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Cytoskeleton changes following differentiation of N1E-115 neuroblastoma cell line

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Summary. No systematic approach to detect expression of differentiation-related elements was published so far. The undifferentiated N1E-115 neuroblastoma cell line was switched into a neuronal phenotype by DMSO treatment and used for proteomic experiments. We used two-dimensional gel electrophoresis followed by unambiguous mass spectrometrical identification of proteins to generate a map of cytoskeleton proteins (CPs), i.e., to search for differentiation-related structures. Alpha-actin, actin-like protein 6A, gamma-tubulin complex component 2, tubulin alpha 3/alpha 7, CLIP associating protein 2, B4 integrin interactor homolog were detectable in the undifferentiated cell line exclusively and neuron-specific CPs drebrin and presynaptic density protein 95, actin-related protein 2/3, alpha and beta-centractin, PDZ-domain actin binding protein, actinin alpha 1, profilin II, ezrin, coactosin-like protein, transgelin 2, myosin light polypeptide 6, tubulin alpha 2, 6 and 7, beta tubulin (94% similar with tubulin beta-2), tubulin beta 3, tubulin tyrosine ligase-like protein 1, lamin B1 and keratin 20 were observed in the differentiated cell line only. We herein identified differentiation-related expressional patterns thus providing new evidence for the role of CPs in the process of neuronal differentiation.

Keywords: Cytoskeleton proteins – Actin/actin binding protein – Tubulin – Proteomics – Differentiation – N1E-115 cell line

Introduction

Differentiation of neurons from a simple sphere is characterised by three steps: First, the original round shape disappears and a bud is formed; second, the bud is transformed into a neurite; and third, the neurite is converted into an axon or dendrite (Da Silva and Dotti, 2002).

It is obvious that the change of cell shape and polarity must be preceded and accompanied by modification of the scaffold, the cytoskeleton proteins (CPs). Microfilaments, microtubules and intermediate filaments/neurofilaments work in a concerted action to induce and maintain the differentiation processes. Involvement of microfilaments (MFs) as actin and tropomyosin in neuronal differentiation (ND) has been reviewed by several authors and is recognised as temporally and spatially regulated appearance and disappearance of microfilaments including the many actin-binding proteins (Da Silva and Dotti, 2002; Weinberger et al., 1996; Hannan et al., 1998; Gunning et al., 1998; Luo, 2002). Microfilaments strongly interact with microtubules (MTs) and this process is essential for differentiation (Rodriguez et al., 2003). The fact that several individual tubulins are differentially regulated during neuronal maturation has been described by Miller and coworkers (Miller et al., 1987) at the transcriptional level.

In a comprehensive review Laferriere and coworkers (Laferriere et al., 1997) were able to summarise the importance of the individual tubulins and their posttranslational modifications as well as microtubule-associated proteins for ND. Therein, the rapid rearrangement and synthesis of individual tubulins in ND is already addressed. The appearance of neuronal specific tubulin beta 4 (syn. class III β-tubulin isotype) following ND has been recognised by several authors (Katsetos et al., 1998, 2001, 2003). The role of intermediate filaments (IFs) for ND has been described for a series of individual IFs including neurofilaments. Ho and Liem (1996) described different IF proteins (alpha-internexin, peripherin, vimentin, etc.), their assembly into IFs, the functions of IFs and their relation to disease with a particular emphasis on the IFs expressed in the nervous system. Furthermore, identification of sites phosphorylated in vivo in high-molecular-weight neurofilament protein (NFP) and properties of NF-associated and neural-specific kinases

phosphorylating specific sites in NFP were described by Pant and Veeranna (1995).

There is abundant literature on changes of individual CPs related to ND but a comprehensive approach to concomitantly determine CPs of all three major classes following ND has not been described so far. It was therefore the aim of our study to apply a method to generate a differential expressional pattern of CPs in neuronal precursors, the well-documented and widely used neuroblastoma cell line N1E-115, before and following differentiation into a neuronal phenotype. The advent of proteomic technologies allowing high-throughput and unambiguous identification of CPs and generation of expressional patterns made us perform the present study. The use of two-dimensional gel electrophoresis along with mass spectrometrical identification showed remarkable differences of the cytoskeleton between a neuronal precursor, the N1E-115 neuroblastoma cell line, before and following ND.

