Journal of Cellular Biochemistry 102:473–482 (2007)

cAMP Induced Alterations of Chinese Hamster Ovary Cells Monitored by Mass Spectrometry

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Abstract Chinese Hamster Ovary fibroblasts (CHO-K1) have shown different protein contents when undergoing differentiation by 3′,5′-cyclic adenosine monophosphate (cAMP), which is known to induce reverse transformation (RT) from malignancy to fibroblast-like characteristics. The mass spectrometry investigation here reported about the behavior of CHO-K1 cells before and after exposure to cAMP reveals a change in the composition of nuclear proteins associated to an inhibition of the protein expression. Possible implications of this finding on the control of cell reverse transformation are discussed. J. Cell. Biochem. 102: 473–482, 2007. © 2007 Wiley-Liss, Inc.

Key words: Mass spectrometry; HPLC; CHO-K1; cAMP

INTRODUCTION

CHO-K1 cell displays the classical features of transformed cells grown in vitro: it has a compact and pleomorphic structure with a surface studded with knobs instead of the smooth, highly elongated spindle-shape characteristic of normal fibroblasts; it grows in a random pattern and it shows no contact inhibition of growth but readily grows in three dimensions. It has been reported that cyclic adenosine monophosphate (cAMP) causes cell reverse transformation [Nicolini and Beltrame, 1982; Puck, 1977; Hatzopoulos et al., 1998] and in particular it causes CHO-K1 cells to lose their characteristics and to assume the morphology and growth characteristics of normal fibroblasts [Hsie and Puck, 1971]. This process is named "reverse transformation" (RT) and appears to involve microtubules [Brinkley et al., 1975], chromatin-DNA structure [Nicolini, 1983; Nicolini et al., 1983] and coupling of nuclei to cell morphometry [Nicolini and Beltrame, 1982], which have impact also other biological processes such as the control of gene expression [Parodi et al., 1979] and the cell cycle progression [Nicolini and Beltrame, 1982; Belmont et al., 1984].

To clarify the molecular mechanisms CHO mutants were selected for their unresponsiveness to the reverse transformation reaction of cAMP derivatives. Phosphorylation analysis was carried out by 2D gel electrophoresis. Seven differences in protein phosphorylations in the parental CHO cell were identified as a result of treatment with dibutyryl cAMP [Schliwa, 2002]. Unresponsive mutants of two kinds were found. One had lost all seven of the phosphorylation changes induced by dibutyryl cAMP in the wild-type cell. However, the other type, equally resistant to reverse transformation by cAMP derivatives, differs from the parental cell in only one (or possibly two) phosphorylation events involving a 55,000-dalton protein. Phosphorylation of this protein, which is not yet identified, may therefore be directly related to transformation and its reversal in the CHO cell [Schliwa, 2002]. However, the changes in the concentration of the different metabolites and the detailed different protein composition associated with morphological changes observed [Hsie et al., 1971] are still largely unknown.

Grant sponsor: Ministero dell'Istruzione, Università e Ricerca (MIUR); Grant number: RBNE01X3CE.

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Received 16 October 2006; Accepted 24 January 2007

DOI 10.1002/jcb.21308

The detailed proteomic changes associated to microtubules disruption [Brinkley, 1982], nuclei-cell uncoupling in [Belmont and Nicolini, 1982; Nicolini and Beltrame, 1982] and changes in cell membrane [Gabrielson et al., 1982; Puck et al., 1998] during reverse transformation, indeed, is yet obscure. We therefore analyzed the CHO-K1 cell protein composition to identify eventual changes in the protein pattern before and after the reverse transformation, likewise the induction of collagen synthesis [Hsie and Puck, 1971].

We investigated by mass spectrometry and HPLC particularly the nuclear envelope proteins, yet largely unknown, that can be involved in cell differentiation and in the nucleus reorganization [Puck, 2002; Schirmer et al., 2003].

EXPERIMENTAL PROCEDURES

Cell Preparation

Cells used were the CHO-K1, which is a stable hypodiploid line derived by spontaneous transformation from a fibroblast culture [Misteli, 2001]. Chinese hamster ovary fibroblasts (CHO clone K1) were supplied by American Type Culture Collection, Rockville, MD. Cells were cultured in F12 medium supplemented with 10% fetal calf serum and 0.2% gentamycin at 37°C in 5% CO_2 atmosphere. To reverse transform the cells they were treated with a solution 10^{-3} M cyclic 3′,5′-monophosphate adenosine sodium salt (cAMP, Sigma Chemical Co., St. Louis, MO) that was added to the normal culture medium for 6 h before the analysis. It has been shown that single cells of CHO-K1 in the native state grow equally well on plastic surfaces or in suspension. In the presence of reverse transformation conditions, however, excellent growth is still achieved on the plastic surface but no growth whatever occurs in suspension [Hsie and Puck, 1971]. We then monitored CHO-K1 reverse transformation monitoring their growing behavior by a phase contrast microscope (Wilovert, Wesco).

