charged

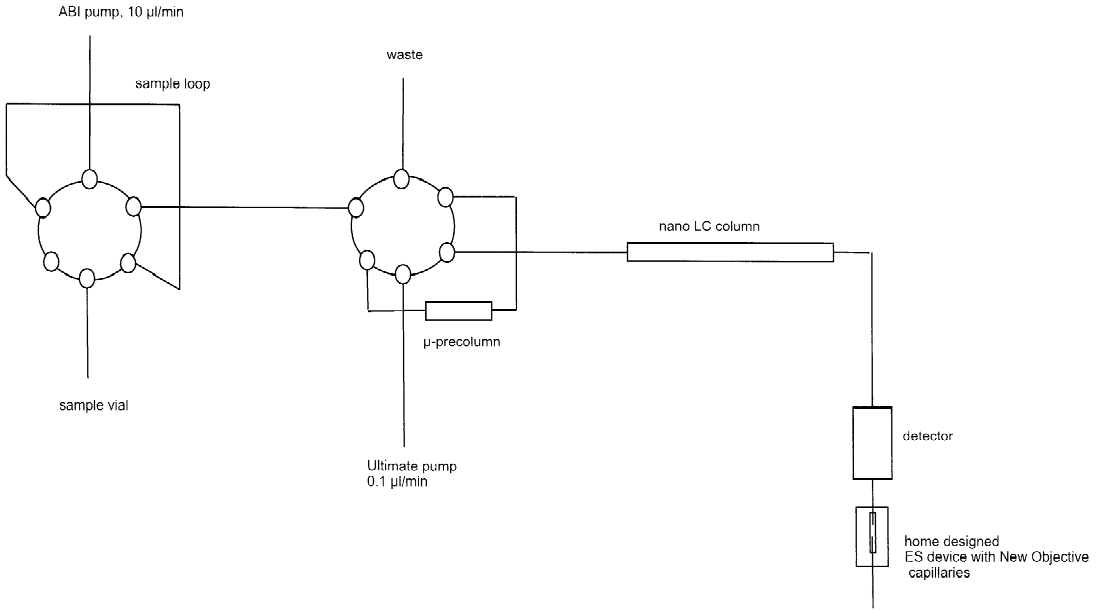

Fig. 1. Schematic drawing of the nano-LC–MS setup used in this work (not to scale).

fragment ions to singly charged species, and the PepSeq tool which was used to help in finding the fragment ion series. In many examples, large parts of the sequences could easily be read immediately from the MaxEnt converted spectra.

The sequence information on *X. laevis* proteins in the non-redundant databases accessible for searches using mass spectrometric data is limited. Therefore, typical tools such as peptide mass fingerprinting and peptide sequence tag search were not successful. Generally, we did a preliminary database screening using PMF with intact peptide masses using ProteinProbe (Micromass) against the SwissProt database. If not successful, we used the National Center of Biotechnology Information (NCBI) Blast program to search for homology between a de novo determined peptide sequence and protein sequences available in the database.

3. Results and discussion

3.1. Results

The 2D-PAGE gels of the protein extracts of the neurointermediate lobes obtained from black- and white-background adapted *Xenopus* are shown in Fig. 2. A number of protein spots showed clear differences in intensity that could be revealed even visually, without the need of gel image analysis. These spots were excised from the gel and digested with trypsin. As indicated in the figure, 10 of these proteins were selected for further analysis on our nano-LC–MS–MS system. Spots 1–9 were clearly overexpressed in black-background adapted animals, whereas spot 10 showed higher expression in white-adapted animals. As typical examples, Fig. 3a shows the total ion current (TIC) chromatogram of the automated LC–MS run of spot 3, whereas Fig. 4 shows the MS–MS spectrum of the marked peak on the chromatogram. In Table 1, we summarize the peptide sequences we obtained through this approach, and the results of the best match we obtained from database similarity searches. Two proteins could be identified based on the mass of the intact peptides, thus based on PMF using the LC–MS data, i.e. calreticulin and the M_r 78 000 glucose-regulated protein, also called BiP protein. For these proteins, the MS–MS data confirmed their identification (data not shown). Except for calreticulin and BiP, only the DNA sequence for POMC and cofilin-2 of *X. laevis* in this list are available in the protein databases. We would therefore not have been able to identify most of the proteins in this study using PMF. Moreover,

