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Identification of biomarkers indicating cellular changes after treatment of neuronal cells with the C3 exoenzyme from *Clostridium botulinum* using the iTRAQ protocol and LC–MS/MS analysis☆

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ABSTRACT

Proteomic approaches are used to identify biomarkers, to monitor pathological changes inside of cells and for a better diseases diagnosis. Comparable changes in protein homeostasis also occur in differentiating cells and proteomic techniques should be suitable to identify biomarkers that indicate different steps of cellular development. The C3 exoenzyme from *Clostridium botulinum* (C3*bot*) inactivates Rho GTPases and induces morphological cellular changes like cell rounding and neurite outgrowth [G. Ahnert-Hilger, M. Höltje, G. Grosse, G. Pickert, C. Mucke, B. Nixdorf-Bergweiler, P. Boquet, F. Hofmann, I. Just, J. Neurochem. 90 (2004) 9]. To investigate these observations further a comparative proteomic approach has been chosen to elucidate C3*bot* effects in the neuroblastoma cell line model SH-SY5Y. The screening method applied for biomarker detection was based on the stable isotope approach isobaric tagging for relative and absolute quantification (iTRAQ). Proteins of C3*bot*-treated and untreated cells were digested and peptides were labeled by the iTRAQ reagent, combined, and separated by means of a two-dimensional nano-HPLC system. Peptide analysis was performed in a MALDI-TOF/TOF mass spectrometer. Identification and quantification of peptides and their corresponding proteins were accomplished by MS/MS spectra analysis. Overall, five replicate measurements identified 355 different proteins of which 235 were accessible for quantification. C3*bot* altered the concentration of 55 proteins (at least 1.3-fold) and several proteins were identified as possible biomarker candidates that indicate C3*bot*-induced cellular changes.

1. Introduction

Within the last 20 years, two main fields of research received central attraction in life science: genomics, the field of genome research and proteomics, which covers the analysis of all proteins expressed in a cell, tissue, body fluid, or whole organism [2]. Whereas genomes cover the construction plan of the biological system, proteomes represent the molecules liable for its realization and regulation. The proteome of a cell is highly dynamic and changes over time. Proteome analyses are therefore not limited to the description of a protein composition of a current state but are often used to identify and characterize qualitative or quantitative changes in cellular protein patterns. Such experiments aim at the identification of biomarkers, which define a particular state of a cell, tissue or organism. Thus, a biomarker is a protein (or other biological compound) that can be evaluated as an indicator of a normal biological or a pathogenic process as well as a pharmacological response to a therapeutic intervention. Research interests focus mainly on the identification of biomarkers for early detection or better diagnosis of diseases [2–6]. These studies require the ability to perform high quality and high throughput analysis, monitoring many proteins with high confidence at a given time point. Standard techniques in proteome research based on two-dimensional gel electrophoresis have been successfully applied for biomarker identification [7–9]. However, due to the known shortcomings of gel-based approaches, e.g. identification of basic and acidic proteins, methods for protein tagging based on stable isotopes have been established. In these techniques, mass spectrometric protein identification is directly combined with highly sensitive protein quantification allowing improved identification of new biomarkers [10–12]. Methods based on this principle include isotope coded affinity tags (ICAT) [13], isotope coded protein labels (ICPL) [10], stable isotope labeling by amino acids in cell culture (SILAC) [14], ExacTag (PerkinElmer), and isobaric tagging and relative quantification (iTRAQ) [15]. In the work described here the iTRAQ technique is used. In this approach proteins from up to eight samples can be analyzed. Proteins are digested by trypsin and the generated peptides are tagged with different isobaric reagents containing varying

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amounts of heavy stable isotopes. Peptide samples are combined and analyzed in a mass spectrometer. All tags are composed of a reporter group and a balance group. Upon fragmentation in MS/MS analysis, the bond between the reporter group and the balance group is cleaved. The balance group remains attached to the peptide and the reporter groups give a defined MS signal. Reporter groups from the labeled samples differ in their mass due to the different stable isotopes. Thus, a specific reporter group can be assigned to each sample. Since all physicochemical parameters (except the mass) of these reporter groups are identical, the different abundance of the reporter groups correlates to the abundance of the labeled peptides from each sample. The relative concentrations can be calculated by the peak area of the reporter groups. A highly precise measurement of the relative abundance of the respective protein is obtained because several peptides of one protein are quantified. We applied the iTRAQ protocol to study the proteome of neuronal cells before and after treatment with the C3 exoenzyme from *Clostridium botulinum* (C3*bot*) to identify biomarkers that respond to the C3*bot* treatment.

C3*bot* is the prototype of C3-like ADP-ribosyltransferases, which exclusively modify the Rho subfamily GTPases RhoA, RhoB and RhoC by recruiting the ubiquitous co-substrate NAD^+ for ribosylation of asparagine-41 [16,17]. Rho-GTPases are molecular switches that control a variety of signal transduction pathways in eukaryotic cells [18–20]. They are master regulators of the actin cytoskeleton and cell polarity. Additional Rho proteins are involved in cell cycle progression, transcriptional activity, vesicular transport pathways, cell migration, and microtubule dynamics [21,22]. The specific inactivation of Rho-GTPases results in reorganization of the actin cytoskeleton [16]. Rho proteins are involved in neuronal development [23]. During neuronal growth, neurons undergo dramatic morphological changes, which result in the differentiation of axons and dendrites. Neuronal growth appears to depend upon a balance between activation (Rac and Cdc42) and inactivation (RhoA) of a subset of Rho GTPases [24]. Recently, it has been shown that C3*bot* exhibits neurotrophic effects on murine hippocampal neurons to result in axonal growth. However, similar effects were observed with the enzymatically inactive C3*bot*, suggesting a novel intrinsic neurotrophic function of C3*bot* that is not mediated by a direct inhibition of RhoA, RhoB and RhoC [1].