Protein Extraction

A protein extraction kit (Subcellular Proteome Extraction Kit, Calbiochem) was chosen for the total protein extraction. It is designed for extraction of cellular proteins from adherent and suspension-grown cells according to their subcellular localization. For the sequential extraction of the cell content, the kit takes advantage of the differential solubility of certain subcellular compartments in special reagent mixtures.

Upon extraction of culture cells, four partial proteomes of the cells are obtained:

- cytosolic proteins,
- membrane and membrane organelle proteins,
- nucleic proteins,
- cytoskeleton proteins.

The kit contains four extraction buffers, a protease inhibitor cocktail to prevent protein degradation and a nuclease (Merck) to achieve an efficient removal of contaminating nucleic acids. The subcellular extraction was performed according to the included protocol for freshly prepared adherent culture cells. We performed extraction from 10^6 CHO-K1 cells in logarithmic phase at 80% confluence. To monitor the extraction procedure, morphological changes of the cells were examined by a phase contrast microscope (Wilovert, Wesco). The protein fractions were stored at −80°C until the MS analysis. The amount of total proteins recovered from each extraction step has been evaluated by Bradford assay [Bradford, 1976; Tjio and Puck, 1958], using bovine serum albumin as standard.

Mass Spectrometry Experiments

The identification of different protein patterns in CHO-K1 cells grown with and without cAMP, is performed with a RP-HPLC-ESI MS apparatus, a ThermoFinnigan (San Jose, CA) Surveyor HPLC connected by a T splitter to a PDA diode-array detector and to Xcalibur LCQ Deca XP Plus mass spectrometer. The mass spectrometer is equipped with an electrospray ion source (ESI). The chromatographic column is a Vydac (Hesperia, CA) C8 column, with a 5 μm particle diameter (column dimensions 150 × 2.1 mm). For HPLC-ESI MS analysis the protein solutions, stored at −80°C, were heated at room temperature and then lyophilized. The lyophilized proteins were immediately dissolved in aqueous 0.2% TFA and centrifuged at 12,000 rpm for 5 min. After centrifugation, the supernatant was analyzed by HPLC-mass spectrometry. The following solutions were utilized for reversed-phase chromatography: eluent A, 0.056% aqueous TFA, and eluent B,

0.05% TFA in acetonitrile/water 80:20 (v/v). The gradient applied was linear from 0 to 55% in 40 min, at a flow rate of 0.30 ml/min. The T splitter gave a flow rate of about 0.20 ml/min toward the diode array detector and a flow rate of 0.10 ml/min toward the ESI source. The diode array detector was set in the wavelength range 214–276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 4.50 kV, and the capillary temperature was 22°C. The deconvolution of the averaged ESI mass spectra was performed by Mag-Tran1.0 software. A preliminary proteins identification from the mass values of the intact protein was obtained through a search in Swiss-Prot Data Bank (http://www.expasy.org).

The identification of CHO-K1 proteins via mass fingerprint is performed coupling HPLC (Varian, Inc.) to separate the proteins, and MALDI TOF MS, a Bruker Autoflex (Bruker Daltonics, Leipzig, Germany) to analyze the tryptic digest of these samples. The HPLC measurements were carried out on a Varian Star HPLC system which includes: 9012 Gradient Solvent Delivery System, 9050 UV-VIS Detector, 9300 Refrigerated AutoSampler (fitted with a 20 μl loop), and a Star Chromatography Workstation (Varian, Inc., IL, USA). Proteins will be separated on a C8 (250 × 4.6 mm; 5 μm particle size) reverse phase column (Macherey-Nagel, Germany). For reversed-phase chromatography we utilized the following solutions: eluent A, 0.056% aqueous TFA, and eluent B, 0.05% TFA in acetonitrile/water 80:20 (v/v). The gradient applied was linear from 0 to 50% in 30 min, at a flow rate of 0.30 ml/min. For HPLC analysis the protein samples stored at −80°C, were heated at room temperature and then 100 μl of solutions were injected. We used three samples of Lysozyme from chicken egg white (Sigma Chemical, Co., St. Louis, MO) at different concentrations to standardize the HPLC results and the protein amounts loaded to MS.

For protein fingerprint, the fraction collected from HPLC were digested with trypsin overnight, according to the enzyme supplier (Sigma-Aldrich).