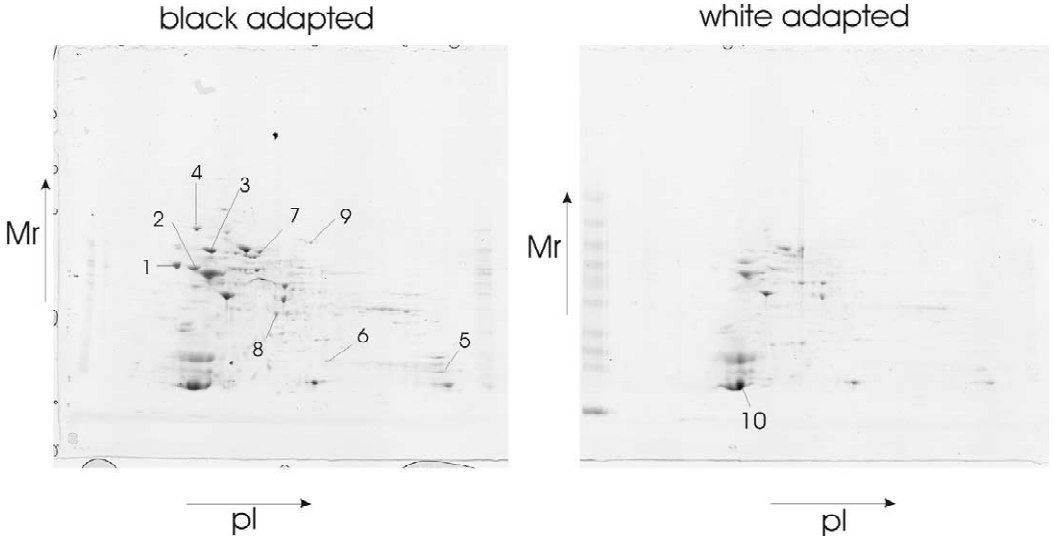

Fig. 2. 2D-PAGE of proteins isolated from the neurointermediate pituitary lobes of white and black background adapted *X. laevis*. The indicated spots are identified in this work.

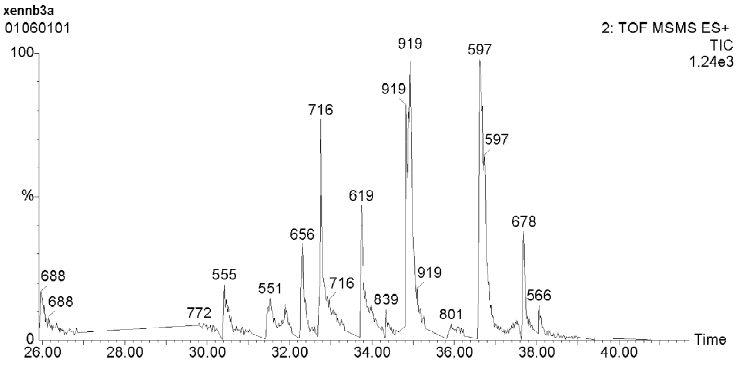

Fig. 3. TIC chromatogram of the LC–MS separation of the tryptic digest of spot 3. Time scale in min.

proteins such as POMC are heavily glycosylated, which would have given difficulties to identify this protein by PMF as well.