Materials and methods

Cell culture

N1E-115 (mouse neuroblastoma cells) cells were obtained from ATCC (CRL 2263) and maintained in DMEM (Gibco) containing 4500 mg/l glucose, L-glutamine, without pyruvate and with 10% fetal bovine serum (FBS), antibiotics penicillin, streptomycin at concentrations of 60 µg/ml and 100 µg/ml, respectively and incubated in a humidified incubator with 5% CO_2 at 37 °C.

Neuronal differentiation

Undifferentiated N1E-115 cells were grown in 150 mm dishes in normal DMEM (Gibco) with 10% FBS and ND was induced by a change to DMEM that contained 2% FBS and 1.25% DMSO. Complete ND was observed after 5 days in the differentiation medium and the cells were harvested by using a cell scraper (Nalge Nunc, Rochester, NY).

Sample preparation

Both undifferentiated and differentiated N1E-115 cells were washed three times in 10 ml of phosphate-buffered saline (Gibco BRL), centrifuged for 10 min at $800 \times g$ at room temperature and subsequently homogenised with 1.0 ml of sample buffer consisting of 7 M urea (Merck, Darmstadt, Federal Republic of Germany), 2 M thiourea (Sigma, St. Louis, MO), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate) (Sigma), 65 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetetraacetic acid) (Merck), 1 mM PMSF, 0.5% carrier ampholytes and protease inhibitor complete (Roche, Basel, Switzerland). After homogenization samples were left at room temperature for 1 h and centrifuged at $150{,}000 \times g$ for 60 min and the supernatant was transferred into Ultrafree-4 centrifugal filter units (Millipore, Bedford, MA), for desalting and concentrating proteins. Protein content of the supernatant was quantified by the Bradford protein assay system (Bradford, 1976).

The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm. All experiments were carried out in triplicate.

Two-dimensional gel electrophoresis (2-DE)

Samples were subjected to 2-DE as described elsewhere (Langen et al., 1999; Oh et al., 2004). 0.8 mg of protein was applied on immobilised pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 8000 V at 4 V/min and kept constant for a further 3 h (approximately 150,000 Vh totally). After the first dimension, strips (13 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DDT. After equilibration, strips were loaded on 9–16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. Gels (180 by 200 by 1.5 mm) were run at 40 mA per gel. Immediately after the second dimension run, gels were fixed for 12 h in 50% methanol containing 10% acetic acid and stained with colloidal Coomassie blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA), covering the range of 10–250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and gels were scanned with Imagescanner (Amersham Bioscience). Electronic images of the gels were recorded using Photoshop (Adobe) and PowerPoint (Microsoft) software.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)

Spots were excised with a spot picker (PROTEINEER sp, Bruker Daltonics, Bremen, Federal Republic of Germany), placed into 96-well microtiter plates and in-gel digestion and MALDI sample preparation were performed by an automated procedure (PROTEINEER dp, Bruker Daltonics). Briefly, spots were excised and washed seven times with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/µl trypsin (Promega, Madison, WI) in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30 °C. Peptide extraction was performed with 10 µl of 1% TFA in 5 mM OGP and directly applied onto a target (AnchorChip™, Bruker Daltonics) that was spotted with α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex TOF/TOF (Bruker Daltonics) operated in the reflector mode. An accelerating voltage of 25 kV was used. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 50–200 consecutive laser shots. Spectra were interpreted with the aid of the Mascot Software (Matrix Science Ltd, London, UK). For protein search, a mass tolerance of 100 ppm and 1 missing cleavage site were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criteria for correct identification.