To obtain more information about the effects of C3*bot* on neuronal cells, we used the human neuroblastoma SH-SY5Y cell line as a model system. SH-SY5Y cells respond to C3*bot* treatment by actin cytoskeleton reorganization and neurite formation [1,16]. Cellular proteins from C3*bot* treated and untreated cells were extracted and were digested with trypsin. The resulting peptides were labeled with the iTRAQ reagent and separated by an HPLC-based two-dimensional chromatography system that was composed of cation exchange and reversed phase chromatography. Separated peptides were subjected to MALDI-MS/MS analysis for identification and relative quantification.

2. Experimental

2.1. Reagents and materials

Trifluoro acetic acid and formic acid (99% purity) were from J.T. Baker (Mallinckrodt Baker, Griesheim, Germany). The MS peptide calibration standard and α-cyano-4-hydroxy-cinnamic acid (CHCA) were purchased from Bruker Daltonik GmbH (Bremen, Germany). Chemicals for cell culture were from Invitrogen GmbH (Karlsruhe, Germany). All aqueous solutions were prepared with deionized water having a resistance of at least 18 MΩ (Millipore, Schwalbach, Germany). Fluka (Sigma–Aldrich GmbH, Seelze, Germany) offered the sodium hydroxide, ammonium dihydrogenphosphate, Coomassie brilliant blue, the solvents ethanol, acetonitrile and all other chemicals in the highest purity available.

2.2. Cell culture and preparation of crude extracts

SH-SY5Y cells were cultured in Dulbecco's minimum essential medium containing 14% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium pyruvate and maintained at 37 °C in an atmosphere of 5%CO_2 in air. The medium was changed every 2 days and cells were split every 6 days. Before C3*bot* treatment, cells were grown for 1 day and then C3*bot* was added to the medium at a concentration of 1 μM. After 72 h, the medium was removed and cells were washed three times with PBS. The cells were scratched from the culture flasks with a cell scraper and were resuspended in a small volume of lysis buffer (6 M urea, 2 mM DTT, 50 mM NH_4HCO_3). Supersonic disruption was performed in a cycle of 10×5 s for 5 times at 10% sonic energy using a sonotrode (Bandelin Electronic, Berlin Germany). To remove cell debris, lysates were centrifuged at $13{,}000 \times g$ for 30 min at 4 °C. Protein concentration was determined using Coomassie brilliant blue with BSA as a standard [25]. Usually protein concentrations of 1.5 mg/ml were obtained with a yield of 0.75 mg protein per 1×10^7 cells.

2.3. iTRAQ labeling of proteins

The iTRAQ labeling was done using the iTRAQ™ kit, from Applied Biosystems (Framingham, MA, USA) as described in the manual supplied. Before labeling protein samples were concentrated by chloroform/methanol precipitation [26], dried and dissolved in 30 μL iTRAQ-dissolution buffer containing 0.5 M triethylammonium bicarbonate at pH 8.5 and 1.5 μL 2% sodium dodecyl sulfate (SDS) with a protein concentration of 3 μg/μL. The maximum protein amount suitable for the kit was 100 μg protein per sample. For test analysis, a standard mixture containing four proteins was prepared. Bovine serum albumin (BSA), ovalbumin, Toxin A from *C. difficile* and recombinant C3*bot* were dissolved as stock solutions of 1.0 mg/ml in 40 mM NH_4HCO_3 containing 10% acetonitrile. Two mixtures of the standard proteins were prepared for iTRAQ labeling. Protein amounts were adjusted such that BSA was present in a heavy to light (H/L) ratio of 2:1. C3 was also present in a H/L ratio of 2:1, ovalbumin in a ratio of 3:1 and Toxin A in a ratio of 1:1. A total amount of 20 μg of standard proteins was used for labeling. The protein mixture and cell lysates were reduced by adding 2 μL of 50 mM reduction solution (TCEP-HCl, tris(2-carboxy-ethyl)phosphine) and the samples were incubated for 1 h at 60 °C. The cysteines were alkylated using 1 μL of 200 mM methyl-methanthiosulfonate (MMTS) for 10 min at 25 °C. 4 μL of 1 μg/μL modified trypsin (Promega, Madison, USA) in 50 mM acetic acid was added to the samples and cleavage was performed over night at 37 °C. Digests were dried in a SpeedVac, resuspended in 30 μL of 0.5 M triethylammonium bicarbonate at pH 8.5, amino-labeled with one of the four iTRAQ™ tags at 25 °C for 2 h and then combined in equal amounts. Samples were dried in a SpeedVac and redissolved in 100 μL 2% acetonitrile, 0.1% formic acid (pH 3.5), for further purification and separation of the labeled peptides using 2D-chromatography.

2.4. Peptide separation by two-dimensional HPLC

Peptide separation was performed by strong cation exchange chromatography followed by reversed phase chromatography using a nano-HPLC system (Dionex GmbH, Idstein, Germany) that consisted of an autosampler (Famos), a loading pump (Switchos), a gradient pump (Ultimate), and a microfraction collector (Probot).