3.2. Technical aspects

Most proteomic papers deal with either MALDI-TOF-MS, which was not suited for our research, or nano electrospray MS analysis in an off-line approach using coated borosilicate capillaries, with or without prior desalting. We found a coupling of nano-LC to our Q-TOF-MS system very useful, since it highly automates both sample preparation (desalting) and sample injection (using the autosampler) without further manual interference. Nano-LC was proven to be essential to provide the sensitivity needed to detect the peptides obtained from tryptic digestion of a protein separated on 2D-PAGE, of which we estimated that most of them are present at a level of 50–100 fmol/μl. Compared to our previously published set-up where we used a commercially available nano-LC–MS interface [11],

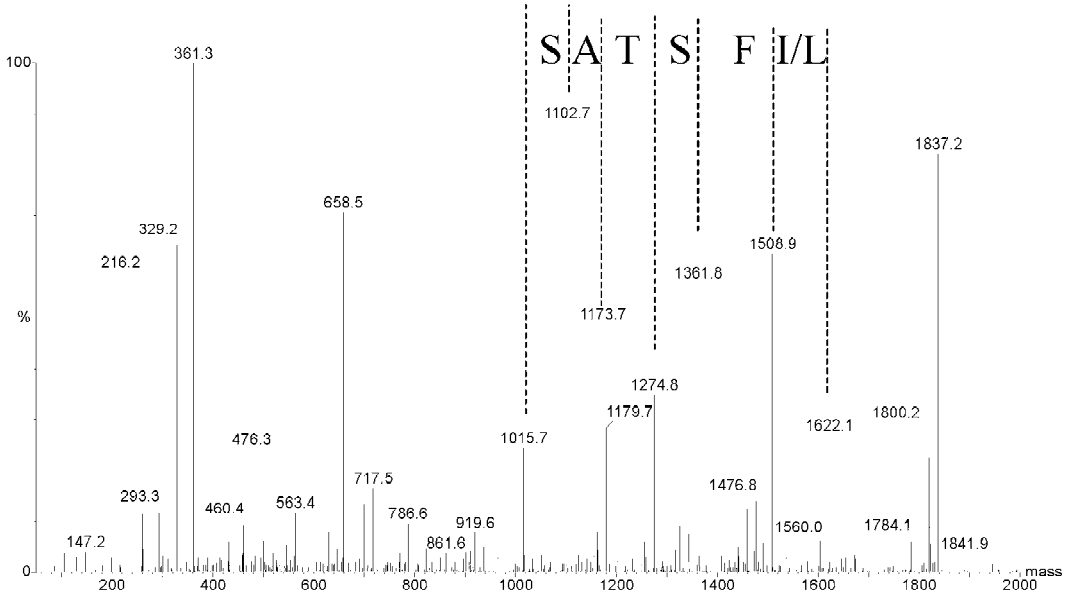

Fig. 4. MS–MS spectrum of a tryptic peptide obtained from spot 3 after LC–MS separation. The partial sequence identified led, together with other peptides, to the identification of this protein.

Table 1
Identification of Xenopus laevis proteins using nano-LC–MS–MS

Spot	Protein identity (SwissProt accession)	Method of identification[a]	Sequences derived
1	Calreticulin (Q19710)	PMF +PST	
2	Protein disulfide isomerase A1 (P07237)	Similarity (human)	LITLEEENTK EESDIAQEFRR YVSTVNELLVSK
3	M_r 78 000 glucose regulated protein (Q91883)	PMF+PST	
4	HSP90/endoplasmin (P14625)	Similarity (human)	VDSDDLPLNVSR
5	Cyclophilin-5 (P52013)	Similarity (*C. elegans*)	GSFFITTVK
6	Cofilin 2 (P45593)	Similarity	VVSLEGK
7	Protein disulfide isomerase A3(P30101)	Similarity (human)	LAPEYLEAATK TANDVP TLYFA
8	Pro-opiomelanocortin (P06298)	Similarity	SARSPVFPG DLSSED
9	Serotransferrin (P20233)	Similarity	VNQQSLYGR
10	Vasotocin/neurophysin (P08163)	Similarity (*Bufo japonicus*)	TVMDGSASDFLR

[a] PMF = peptide mass fingerprinting, PST = peptide sequence tag. If the sequence of the corresponding *X. laevis* protein was not available, the organism used to identify the protein by similarity is mentioned. In these cases, the SwissProt accession number refers to the homologous protein.

we find our current interface to be much more robust, mainly because the simple use of PTFE sleeves to connect the different tubing is more reliable than the connections using zero-dead volume unions which require repeated plumbing. The use of the commercially available PicoTip provides a much more stable spray. Generally, over 40 runs can be performed without replacing any component without loss of sensitivity.