For MALDI-TOF MS the tryptic digest samples were diluted to 4 nM protein concentration in a 0.1% TFA solution. The matrix used for the mass spectrometric analysis was a saturated solution of acid (α-Cyano-4-hydroxycinnamic acid for peptides and light proteins and sinapinic acid for heavy proteins, Bruker Daltonics) dissolved in 2/3 of 0.1% TFA and 1/3 of acetonitrile. For the analysis 1.5 μl of matrix solution was mixed with 1.5 μl of sample, then 1 μl of this mixture was spotted onto a suitable aluminum plate and air-dried. MALDI-TOF MS was externally calibrated using protein and peptide calibration standard solutions (Bruker Daltonics), resulting in a mass accuracy <100 ppm for intact proteins and <10 ppm for peptides.

The mass lists obtained were submitted to a data bank search. We use a specific software for protein data interpretation, Biotools (Bruker Daltonics), that allows an automated protein identification via library search and that has a MASCOT Intranet search software (Matrix Sciences, Ltd. www.matrixscience.com) fully integrated.

RESULTS

MS analysis started from the HPLC-ESI MS analysis of nuclear proteins fraction from CHO-K1 cells and from cAMP treated cells. The total ion current (TIC) and the UV absorbance at 214 and 276 nm (not reported) profiles obtained during chromatographic analysis of the two protein solutions are reported in Figure 1. TIC and UV profiles did not reveal significant signals between 0 and 10 min. The highest variations are identifiable in the TIC plots (see enlargement in the box) and show an inhibition of the nuclear protein expression after treatment with cAMP.

In particular:

- in the untreated CHO-K1 plot there is a peak at 42.45 min not observable in the cAMP treated CHO-K1 plot;
- the intensity of the 44.90 min peak in the untreated CHO-K1 TIC plot is double regarding the cAMP treated cells plot peak.

The TIC of proteins of interest was used for their relative quantification. Since the TIC depends not only from concentration but also from the charge of the analyte, sample treatment was standardized both in dilution and HPLC-ESI-MS procedures, to ensure similar bias, namely same ion suppression, pH and organic solvent effects on the analyte charge. Under these conditions, the TIC can be roughly considered proportional to protein