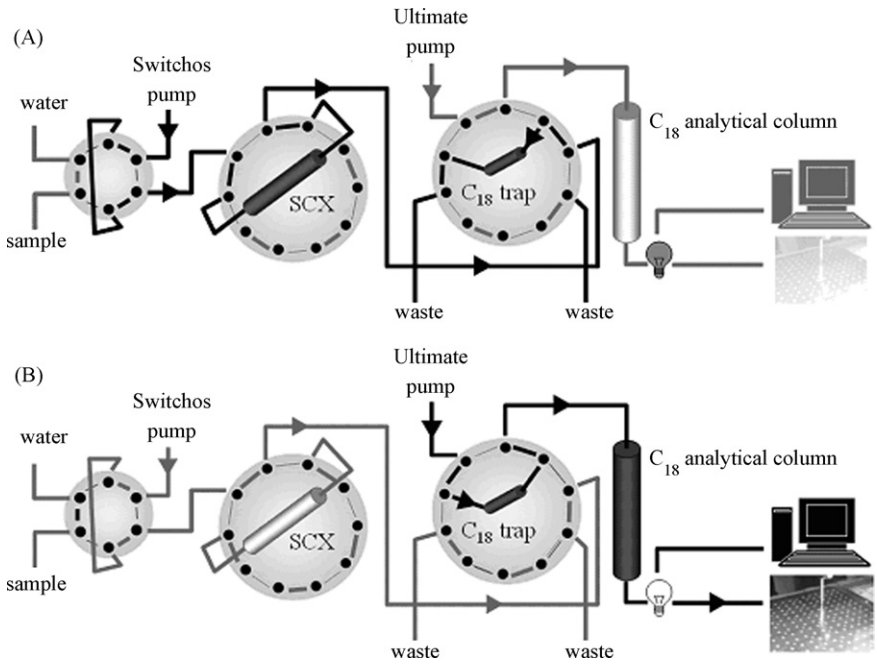

Fig. 1. Schematic of the two-dimensional HPLC-system. Panel (A) shows the system operating in the SCX-mode with a flow of 15 μL/min from the Switchos pump. Peptides not retained at the SCX column were trapped with a C18-trap column. Panel (B) displays the separation mode in the second dimension. Peptides bound to the trap column were eluted using an acetonitrile gradient with a flow of 250 nL/min produced by the Ultimate pump, monitored and directly spotted onto a MALDI target.

In the first step of the separation, 10 μL of the peptides (2 μg) generated from the standard proteins or 19 μL of the peptide mixture (10 μg) of cell lysates were loaded onto a ProSpere P-SCX-NP strong cation exchange column (2.5 μm, 50 mm, ID 300 μm; Alltech Grom GmbH, Rottenburg-Hailfingen, Germany) with a flow of 15 μL/min 2% acetonitrile in 0.1% formic acid. 40 μL salt plugs containing increasing concentrations of KCl (15 mM, 30 mM, 60 mM, 100 mM, and 500 mM) were used to elute the retained peptides from the SCX onto a C18 trap column (PepMap 300 μm × 5 mm, 3 μm, 100 Å, Dionex). Finally, the column was regenerated with 2 M KCl. The schematic workflow of the two-dimensional LC approach is presented in Fig. 1. Panel A shows the switching of the 10-port valves during injection or elution with the salt plugs. Peptides which flowed through or which were eluted from the SCX are trapped on a short RP-trap-column. After 6 min the SCX column was closed out of the flow and the trap was washed with 2% acetonitrile in 0.1% formic acid to remove salts and detergent. After 3 min the trap column was integrated into the nano-flow (250 nL/min) of the gradient pump (Fig. 1B). The peptides were washed onto a separation column (PepMap, C18 reversed phase material, 75 μm × 150 mm, 3 μm, 100 Å, Dionex GmbH, Idstein, Germany) and eluted using eluent A with 5% acetonitrile in 0.1% TFA and eluent B with 80% acetonitrile in 0.1% TFA. A gradient from 12% to 40% eluent B in 70 min, 40% to 100% eluent B in 3 min was applied. 256 fractions of ca. 72 nL were collected directly onto a MALDI target plate with a continuous sheath flow of 3 μL/min 5% acetonitrile in 0.1% TFA using a microfraction collector.

2.5. MALDI target preparation

A 4 mg/mL CHCA in 50% acetonitrile, 0.1% TFA and 10 mM $NH_4H_2PO_4$ matrix stock solution was diluted 1:4 with ethanol. A MALDI Anchor Chip 600/1536 target plate (Bruker Daltonik GmbH) was used and 0.6 μL of matrix solution was applied to each spot. For MS calibration 0.3 μL of peptide calibration standard (Bruker Daltonik GmbH) was added on the target calibration spots. Subsequently, recrystallization of the sample and standard was done with 0.2 μL of 0.1% TFA in ethanol.

2.6. MALDI analysis

MALDI analysis was performed with an Ultraflex TOF/TOF I from Bruker Daltonik GmbH (Bremen, Germany) operating with FlexControl 2.4, FlexAnalysis 2.4, BioTools 3.0 and Warp-LC 1.1 software. For protein identification searches against the MSDB database using MASCOT software version 2.2 (Matrix Science, UK) were carried out. MS and MS/MS spectra were taken with a 50 Hz nitrogen laser operating at a wavelength of 337 nm. 300 shots were collected for MS spectra and compounds with a signal/noise ratio above 20 were taken for MS/MS measurements. 300 shots were collected for the parent ion counting only spectra with a half-width <1.3 and an intensity of 10× laser energy. 700 shots were summed up for the fragments.

2.7. Identification and quantification criteria

Mascot searches were performed for protein identification. Therefore, MS/MS-spectra were matched with the MSDB database. Searches accomplished out for the SH-SY5Y samples were limited to human proteins. Samples were analyzed for modifications like oxidation (M), hydroxylation (P) or phosphorylation (STY) in addition to the iTRAQ modification. MS/MS-spectra were utilized if the Mascot peptide ion score was above 25. For protein identification, two peptides per protein had to be identified and the Mascot protein score had to be above 30. All data were checked using a decoy database containing nonsense proteins. The highest protein scores generated using this database were below 30. If only one peptide was identified for a particular protein, the peptide ion score had to be above 30 and the investigators checked the MS/MS spectrum individually. If one MS/MS spectrum fit to more than one protein, only that protein with the highest score was taken. Quantification was carried out by calculating the ratio between the peak areas of the 114/115/116 or 117 iTRAQ reporter groups. To calculate the