Results

As shown in Fig. 1A and B, undifferentiated cells were differentiated into a morphologically neuronal phenotype with neuritic outgrowth and a neuronal network (Clejan et al., 1996). Morphologically differentiated cells were judged by the length of neurites with a length more than

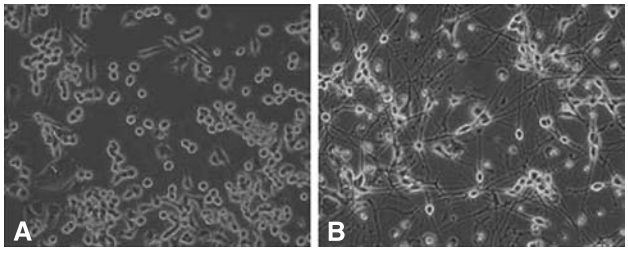

Fig. 1. Morphological difference between undifferentiated N1E-115 cells (**A**) and differentiated N1E-115 cells presenting with a neuronal network (**B**) (A, B: ×10, objective lens A-plan; Zeiss axiovert 200 microscope)

2 times the cell body diameter. These cells were subjected to analysis and revealed >90% cells with a neuronal phenotype (Soucek et al., 1998; Kubista et al., 2002).

From the maps generated (not shown), CPs were selected and maps were produced for undifferentiated (Fig. 2) and differentiated cell lines (Fig. 3) from three master gels each. Only high-abundance, i.e., Coomassie blue-stained spots were used for identification and differences were presented as ''present'' (detectable) or ''not present'' (undetectable) thus defining the level of difference.