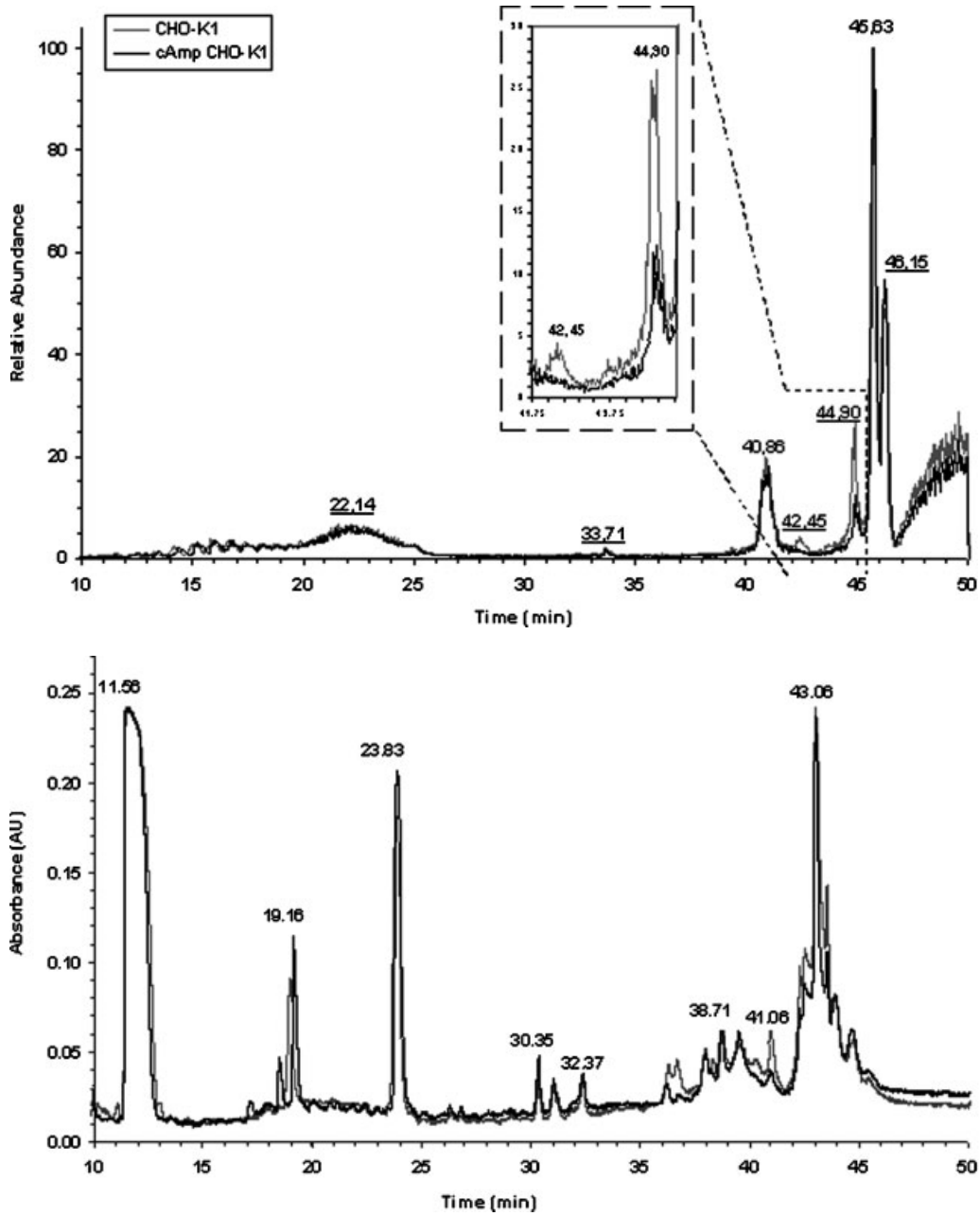

Fig. 1. HPLC-ESI-MS TIC and UV profile of nuclear protein fraction of CHO-K1 cells (gray line) and of CHO-K1 cells treated with cAMP (black line). **Upper plot**: HPLC-ESI-MS TIC profile collected by ion trap mass spectrometer (elution time 10–50 min). Enlargement of the 41.7–45.5 min elution range showing the TIC profile is represented in the box where the greater differences between the two plots are shown: the 42.45 min peak (absent in the treated CHO-K1 plot) and the 44.90 min peak (its relative abundance is double in the untreated CHO-K1 plot). **Bottom plot**: UV (214 nm) profile (elution time 10–50 min).

concentration, and it can be used to evidence correlations existing among different proteins in different samples [Messana et al., 2004].

Regarding the first identified variation we analysed the averaged ESI mass spectrum (300–2000 m/z range) recorded in the elution range 42.25–42.68 min, shown in the 2^{nd} *panel* of Figure 2A. From the Gaussian deconvolution of this spectrum, performed by MagTran1.0, we identified three protein species (see Fig. 2B). We performed a first tentative protein identification on the basis of these weights through a search in Swiss-Prot data bank. We restricted our search to rodent proteins.