3.3. Biological relevance

Several of the identified proteins, such as calreticulin, protein disulfide isomerase and BiP reside in the luminal part of the endoplasmatic reticulum (ER) where they aid in protein folding, facilitate the assembly of multimeric protein complexes or catalyse the interconversion of peptidyl-propyl imide bonds in peptide and protein substrates (e.g. cyclophilin). Their higher protein expression levels detected in black-background adapted animals are not surprising. From our earlier differential hybridization screening approaches comparing the expression levels of the mRNA populations in melanotropes of white- and black-background adapted animals, we know that the expression of POMC and several of the transcripts involved in the regulated secretory pathway can be 30 times higher in the neuroendocrine cells of the black-background adapted animals when compared to those in white animals [10,15–17]. The physiological relevance of the coexpression of the above proteins with POMC in *Xenopus* melanotropes is therefore obvious. Besides, morphometric analyses at the ultrastructural level have shown that the melanotrope cells of white-background adapted animals can be categorized as secretory inactive (numerous round to oval-shaped secretory granules, poorly developed Golgi complexes and ER dilatations) while the neurointermediate lobe of black-background adapted toads consists primarily of secretory active melanotrope cells (much larger cells with well developed Golgi areas and large amounts of rough ER) [18].

The identification of HSP-90 in the active melanotropes is of interest. HSP-90 is widely observed in stress response analyses and is localized in the cytosol of ‘‘hyper’’ active cells, which play a role in signal transduction pathways. It may influence in

this way the regulation of the transcription of a discrete set of genes (the so-called ''folding catalysts'').

Cofilin 2 and serotransferrin are involved in actin polymerization and in the delivery of Fe-ions to cells for growth and cell proliferation, respectively. Their role in cellular adaptation of the melanotropes of black-background adapted animals is at present not obvious. Serotransferrin is found in the blood serum but also in the lumen of early endosomes after receptor-mediated endocytosis. As such, this protein may be incorporated in the endosomes of the active melanotropes to liberate the free Fe-ions for growing of the cells. However, we may not exclude the possibility that the identification of serotransferrin is due to the higher amounts of this protein in the blood circulation around the neurointermediate neuroendocrine cells in the black animals. Clearly, further studies (e.g. immunocytochemical localization of the protein) are needed.

The identification of the vasotocin/neurophysin precursor in the neurointermediate lobes of the white-background adapted animals is also interesting. This precursor is one of the typical neurohypophyseal neurohormone complexes that are synthesized in the cell bodies of the hypothalamus, then transported along axons, and stored in the terminals of the axons in the neurohypophysis or pars nervosa of the pituitary. The precursor is further processed by endoproteinases to generate the hormonal bioactive peptide vasotocin. This neuropeptide is an orthologue of the mammalian vasopressin that controls the permeability of the skin in amphibians and is therefore essential for the maintenance of the water balance. The higher expression in the neurointermediate lobe of white-background adapted animals is in line with earlier morphometrical studies on the dynamics of synaptic and endocrine communication in our model system. These studies have clearly demonstrated profound effects of background adaptation on the morphology of the neuronal network in the neurointermediate lobe [19]. The size of the single and clustered varicosities of the axon terminals of the pars nervosa around the melanotropes of white-adapted animals are much higher than in black-adapted ones, reflecting the strong plasticity of the active synaptic zones. The physiological importance of the locally higher amounts of vasotocin need further investigations.

4. Conclusion

An automated nano-LC–MS–MS is demonstrated to allow the unambiguous identification of proteins from 2D-PAGE separations of total cell extracts. The present data, furthermore, show the complementarity of differential display analysis at the mRNA level and at the protein level. Currently, this approach is used to identify all other proteins that are differentially expressed in this system.

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