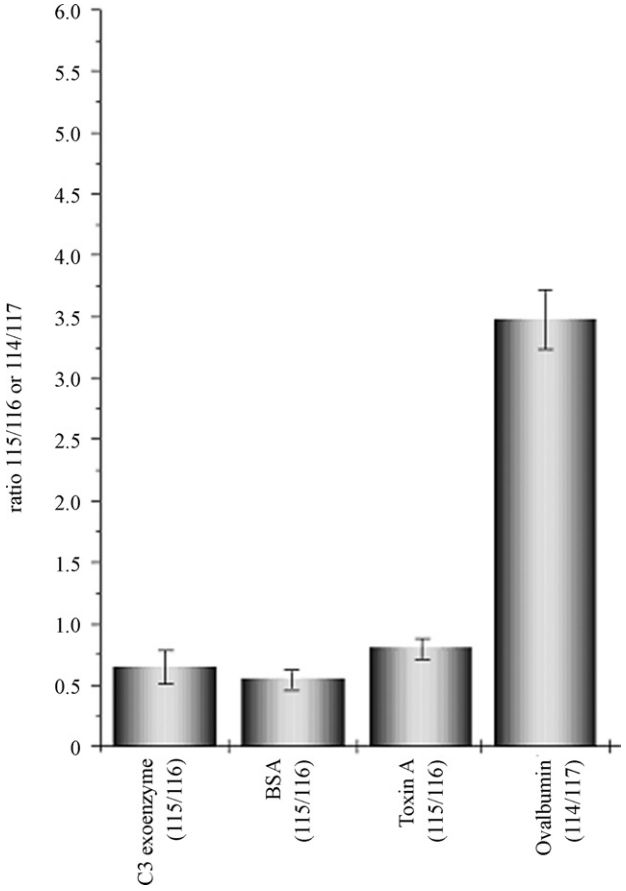

Fig. 2. Quantification of standard proteins. C3*bot* as well as BSA were labeled in a H/L ratio of 2:1 in the standard mixture, Toxin A with a H/L ratio of 1:1 and ovalbumin with an H/L ratio of 3:1. The iTRAQ reporter reagents used for the light version were "114" and "115", the reporters the heavy version were "116" and "117" as indicated on the left side. The expected regulation factors for C3*bot* and BSA are 0.5, for Toxin A 1.0 and for ovalbumin 3.0. The data are representative of four independent experiments.

heavy/light (H/L) ratios of a protein, all ratios determined for its peptides were used and the average ratio was determined.

3. Results

In this study, the iTRAQ method was used to identify biomarkers in neuronal cells which indicate cellular changes after treatment with C3*bot*. The neuroblastoma cell line SH-SY5Y was chosen as model a system. These cells respond to the C3*bot* treatment by reorganization of the actin cytoskeleton and formation of neurites. The uptake of C3*bot* proceeds relatively slowly [1,16]. To detect considerable changes at the proteome level, high concentrations (1 μM) of this exoenzyme were added to the cells and an incubation period of 72 h was chosen. The functional integrity of the SH-SY5Y cells was maintained throughout this incubation time (and even longer). After C3*bot* treatment, all cells appeared round and neurites were formed (data not shown). To elucidate these effects at the protein level, crude extracts were prepared from C3*bot* treated and untreated SH-SY5Y cells. Both extracts were then digested with trypsin and the generated peptides were labeled with the iTRAQ reagent. This highly complex peptide mixture was analyzed by two-dimensional HPLC and MALDI techniques. Each experiment consisted of 1536 spots per MALDI target, the maximum number of spots possible per plate. The available software cannot combine MS spectra from two or more experiments. To take advantage of all MALDI target spots for peptide analysis, only those fractions of the two-dimensional HPLC that contained peptides were collected.

3.1. Analysis of standard protein mixtures

To establish the iTRAQ method, a standard protein mixture was applied containing BSA, Toxin A from *Clostridium difficile*, ovalbumin, and recombinant C3*bot*. Proteins were cleaved by trypsin and labeled with the iTRAQ reagent. C3*bot* and BSA were added each in a ratio of H/L of 2:1, ToxA in a ratio H/L of 1:1 and ovalbumin in a ratio of H/L 3:1. Four replicate experiments were carried out and peptides were separated by two-dimensional HPLC and analyzed by MALDI-MS/MS (Fig. 2). All four proteins were identified by database analysis with a high Mascot score (C3*bot* 83, BSA 868, Toxin A 202, ovalbumin 69). All peptides were identified only in the iTRAQ labeled form, except one peptide of Toxin A that was unlabeled. The corresponding masses of the unlabeled peptides were completely absent on MS level, demonstrating that the labeling reaction was nearly 100% efficient (data not shown). When unlabeled BSA, Toxin A, C3*bot*, and ovalbumin were analyzed with the same procedure a similar although unlabeled, pattern was obtained and nearly identical Mascot identification scores were determined (data not shown). This control confirms the compatibility of the iTRAQ labeling method with two-dimensional HPLC separation and MALDI-MS/MS identification. C3*bot*, Toxin A, BSA, and ovalbumin were found in the expected ratios with a variation between 10% and 20% for all identified labeled peptides (20% for C3*bot*, 12% for BSA, 10% for Toxin A, 11% for ovalbumin) (Fig. 2). The variation for all labeled peptides of a single standard protein was identified to be between 10% and 20%, as previously described by Ross et al. [15]. In keeping with this, in the present analysis proteins were designated up-regulated if the H/L ratio was determined to be above 1.3 (Table 1). Only few of the identified proteins were down-regulated as evident from the H/L ratio and no threshold was used for down-regulated proteins (s.b.).

3.2. Analysis of C3bot-induced changes of the SH-SY5Y proteome

Proteins from crude extracts of C3*bot* treated and untreated SH-SY5Y cells were digested by trypsin. The peptides were labeled with