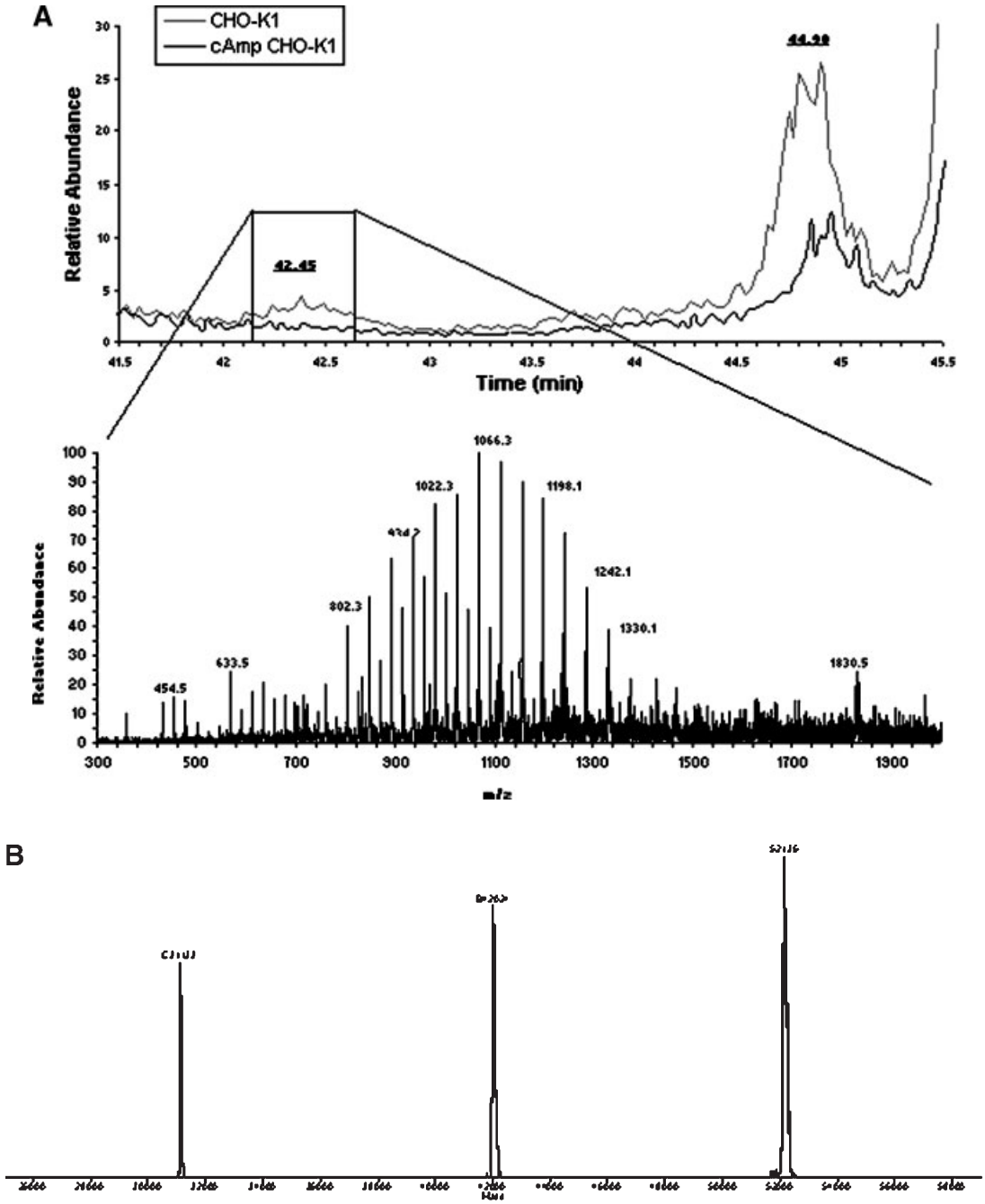

Fig. 2. **A**: 1st panel (from the top), HPLC-ESI-MS TIC profile (elution range 41.5–45.5 min). 2nd panel, averaged ESI mass spectrum (300–2000 m/z range) of CHO-K1 nuclear protein fraction recorded in the elution range 42.25–42.68 min. **B**: deconvoluted spectrum of averaged ESI mass spectrum reported in the second panel.

In Table I, we report the experimental and theoretical weights and the function of the identified proteins. Among these proteins, Guanine nucleotide-binding protein (Gna11) and Myosin heavy chain 10, non-muscle (Myh 10), are particularly interesting because they are both involved in cellular regulation. Heterotrimeric guanine nucleotide-binding proteins (G proteins) are integral to the signal transduction pathways that mediate the response of the cell to many hormones, neuromodulators, and a variety of other ligands [Strathmann and

TABLE I. Proteins Detected in the CHO-K1 Protein Nuclear Fraction Correspondently to the 42.54 min TIC peak (shown in Fig. 2B)

Exp. mass (Da)	Th. mass (Da)	Protein	Function
31133	31133	Myh10 (fragment B) Myosin heavy chain-B (Fragment), non-muscle	Cellular myosin appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping.
42024	42024	Gna11 Guanine nucleotide-binding protein, alpha-11 subunit	Guanine nucleotide-binding proteins (G proteins) are involved as modulators or transducers in various transmembrane signaling systems. Acts as an activator of phospholipase C.
52136	52134	S61A1 Protein transport protein Sec61 alpha subunit isoform 1	Plays a crucial role in the insertion of secretory and membrane polypeptides into the ER. Required for assembly of membrane and secretory proteins. Tightly associated with membrane-bound ribosomes, either directly or through adaptor proteins.

Simon, 1990]. Nonmuscle myosins play a role in diverse cellular functions, for instance cytokinesis, proliferation, secretion, and receptor capping. Two isoforms of the nonmuscle myosin heavy chain (nmMHC), chain A (nmMHC-A) and chain B (nmMHC-B) have been identified. The nmMHC-B in particular is involved in cell growth regulation and transformation [Strathmann and Simon, 1990].

Regarding the 44.90 min TIC peak variation-relative abundance passed from 26.6 for the untreated cells plot to 12.3 for the cAMP treated cells plot (Fig. 3A, *1st panel*)—we analyzed the averaged ESI mass spectra recorded in the elution range 44.40–45.20 min (Fig. 3A, *2nd panel*). The two spectra are clearly identical: the difference of intensity in the TIC peaks can be explained by a different amount of the same proteins in the two samples. We performed a Gaussian deconvolution of this spectrum and identified three protein species (Fig. 3B). Through a search in Swiss-Prot data bank we have been able to identify only the 29628 Da peak as CD82_MOUSE protein. This protein, associates with CD4 or CD8 glycoprotein delivers co-stimulatory signals for the TCR/CD3 pathway [Itoh and Adelstein, 1995] involved in apoptosis regulation. For 6632 Da peak and 20739 Da peak it was not possible to identify any protein.