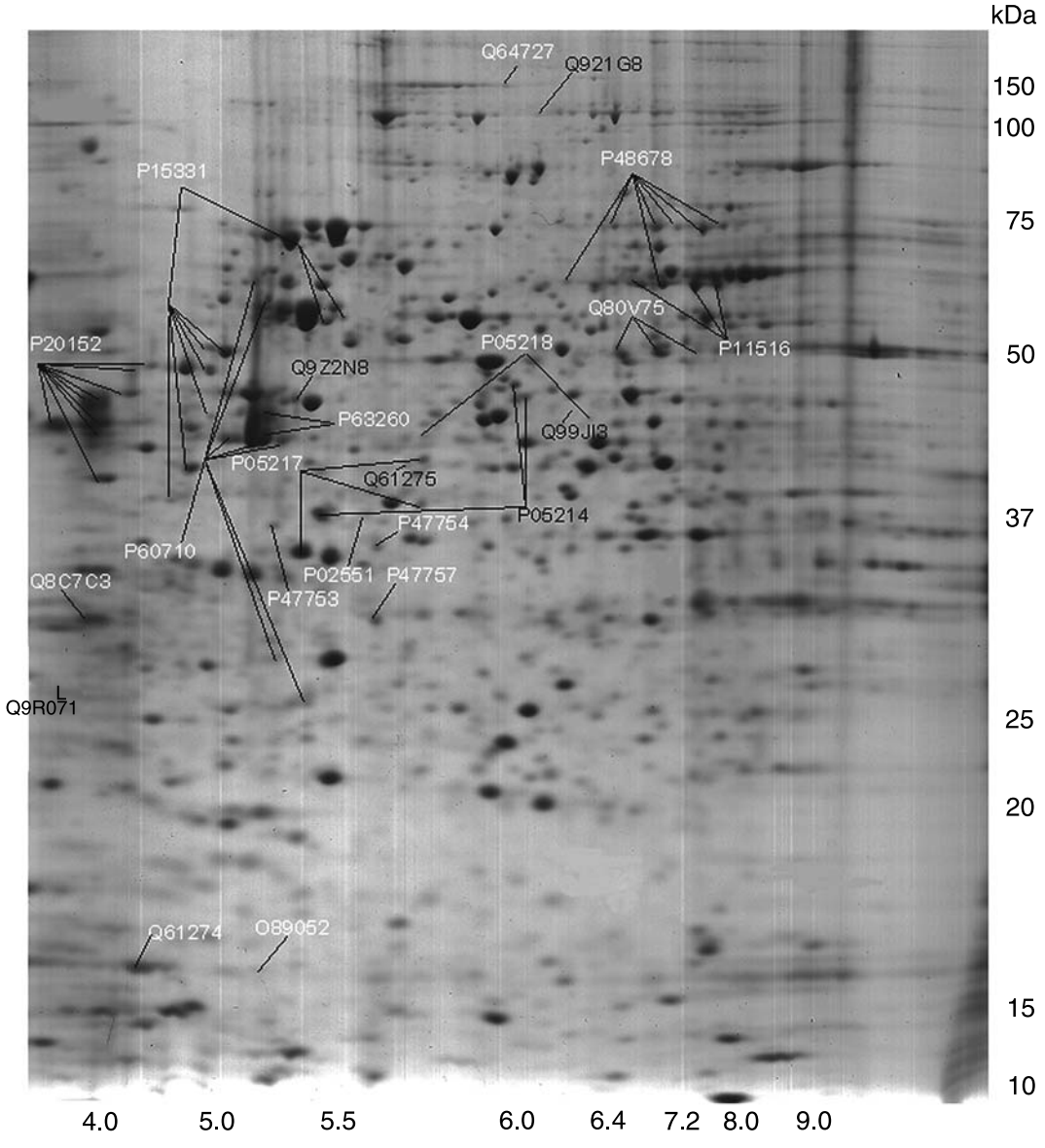

Fig. 2. Master map of the undifferentiated cell line. This map is shown with accession numbers from Swissprot database. 17 out of 23 proteins were observed in both the undifferentiated and differentiated cell lines (white numbers)