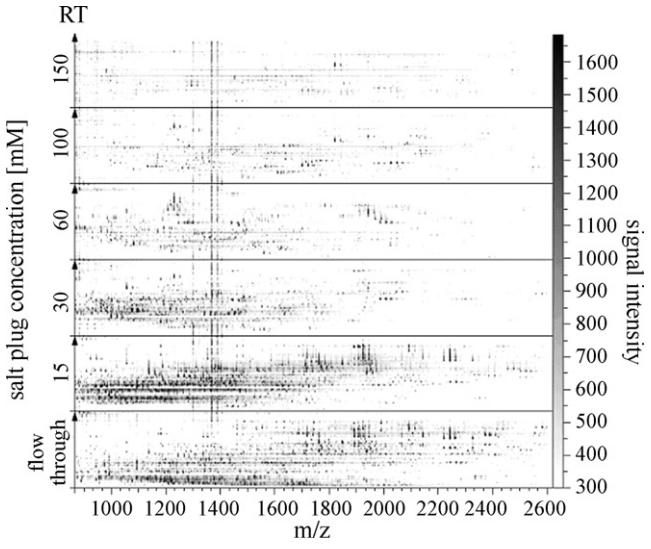

Fig. 3. Two-dimensional mass to charge plot of the analyzed HPLC fractions. Peptides were loaded onto the SCX column and were eluted by the indicated salt plugs. All SCX eluted peptides bound to the reversed phase column and were eluted by an increasing acetonitrile gradient. All fractions eluting from the RP column were analyzed in the MS and all spectra are displayed one on top of the other in a gel view mode. Thus, all peptides are indicated by small grey bars. The relative intensity of the MS signal is indicated on the right side. On the left, the 6 SCX eluted fractions are indicated by the different salt concentrations. Below, the *m*/*z* values are given.

Table 1
Regulated proteins in C3*bot* treated SH-SY5Y cells.

#	Protein group/protein name	Accession[a]	Score[b]	MW[c] (kDa)	IEP[c]	# of (H/L)[d]	Regulation (H/L)[e]	Variation (%)[f]
Cell death								
1	Exportin-2	P55060	33	110.4	6.2	8	1.33	2.97
Biological processes								
2	Alpha-2-HS-glycoprotein	P02765	33	39.3	5.4	1	**0.90**	–
3	Heat shock protein 10 kDa	P61604	44	6.4	9.6	1	1.30	–
Cell cycle								
4	Peroxiredoxin-2	P32119	64	21.7	5.6	1	1.36	–
Cell growth								
5	Macrophage migration inhibitory factor	P14174	143	12.3	9.3	15	**0.91**	1.08
6	Striated muscle preferentially expressed protein kinase	Q15772	36	348.9	8.3	9	1.35	1.72
Cell morphology								
7	14-3-3 protein beta/alpha	P31946	62	27.9	4.6	2	1.31	2.56
8	14-3-3 protein gamma	P61981	116	28.4	4.5	6	1.31	3.75
9	ACTA2	Q13707	159	36.8	5.1	8	1.56	4.16
10	Actin alpha 2	P62736	190	42.1	5.1	4	1.52	16.10
11	Actin beta variant	Q53G99	445	41.7	5.3	23	1.48	–
12	Actin gamma	P63261	267	41.6	5.2	20	1.41	18.31
13	Actin kappa	Q562R1	37	42.0	5.7	3	1.57	11.52
14	Actin alpha cardiac muscle 1	P68032	276	42.0	5.1	9	1.52	6.44
15	Stathmin	P16949	101	20.6	5.7	10	**0.85**	14.93
16	Tubulin alpha-4 chain	Q9H853	138	27.5	9.4	5	1.37	13.69
17	Tubulin alpha-4A chain	P68366	227	49.8	4.8	15	1.33	8.54
Chaperoning								
18	Histone H2A type 1	P0C0S8	242	14.1	11.4	26	1.57	0.86
19	Histone H2A type 1-A	Q96QV6	187	14.1	11.3	6	1.56	6.45
20	Histone H2AV	Q71UI9	138	13.4	11.1	6	1.46	18.42
21	Histone H2B type 1-B	P33778	266	13.8	10.8	12	1.33	4.52
22	Histone H2B type 1-C/E/F/G/I	P62807	148	13.7	10.8	5	1.36	9.07
23	Histone H2B type 1-D	P58876	308	13.8	10.8	34	1.35	3.88
24	Histone H2B type 1-J	P06899	210	13.6	10.5	17	1.40	7.44
25	Histone H3.1t	Q16695	110	15.4	11.9	16	1.32	12.69
26	Histone H4	P62805	325	11.4	11.8	43	1.78	7.65
DNA synthesis and processing								
27	Structural maintenance of chromosomes protein 5	Q8IY18	35	144.1	9.4	1	**0.95**	–
Metabolism								
28	ATPase type 13A1	Q9EPE9	60	108.5	9.4	1	1.35	–
29	Bifunctional protein NCOAT	O60502	37	67.8	4.6	4	1.39	2.00
30	Glutathione-S-transferase P	P09211	48	23.2	5.3	9	1.70	3.32
31	Putative Xaa-Pro aminopeptidase 3	Q9NQH7	32	57.0	6.4	3	1.35	3.58
32	Pyruvate kinase isozymes M1/M2	P14618	128	57.8	9.0	6	1.36	1.95
Nucleotide metabolism								
33	Poly(A) binding protein cytoplasmic pseudogene 2	Q6NV95	44	29.9	9.7	1	**0.91**	–
34	Zinc finger protein 803	O95785	35	178.7	6.6	1	1.30	–
Protein synthesis and processing								
35	Heat shock protein 60 kDa	P10809	44	6.0	4.6	3	1.41	4.68
36	Lamin-A/C	P02545	68	80.0	6.6	2	**0.95**	6.73
37	Peroxiredoxin-4	Q13162	31	30.5	5.8	1	1.30	–
38	Williams–Beuren syndrome chromosomal region 20B protein	Q3KNT7	33	16.5	11.3	3	**0.89**	4.33
39	Zinc finger CCCH domain-containing protein 4	Q9UPT8	43	31.0	9.9	3	1.35	8.42
RNA synthesis and processing								
40	Pygopus homolog 2	Q5T170	55	41.2	7.7	2	**0.82**	5.72
41	Doublesex and mab-3 related transcription factor 1 c	Q6T1H8	49	18.3	12.3	1	1.31	–
42	Elongation factor 1-alpha 1	P68104	149	50.1	9.7	14	1.37	5.72
43	General transcription factor II-I	Q86UP8	41	107.2	8.5	10	2.08	10.92
44	Homeobox protein EMX2	Q04743	47	28.3	10.3	2	1.35	29.86
45	Zinc finger protein ZFPM1	Q8IX07	41	104.5	9.2	1	**0.92**	–
Stress response								
46	Heat shock protein 27 kDa	P04792	163	22.8	6.0	12	**0.88**	8.20
47	Heat shock protein 90 kDa Bb	Q58FF8	32	49.1	4.9	1	1.35	–
48	STIP1 protein	Q3ZCU9	32	68.0	8.6	1	1.31	–
Transport								
49	Cytochrome *c*	P99999	36	11.6	10.2	3	1.45	9.08
Ubiquitin cycle								
50	HBxAg transactivated protein 2	Q9Y520	30	186.1	10.2	1	1.36	–
51	MAP7 domain-containing protein 1	Q3KQU3	46	92.8	10.8	1	1.31	–
52	Probable mast cell antigen 32 homolog	Q7Z6M3	30	38.7	8.7	2	2.32	1.81
53	ProSAPiP1 protein	Q8IXX8	31	66.5	6.4	1	1.44	–