Moreover we analyzed 17 other proteins of the nuclear envelop (Fig. 4) present in the same content in the cells before and after cAMP exposure. The relative molecular weight are 2.4 kDa, 11.9 kDa, 17.3 kDa, 29.55 kDa, 35.6 kDa, 41.2 kDa, 46.7 kDa, 52.3 kDa, 52.9 kDa, 58.9 kDa, 64.2 kDa, 70.7 kDa, 60.9 kDa, 82.5 kDa, 93 kDa, 96.2 kDa and 99.3 kDa. It has been possible to identify none of these proteins only from their molecular weight.

We subsequently performed a series of experiments—coupling HPLC (Table II) and MS [Nagira et al., 1994]—to identify all these proteins and to confirm the identity of the proteins via mass fingerprinting (that allows to identify the protein with a very high probability).

This second step was conducted on a MALDI-TOF Mass Spectrometer. For protein fingerprint, the HPLC fractions were digested and the tryptic digest samples were analyzed by MALDI-TOF MS. Until now we have obtained from this analysis the confirm of identification of 31133 Da protein as "Myosin heavy chain-B (Fragment), non-muscle" (see Fig. 5), present only in the CHO-K1 sample (HPLC fraction 3, see Table II).

DISCUSSION

The study here reported about the effect of cAMP on the protein expression of the CHO-K1 cells is continuing structural and functional work started in our laboratory many years ago at the level of nuclei and genes [Nicolini and Beltrame, 1982; Vergani et al., 1992, 2001]. Our RP-HPLC-ESI MS results, confirmed by HPLC measures, show a different protein content in the nuclear protein fractions of the cells after exposure to cAMP, possibly linked to the above early observations. In particular we focused our attention on the two main differences in the TIC plots of the nuclear protein fractions of untreated and treated cells that show a decrease in the nuclear protein amount after cAMP treatment. Through ESI MS analysis we identified a group of three proteins, Myh10,