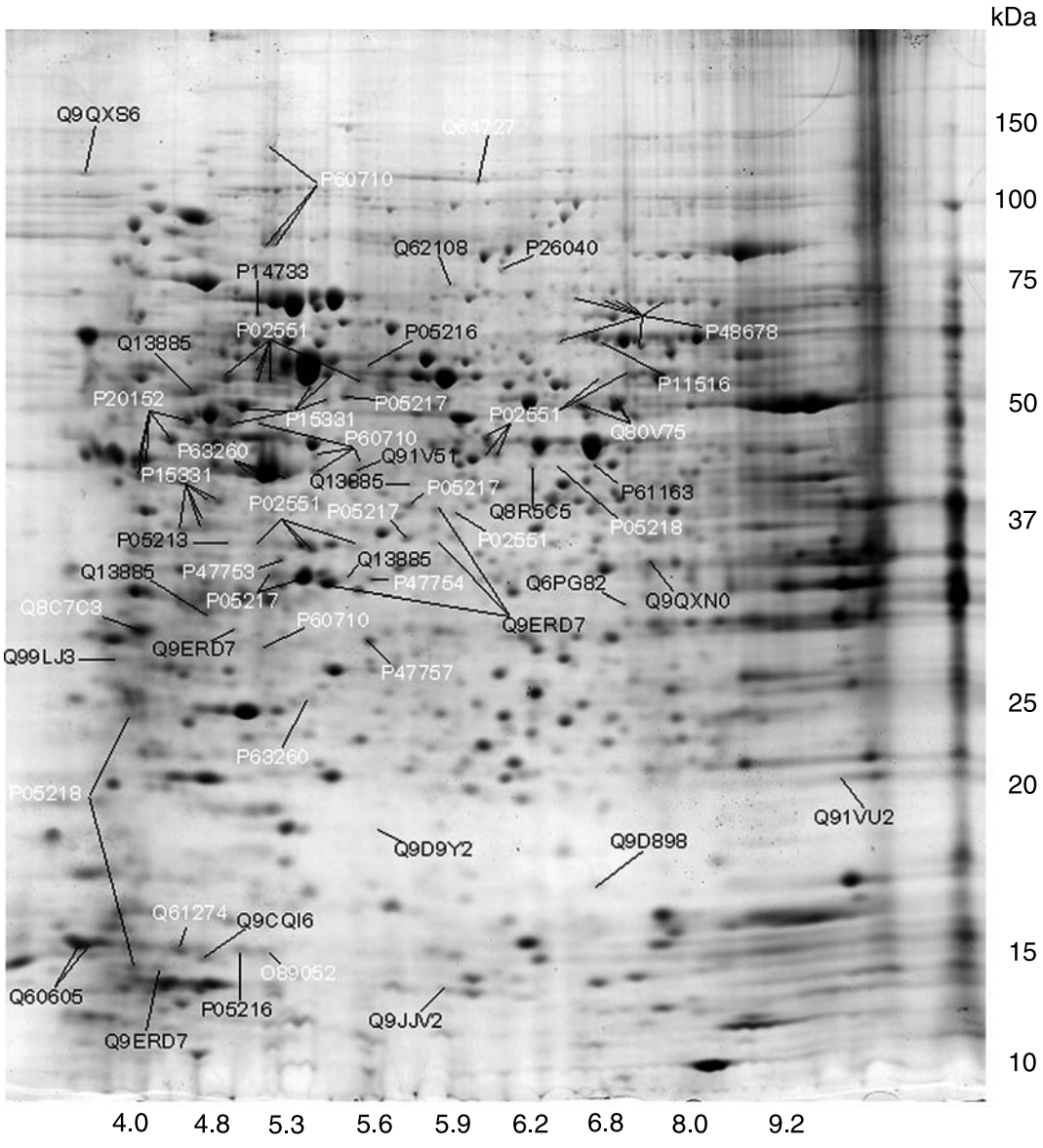

Fig. 3. Master map of the differentiated cell line. This map is presented as above. 37 proteins are shown; 20 proteins were observed in the differentiated cell lines only and 17 proteins (white accession numbers) were comparable between cell lines

In the undifferentiated cell line, 23 cytoskeleton proteins were identified and some IF proteins, such as peripherin and vimentin, were represented by several spots (8 and 9 each; Table 1).

In the differentiated cell line, 37 cytoskeleton proteins were identified and most were represented by a single spot except some proteins; in particular, actin cytoplasmic 1, tubulin alpha-1 chain, peripherin were represented by a series of spots (8, 15, and 10 respectively).

In both cell lines, 17 CPs were detected, and in the undifferentiated cell line, six CPs were identified that were not observed following differentiation (Table 1).

In the differentiated cell line, 20 CPs were observed that were not detectable in the undifferentiated cell line (Table 1).

Some spots of CPs have shown a shift towards acidic. Major shifts towards alkaline were observed for transgelin 2, tubulin alpha-1 chain, tubulin alpha-3/alpha-7 chain, tubulin beta-2 chain, tubulin beta-3, tubulin beta-5 chain, lamin C and C2, and keratin 20.

A major shift towards acidic was observed in PDZ domain actin-binding protein Shroom, tubulin tyrosine ligase-like protein1 and peripherin (Table 1, Figs. 2 and 3).

Table 1. Cytoskeleton proteins expressed in undifferentiated and differentiated cells

Accession nr.	Protein name	pI (T)[a]	Mol. mass (kDa)	Value of characteristic for protein in undifferentiated cells				Value of characteristic for protein in differentiated cells			
				Score	Peptide match	pI (O)[b]	Nr. of spots	Score	Peptide match	pI (O)	Nr. of spots
Q9QXS6	Drebrin	4.45	77.29					167	20	3.9	1
Q62108	Presynaptic density protein 95 (PSD-95) (SAP90)	5.56	80.47					63	15	6.0	1
Q61275	Alpha-actin [Fragment]	5.83	39.25	63	9	5.75	1				
P60710	Actin, cytoplasmic 1	5.29	41.74	220	23	5.1 5.2 5.25 5.3 5.3 5.35 5.45	7	146	21	5.2 5.3 5.35 5.35 5.4 5.45 5.45 5.6	8
P63260	Actin, cytoplasmic 2	5.31	41.79	230	26	5.35 5.35	2	203	21	5.2 5.3 5.35	4
Q61274	Alpha-cardiac actin [Fragment]	5.3	16.77	48	7	4.5	1	102	9	4.6	1
Q9Z2N8	Actin-like protein 6A (baf 53a)	5.38	47.49	148	17	5.4	1				
Q9D898	P16-ARC (90% similarity with actin-related protein 2/3 complex subunit 5-like protein)	6.31	16.98					83	8	6.7	1
P61163	Alpha-centractin	6.19	42.61					182	15	6.9	1
Q8R5C5	Beta-centractin (actin-related protein 1B)	5.98	42.28					237	19	6.35	1
Q9QXN0	PDZ domain actin-binding protein Shroom	8.62	215.26					70	25	7.6	1
Q99LJ3	Actinin, alpha 1 [Fragment]	4.92	45.92					62	15	4.2	1
Q80V75	Fscn1 protein [Fragment]	6.57	51.41	264	29	6.7 7.0 7.4	3	342	34	6.7 7.2 7.25	3
P47753	F-actin capping protein alpha-1 subunit	5.34	32.94	150	13	5.3	1	241	14	5.35	1
P47754	F-actin capping protein alpha-2 subunit	5.58	32.84	197	14	5.65	1	221	17	5.65	1
P47757	F-actin capping protein beta subunit	5.47	31.21	75	12	5.65	1	145	20	5.65	1
Q64727	Vinculin	5.72	117.17	55	17	6.1	1	178	39	6.1	1
Q9JJV2	Profilin II	6.78	14.9					180	9	5.9	1
P26040	Ezrin	5.83	69.28					82	18	6.1	1
Q9CQI6	Coactosin-like protein	5.28	15.94					82	7	4.8	1
Q91VU2	Transgelin 2	8.4	22.4					165	17	9.4	1
Q8C7C3	Tropomyosin 3, gamma	4.71	28.9	84	14	4.1	1	180	21	4.3	1
Q60605	Myosin light polypeptide 6	4.56	16.8					75	15	3.8 3.9	2
P02551	Tubulin alpha-1 chain	4.94	50.14	78	10	5.6	1	385	18	4.9 5.0 5.0 5.1 5.1 5.4 5.45 5.55 5.6 6.0	15