Table 1 (*Continued*)

#	Protein group/protein name	Accession[a]	Score[b]	MW[c] (kDa)	IEP[c]	# of (H/L)[d]	Regulation (H/L)[e]	Variation (%)[f]
54	Putative uncharacterized protein	Q8IYK6	32	14.3	12.0	1	1.31	–
55	SH3 domain-binding glutamic acid-rich-like protein	O75368	34	12.8	5.1	1	1.36	–
56	Uncharacterized protein C8orf12	Q96KT0	37	11.6	10.5	1	**0.62**	–

[a] SwissProt or NCBI accession number.
[b] Mascot search protein score.
[c] Molecular weight (MW) and isoelectric point (IEP) of identified protein.
[d] Number of iTRAQ labeled peptides used for quantification.
[e] Average regulation ratio of the heavy/light labeled peptides determined from the peak area of the corresponding reporter. Bold written H/L values belong to down-regulated proteins.
[f] Variation of the heavy/light ratios of identified peptides from one protein.

the iTRAQ reagent and labeled peptides were separated by means of a two-dimensional HPLC system. Subsequently, identification and quantification was performed in a MALDI mass spectrometer by tandem MS. The SCX separation (first dimension) was used for peptide separation and removal of excess iTRAQ reagent. A significant part of the peptides did not bind to the SCX column and most peptides eluted in a narrow window of 0–30 mM potassium chloride (Fig. 3). This is most likely due to the observation that peptides with the same charge (1+, 2+, . . .) eluted from the SCX column as poorly resolved clusters in order of increasing charge [27,28].

The overall analysis was accomplished in five replicates and 355 proteins were identified (Supplementary Table 1). Of them 233 proteins were identified only in one single experiment (Fig. 4A) and 122 of all identified proteins were found in more than one experiment. Only 12 proteins (3.4%) were identified in all of the five experiments. These are highly abundant proteins in cellular systems and are HSP 70 protein 1, heterogenous nuclear ribonucleoprotein A3 and A2/B1, histone H3.1, histone H4, nucleolin, nucleophosmin, peptidylprolyl cis–trans isomerase A, profilin-1, stathmin, triosephosphat-isomerase, alpha-tubulin and vimentin. 20% of the proteins were identified with a MASCOT score above 100 and nearly 60% of the identified proteins were found with a score below 50 (Fig. 5, Supplementary Table 1).

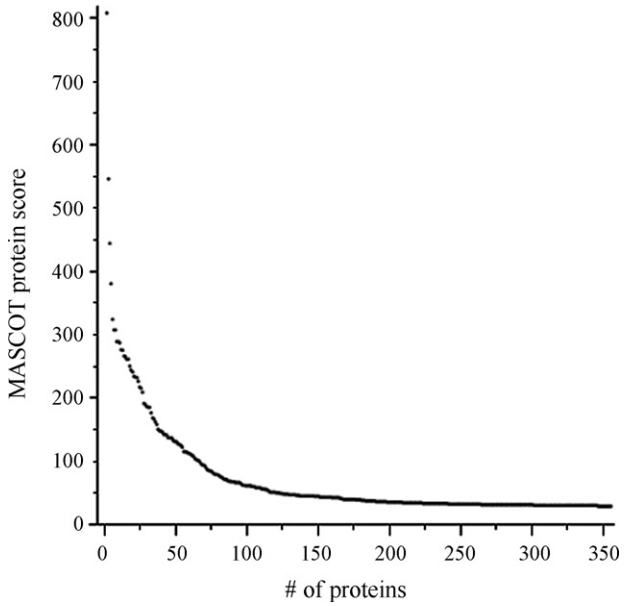

Fig. 4. Comparison of five iTRAQ-LC-MALDI experiments and incidence of the identified and quantified proteins in C3*bot*-treated SH-SY5Y cells. Panel (A) shows the number of identified proteins in five independent experiments. Proteins in the overlapping areas are found in two, three, four or all five experiments. Panel (B) displays the mapping of the identified proteins to different compartments in the SH-SY5Y cells.

The cellular distribution of the 355 different proteins identified is depicted in Fig. 4B. The majority of the identified proteins localize to the cytoplasm (29.3%) and the nucleus (22.9%). 9.7% of the identified proteins have been classified as membrane proteins. Proteins exhibiting high p*I* values such as histones were also detected. The latter two groups are known to be absent using two-dimensional gel electrophoresis and are found herein due to the benefits of the two-dimensional HPLC approach. Only few proteins from other compartments such as endoplasmic reticulum or ribosomes were retrieved. These findings are similar to previous published proteome studies based on LC–MS [29,30]. The proteins were categorized according to common functional features (Supplementary Table 1). This classification is arbitrary, since many of these proteins have more than one function with different roles in diverse cellular processes.