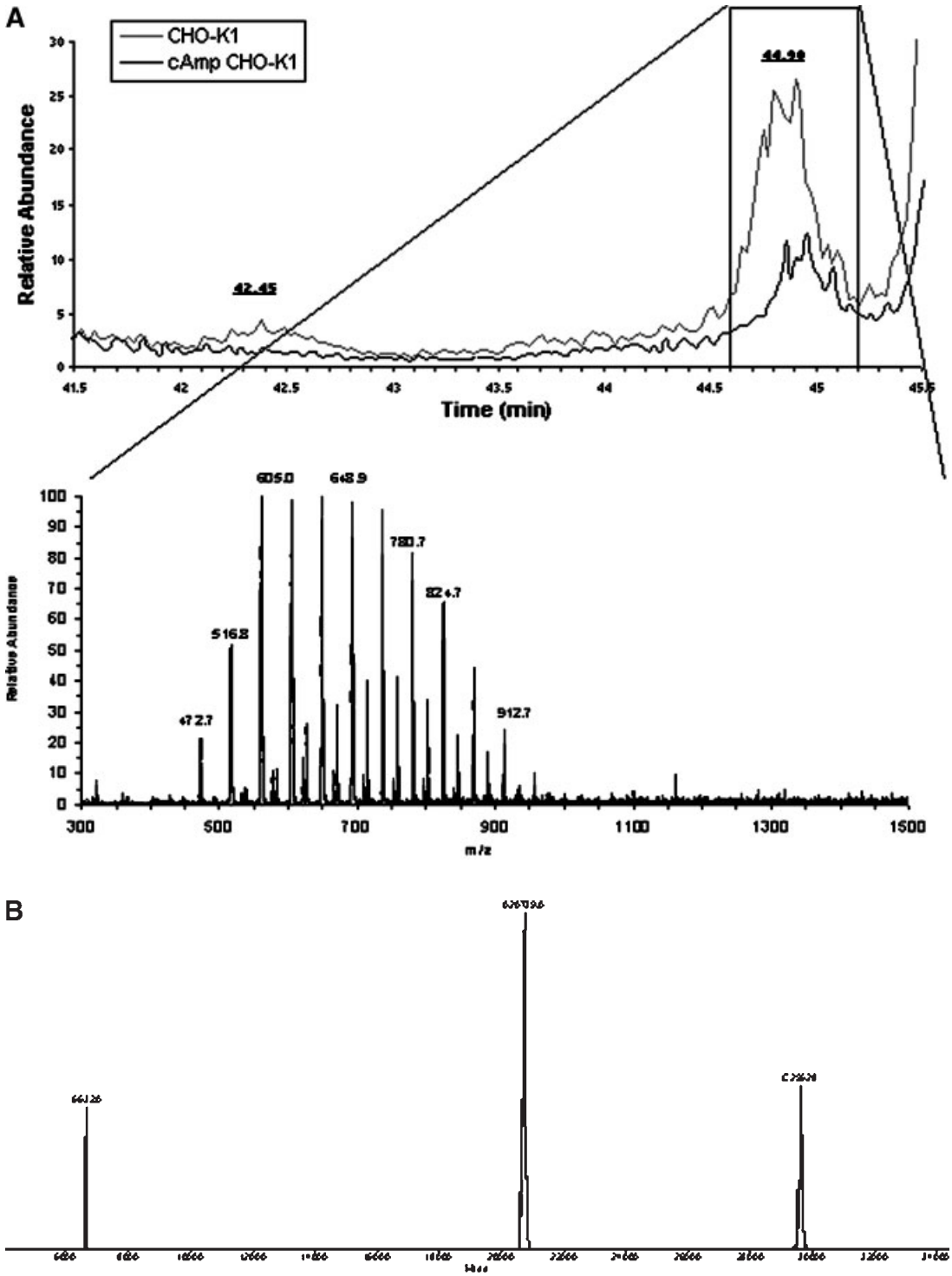

Fig. 3. **A**: 1st panel (from the top), HPLC-ESI-MS TIC profile (elution range 41.5–45.5 min). 2nd panel, enlargement of averaged ESI mass spectra (300–2000 m/z range) of CHO-K1 (gray line) and cAMP treated CHO-K1 (black line) nuclear protein fraction recorded in the elution range 44.40–45.20 min. The spectra are identical. **B**: deconvoluted spectrum of averaged ESI mass spectra reported in the second panel.

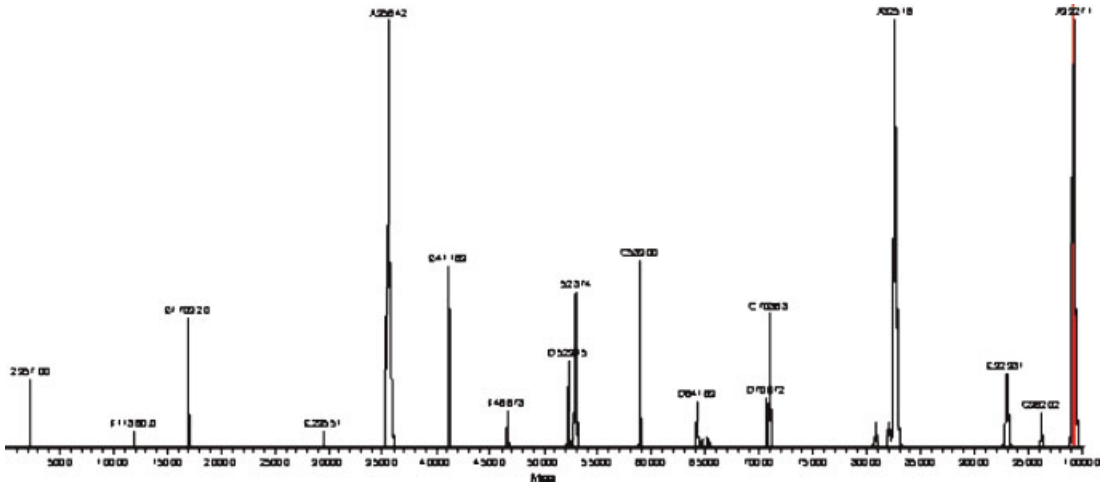

Fig. 4. Sum of the deconvoluted spectra relative to TIC plot 22.00, 45.79, and 46.21 min peak. These proteins are present in the same amount in the cells before and after cAMP exposure (as is visible from Fig. 1). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Gna11 and S61A1 (Fig. 2B), present only in the nuclear protein fraction of untreated cells and involved in cellular regulation processes. The Myh10 protein is involved in the catalysis of movement along a polymeric molecule such as a microfilament or microtubule, coupled to the hydrolysis of adenosine 5′-triposphate (ATP), while the Gna11 protein regulates the cascade of processes by which a signal interacts with a receptor, causing a change in the level or activity of a second messenger.

Moreover we identified another group of three proteins present in both samples but in a double concentration in the nuclear protein fraction of untreated cells (Fig. 3B). Through ESI MS analysis we were able to identify only one of these three proteins, Cd82Mouse, that results involved in apoptosis regulation processes.

Other seventeen proteins present in equal amount before and after cAMP exposure have been identified (Fig. 4). From preliminary HPLC and MS experiments we had a confirmation of the inhibition of the nuclear protein expression after exposure to cAMP; moreover by protein fingerprinting we confirmed the identification of 31133 Da protein as Myh10.