(*continued*)

Table 1 (*continued*)

Accession nr.	Protein name	pI (T)[a]	Mol. mass (kDa)	Value of characteristic for protein in undifferentiated cells				Value of characteristic for protein in differentiated cells			
				Score	Peptide match	pI (O)[b]	Nr. of spots	Score	Peptide match	pI (O)	Nr. of spots
										6.0 6.0 6.1 6.7 7.0	
O89052	Alpha-tubulin [Fragment]	4.85	10.95	84	4	5.2	1	173	7	5.3	1
P05213	Tubulin alpha-2 chain	4.94	50.17					146	14	5.15	1
P05214	Tubulin alpha-3/alpha-7 chain	4.98	49.96	330	13	5.45 6.0 6.05	3				
P05216	Tubulin alpha-6 chain	4.96	49.91					59	8	5.1 5.6	2
Q9D9Y2	Tubulin alpha 7, full insert sequence [Tuba 7]	5.76	44.05					80	7	5.65	1
P05217	Tubulin beta-2 chain	4.79	49.83	433	30	5.4 5.85 5.75	3	141	23	5.1 5.35 5.45 5.75 5.75	5
Q13885	Beta tubulin (94% similarity with Tubulin beta-2 chain)	4.78	49.91					341	32	4.7 4.9 5.55 5.8	4
Q9ERD7	Tubulin beta-3	4.82	50.42					261	35	4.3 5.1 5.45 5.9 5.95	5
P05218	Tubulin beta-5 chain	4.78	49.67	77	18	5.75 6.3	2	128	24	4.2 4.3 6.6	3
Q921G8	Gamma-tubulin complex component 2	6.31	103.8	56	19	6.25	1				
Q91V51	Tubulin tyrosine ligase-like protein 1	8.88	49.11					68	11	5.55	1
Q99JI3	CLASP2 (CLIP associating protein 2)	6.24	66.37	56	16	6.3	1				
P15331	Peripherin	5.4	54.27	269	25	4.8 4.9 5.1 5.1 5.1 5.2 5.5 5.55	8	306	25	4.5 4.7 4.75 4.8 4.85 5.0 5.1 5.4 5.5 5.5	10
P20152	Vimentin	5.06	53.56	324	23	3.8 4.1 4.1 4.2 4.2 4.3 4.5	9	382	33	4.3 4.3 4.35 4.6 4.8	5

(*continued*)

Table 1 (*continued*)

Accession nr.	Protein name	pI (T)[a]	Mol. mass (kDa)	Value of characteristic for protein in undifferentiated cells				Value of characteristic for protein in differentiated cells			
				Score	Peptide match	pI (O)[b]	Nr. of spots	Score	Peptide match	pI (O)	Nr. of spots
						4.6					
						4.8					
Q9R071	B4 integrin interactor homolog	4.56	26.54	82	9	3.8	1				
P48678	Lamin A	6.54	74.21	234	29	6.3	7	115	25	6.4	7
						6.4				6.6	
						6.8				7.0	
						6.8				7.2	
						7.0				7.4	
						7.6				7.4	
						7.9				7.5	
P14733	Lamin B1	5.11	66.65					88	24	5.05	1
P11516	Lamins C and C2	6.37	65.45	254	33	6.7	3	203	31	6.95	1
						7.6					
						7.9					
Q6PG82	Keratin 20	5.24	49.01					64	11	7.4	1