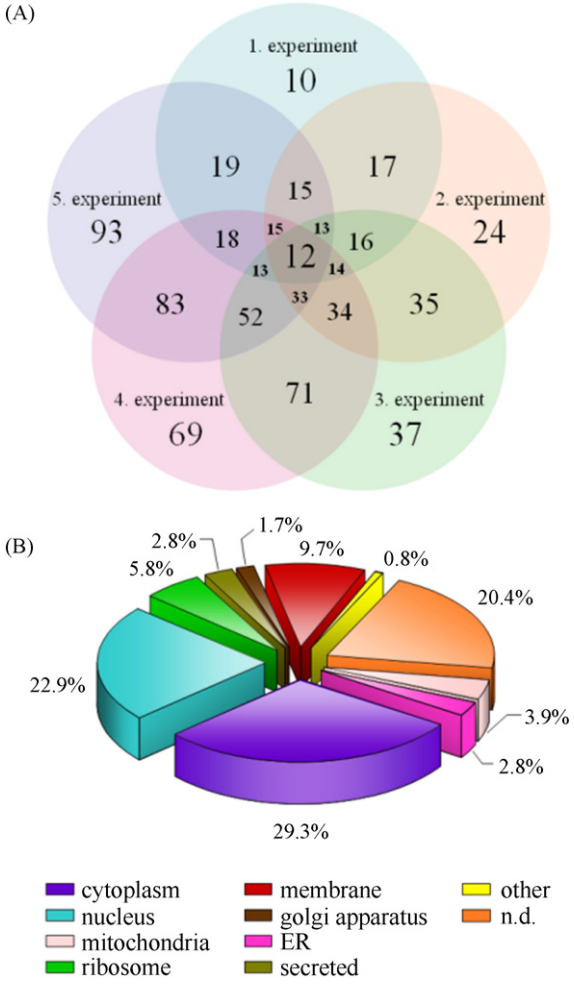

Fig. 5. MASCOT protein score of the identified proteins.

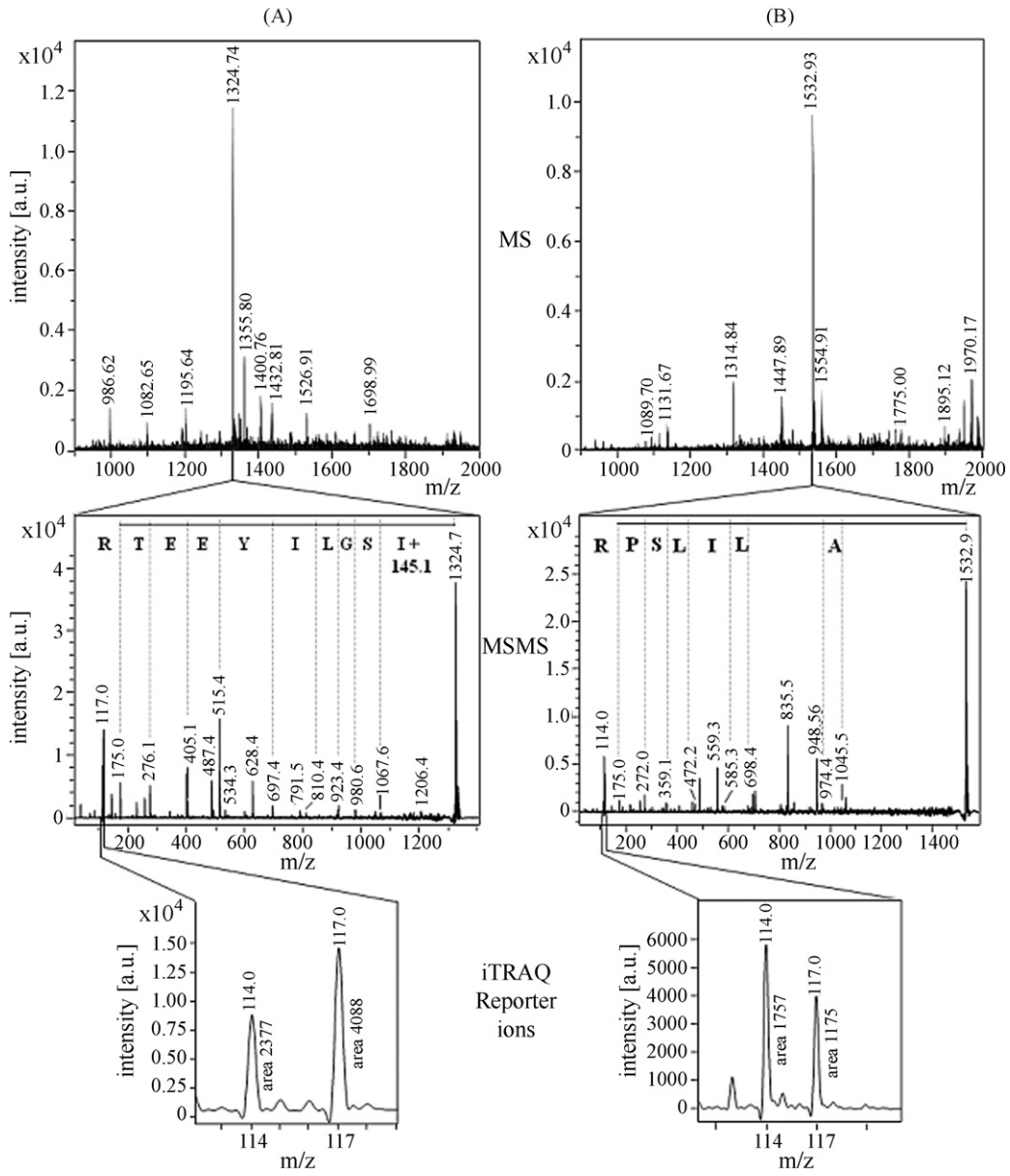

Fig. 6. Mass spectra of up-regulated histone H4 (Panel A) and down-regulated stathmin (Panel B). The MS spectrum of the fraction containing the labeled peptide is displayed in the upper panel. The middle panel represents the MS/MS spectrum of the chosen peptide with the calculated y-ion-series. Panel (A): histone H4 peptide sequence ISGLIYEETR, MW 1324.74 Da; Panel (B): stathmin peptide sequence ASGQAFELILSPR, MW 1532.93 Da; N-terminal labeled with iTRAQ, the areas belonging to the reporter ions 114 and 117 are shown in the lower panel.