The same analysis on the other protein fractions (cytosolic, membrane and membrane organelle and cytoskeleton fraction) is in progress to analyze the entire proteome in presence of cAMP, to obtain a comprehensive understanding of the reverse transformation.

ACKNOWLEDGMENTS

This project was supported by a FIRB International Grant on Proteomics and Cell Cycle (RBIN04RXHS) from MIUR (Ministero dell'Istruzione, Università e Ricerca) to CIRSDNNOB-Nanoworld Institute of the

TABLE II. Results of HPLC Analysis of CHO-K1 and cAMP Treated CHO-K1 Protein Nuclear Fractions. The Reported Data are Relative to the Three Dominant Peaks, which Confirm the Inhibition of the Protein Expression after Exposure to cAMP

	Elution time (min)	Peak area (mAU/sec)	Protein concentration (mg/ml)
Fraction 1			
CHO-K1	9.7	20,329	61
CHO-K1 cAMP	9.5	18,031	54
Fraction 2			
CHO-K1	5.8	2,746	8
CHO-K1 cAMP	6.0	2,109	6
Fraction 3			
CHO-K1	5.1	2,681	8
CHO-K1 cAMP	5.1	0	0

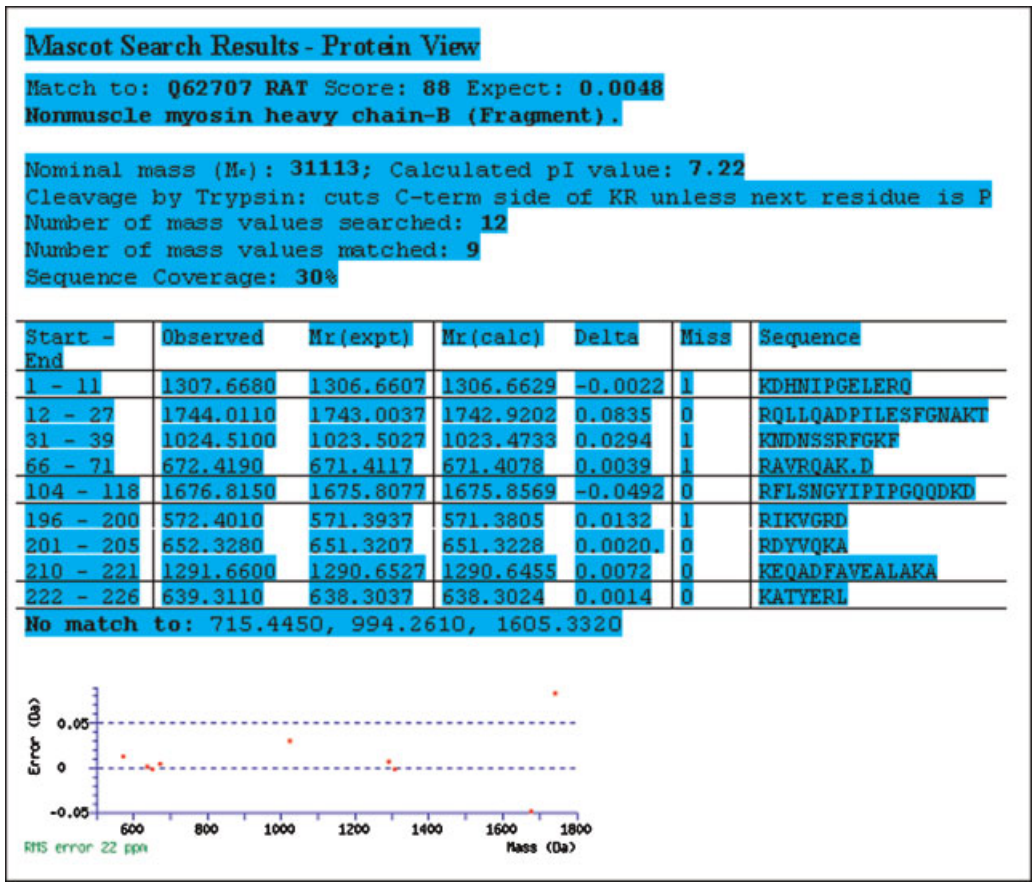

Fig. 5. Mascot results: Mass fingerprint analysis of the fraction 3 obtained from HPLC analysis of CHO-K1 nuclear protein fraction (Table II). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

University of Genova. The authors are grateful to Prof. Bruno Giardina, Prof. Mario Castagnola and Dr. Rosanna Inzitari for the cooperation during HPLC-ESI MS experiments at the Biochemistry Institute of the Catholic University of Rome. We also thank Mr Fabrizio Nozza for technical support.

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