[a] pI(T), theoretical pI
[b] pI(O), observed pI

Discussion

The main finding of this study demonstrates that a number of individual cytoskeleton proteins are differentially expressed in undifferentiated and differentiated cells, reflecting rearrangement of the cytoskeleton and giving us a clue to the concerted action of CPs in the differentiation process. CPs of all categories, microfilament, microtubule and intermediary and neurofilaments, are participating in this mechanism. A series of CPs which have never been reported to be involved in differentiation are described herein along with structures known to be contributing to differentiation. Specificity of this CP expressional pattern is warranted as 17 cytoskeleton elements were observed in both physiological states (Table 1). Six CPs were identified in undifferentiated cells that were not observed in the differentiated cell line, and in the differentiated cell line, 20 CPs were observed that were not detectable in the undifferentiated cell line (Table 1). We discuss herein proteins expressed exclusively either in undifferentiated cells or in differentiated cell lines. CPs identified in both cell lines are not discussed herein.

CPs that have not been linked to ND include alpha-actin (fragment), actin-like protein 6A, protein P16-ARC, alpha- and beta centractin, PDZ domain actin-binding protein Shroom, coactosin-like protein, transgelin 2, myosin-light polypeptide 6, tubulin alpha-2 chain, tubulin alpha-6 chain, tubulin alpha-7, protein 94% similar to tubulin beta-2 chain, tubulin beta-3, gamma-tubulin complex component 2, CLASP2, B4 integrin interactor homolog and keratin 20. These structures are now proposed to be involved in differentiation mechanisms. The following elements were assigned a role for the differentiation process already.

Drebrins (developmentally regulated brain proteins) are neuron-specific F-actin-binding proteins proposed to provide plasticity to the cytoskeleton and to serve as intracellular regulators of neuronal morphogenesis (Ishikawa et al., 1994; Shirao, 1995). Asada and coworkers (Asada et al., 1994) analyzed subcellular distribution of drebrin E in neuroblastoma cells (SH-SY5Y), and according to their results, drebrin E was accumulated in submembranous regions, accompanying filamentous actin (F-actin) in parallel with ND induced by retinoic acid. Like drebrin, alpha-actinin is one of actin-binding proteins which cross-link F-actins and controls actin organization, i.e., actin bundling in dendritic spines (Da Silva and Dotti, 2002). Particularly, alpha-actinin 1 was reported to interact with neural cell adhesion molecule (NCAM), which is an important linker of cell surface receptors with the cytoskeleton (Buttner et al., 2003). Alpha-actinin 2 is involved in anchoring of actin filaments and drebrin inhibits this anchoring event competitively (Shirao and Sekino, 2001). Herein, drebrin was expressed in the differentiated cell

line only along with another F-actin-binding protein, actinin, alpha 1 (detected as a fragment). In consideration of all previously reported findings, our data may support the notion that drebrins and alpha-1 actinin play a modulatory role in the anchoring of actin filaments and formation of dendritic spines thus contributing to morphogenesis during ND.

PSD 95 (Postsynaptic Densities) contains many PDZ domains involved in protein interactions that are often observed in multidomain scaffolding proteins (Kim and Sheng, 2004). Some studies have identified PSD-binding proteins including receptors, adaptors, signaling proteins, and cytoskeletal proteins by proteomic analysis (Yoshimura et al., 2002; Walikonis et al., 2000; Husi et al., 2000). Recently, Takahashi and coworkers reported the cooperation of synaptic clustering of drebrin and PSD-95 in developing neurons and their importance in terms of morphogenesis. In the present study, PSD-95 was expressed in differentiated cells exclusively and this is the first report to reveal differentiation-dependent regulation of this protein. At the same time it represents a marker for ND of N1E-115 cells (Takahashi et al., 2003).