3.3. iTRAQ-based quantification of C3bot-induced changes in SH-SY5Y cells

Among the 355 identified proteins, 235 were accessible for quantification. The remaining 120 proteins did not contain any iTRAQ label and therefore relative concentrations could not be calculated. An altered abundance was detected for 23% of the iTRAQ-labeled proteins after C3*bot* treatment (Table 1). The majority of these proteins (44) displayed a higher abundance after C3*bot* treatment. Only 11 proteins had a lower abundance following C3*bot* treatment and their determined H/L values were 0.95 or lower. These proteins include α-2-HS-glycoprotein, macrophage migration inhibitory factor, stathmin, chromosomal protein 5, poly-A-binding protein pseudogene 2, lamin A/C, chromosomal region 20B protein, pygopus homolog 2, zinc finger protein ZFPM1, HSP 27, and the uncharacterized protein C8orf12.

Labeled peptides derived from one protein had consistent H/L values with a variation of less than 10%, showing the validity of the iTRAQ technique. Comparison of the five iTRAQ experiments reveals the variation for identical proteins to be less than 20%. The average variation was 6%, which is better than the values obtained for the test experiments using standard proteins where a variation of 10–20% was determined for a single protein.

The abundance of 10 proteins was altered more than 50% in response to the C3*bot* treatment, corresponding to less than 6% of the identified labeled proteins. Two proteins were altered by more than 70% and the highest changes were measured for histone H4 (Table 1, No. 26), the transcription factor II-I (Table 1, No. 43) and the probable mast cell antigen 32 homolog (Table 1, No. 52). The most prominent down-regulation was recorded for the uncharacterized protein C8orf12 (Table 1, No. 55) with a H/L ratio of 0.62 and stathmin (Table 1, No. 15) with a H/L ratio of 0.85.

In Fig. 6, examples of MS/MS analysis for a histone-derived peptide (A) an a stathmin-derived peptide (B) are shown. The sequences were determined to be ISGLIYEETR (parent mass 1324.74 Da) for the histone H4 peptide and ASGQAFELILSPR (parent mass 1532.93 Da) for the stathmin peptide. Histone H4 was quantified with 5 peptides and found to be up-regulated (H/L of 1.74) with variation of the identified peptides at 9% (Fig. 6A). The regulation of stathmin (H/L of 0.85) was based on the analysis of six different peptides with a variation of 14% (Fig. 6B).

4. Discussion

We employed the fast and reliable iTRAQ technique, which is based on peptide labeling with stable heavy isotopes, to identify biomarker that indicate C3*bot* responses in neuronal cells.

In total, 355 different proteins belonging to nearly all cellular compartments were identified in SH-SY5Y cells thus, showing the functionality of the combination of two-dimensional HPLC and MALDI MS/MS. A further increase in identified proteins might be obtained upon reduction of sample complexity. To achieve this, additional separation steps on the protein or peptide level should be included. However, we have demonstrated a fast and reliable two-dimensional HPLC method which allows highly automated sample analysis in combination with MALDI-MS/MS. In our experiments, control samples and the C3*bot* treated samples were analyzed five times and the obtained reproducibility is comparable to those of other MS/MS experiments [30].

From the 355 identified proteins, 235 (66%) were quantified using the iTRAQ chemistry. The level of variation for the quantification results was determined to be between 10% and 20%. Those proteins with an H/L value above 1.3 were classified as up-regulated and 44 proteins fulfilled these criteria. Only 11 proteins had a reduced abundance after C3*bot* treatment exhibiting H/L values below 1.

C3*bot* treatment changes the abundance of a large number of proteins involved in cell morphology (Table 1). The inactivation of the RhoA GTPase leads to reorganization of the actin cytoskeleton, the microtubule network, and reduction of cell adhesion accompanied by altered cell motility [1,16]. Indeed, in this study we have found that actin and tubulin were up-regulated. However, stathmin, which promotes disassembly of microtubules and is involved in neuron polarization, was slightly down-regulated. Additionally, stathmin down-regulation may be involved in the effects of Rho-dependent signalling, and thus is a candidate biomarker for cytoskeletal reorganization in this system. Stathmin is regulated by phosphorylation in response to stimulation by neural growth factor [31]. Recently, Berenjeno et al. [32] showed that transcription of stathmin is up-regulated in response to overexpression of constitutively active RhoA. In the experiments described herein, RhoA is inactivated by ADP-ribosylation and indeed the opposite effect was observed. Both findings fit to the Rho-dependent regulation of stathmin. However, other effects might contribute to the apparent up- and down-regulation of proteins, e.g. altered post-translational modification.

A prominent group of proteins exhibiting altered concentrations following C3*bot* treatment are the histones. Histones H2A, H2B, H3.1 and H4 (Table 1) were identified to be up-regulated by an average H/L value of 1.46.

Accordingly, histone up-regulation should indicate an altered homeostasis of the chromatin and thus, the identified histones H2A, H2B, H3.1 and H4 could be considered as biomarkers for C3*bot*-induced changes in neuronal cells. Work is underway in our laboratory to analyze the response of histones to C3*bot* treatment in different neuronal cells and a comparative proteomic analysis of purified nuclei before and after C3*bot* treatment is currently being addressed. Other proteins showing differential expression, such as the probable mast cell antigen (Table 1, No. 52), general transcription factor II-I (Table 1, No. 43), glutathione-S-transferase (Table 1, No. 30), pygopus homolog 2 (Table 1, No. 40), and uncharacterized protein C8orf12 (Table 1, No. 56) are also potential biomarker candidates.

In conclusion, the iTRAQ approach coupled to two-dimensional chromatography and MALDI-MS is a suitable tool for the detection of biomarkers in cell lysates. The method is appropriate for the screening of highly complex protein mixtures in order to identify biomarkers. We have shown that stathmin, histones, and several other proteins are interesting biomarker candidates to indicate C3*bot* induced cellular changes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.12.005.

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