Actin is the major component of microfilaments. Choo and Bray (1978) have identified two different forms of actin, beta and gamma actins, in neurons. Early studies have identified high levels of actin, together with myosin, brain spectrin, tropomyosin, alpha-actinin and calmodulin in growth cones (Bray and Hollenbeck, 1988; Drenckhahn and Kaiser, 1983; Mitchison and Kirschner, 1988; Landis and Reese, 1983). The neuronal actin cytoskeleton is linked to actin-binding molecules and other scaffold proteins by small Rho GTPases (Da Silva and Dotti, 2002). In the present study, we also detected a list of actin-binding and actin-bundling proteins (Table 1) that have been studied in ND.

Profilin is known to be involved in actin filament polymerization (Benlali et al., 2000). In particular, the brain-specific protein profilin IIa is required to modulate actin polymerization with either hindering or facilitating neurite budding (Da Silva et al., 2003). Herein, profilin II was expressed in differentiated cell lines only. In agreement with studies mentioned above, our result confirms the involvement of this CP in ND most probably by acting on actin polymerization during cytoskeleton rearrangement.

Birgbauer and coworkers showed that the abundance and the proportion of ezrin increased upon induction of ND with retinoic acid in embryonal carcinoma cells (Birgbauer et al., 1991). In agreement with this finding, we observed expression of ezrin in DMSO-induced differentiation thus extending and verifying previous work.

Microtubules are a predominant component of the neuronal cytoskeleton and functioning in the mitotic division of differentiating neuronal precursors, in neurite outgrowth and migration, as well as substrates for the intracellular transport of organelles and proteins (Laferriere et al., 1997). Evaluating the role of microtubules and their interactions with actin will also contribute to our understanding of neurite outgrowth and cellular morphogenesis.

Tubulin alpha-1 was highly expressed in differentiated cells revealing 15 spots versus a single spot in undifferentiated cells. According to previous data and our result, overexpression and diversity of tubulin alpha-1 expression may be used as a marker for ND in mouse cell lines. Tubulin alpha-1 is highly expressed at the mRNA level during the extension of neuronal processes (Miller et al., 1987) and overexpression of the protein in our differentiated cell line may represent extension of neuronal processes, i.e., formation of the neuronal network (Fig. 1).

Tubulin alpha-3/alpha-7 was observed in embryonic stem (ES) cells and was down regulated along with vimentin during embryonic stem cell differentiation into neuronal cells (Guo et al., 2001). This is in agreement with our results revealing that tubulin alpha-3/alpha-7 was observed in undifferentiated cell lines only.

Tubulin tyrosine ligase (TTL) is known to catalyze ligation of tyrosine residues to the COOH terminus of the detyrosinated form of alpha-tubulin. Kato and coworkers suggested that deregulation of the tubulin tyrosination–detyrosination cycle by decreased expression of TTL is associated with inhibition of ND and enhancement of cell growth (Kato et al., 2004). In the present study, TTL-like protein 1 was observed in differentiated cells exclusively and this is confirming a role for TTL in ND.

There is poor information on the role of lamins during ND. Pierce and coworkers demonstrated that ND of retinoic acid-treated NT2/D1 cells is accompanied by a significant increase in the amount of lamin B1, along with coordinated loss of lamin A/C expression (Pierce et al., 1999). Our findings show that lamin A and lamin C/C2 protein were observed in both cell lines, though presenting with three spots for lamin C/C2 protein in undifferentiated and a single spot in differentiated. Lamin B1 was expressed in the differentiated cell lines only which is in agreement with the previous report.

In conclusion, we herein describe CPs which were reported to be involved in differentiation and a list of CPs that have never been published in respect to the

differentiation process. We were considering CPs present or absent, detectable or undetectable by the method, in the gel exclusively and we cannot rule out that there may have been more up or down regulated proteins in this DMSO-promoted differentiation process. We unambiguously identified proteins by a proteomic approach rather than by immunochemical methods, independent of antibody availability and specificity. The results form the analytical basis for the now arising questions including modifications of posttranslational modifications, the morphological impact, performance of in vitro and in vivo knock-out and transgenic mouse systems in order to study functional relevance of aberrant proteins detected in this system of ND.

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