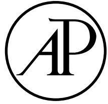
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Reduction of actin-related protein complex 2/3 in fetal Down syndrome brain

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Abstract

Down syndrome (DS) patients present with morphological abnormalities in brain development, leading to mental retardation. Given the importance of actin cytoskeleton to form the basis of various cell functions, the regulation of actin system is crucial during brain development. We therefore aimed to study the expression levels of actin binding proteins in fetal DS and control cortex. We evaluated the levels of eight actin binding proteins using the proteomic approach of two-dimensional gel electrophoresis with subsequent mass spectroscopical identification of protein spots. In fetal DS brain we found a significant reduction of the actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit and the coronin-like protein p57, which are involved in actin filament cross-linking and nucleation and capping of actin filaments. We conclude that deficient levels of these proteins may, at least partially, be involved in the dysgenesis of the brain in DS. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Actin; Cytoskeleton; Actin binding proteins; Coronin-like protein p57; Actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit; Fetal; Down syndrome

In Down syndrome (DS) brain, reduced neuronal density in different regions of the brain and a decrease in dendritic spines and also dendritic branches in early infancy have been reported [1,2] as well as abnormal neuronal differentiation, migration, and synaptogenesis [3–5], leading to mental retardation in these patients. Different basic cell functions such as cell motility, shape, and cytokinesis are based on the exact regulation of the actin filament network through, e.g., actin binding proteins [6–8], which are able to alter the actin filament length or the formation and stiffness of the filament network. Furthermore, actin and proteins regulating the actin network formation play a role in the generation of memory as, e.g., dynamic actin filaments were found to be required for stable long-term potentiation (LTP) in CA1 area of the hippocampus [9]. In addition, depolymerization of dynamic actin might block the insertion of AMPA receptors at synapses [10], which is important during early LTP maintenance [11].

We recently reported a manifold decrease of two actin binding proteins, moesin and drebrin, which were even absent in the majority of fetal DS samples [12,13].

We therefore decided to quantify the levels of eight actin binding proteins with various modulatory effects on the actin system, such as actin filament branching, bundling and cross-linking, nucleation and severing, and capping of actin filaments (see Table 1 and Fig. 1). We aimed to provide further information on molecular mechanisms, leading to abnormal wiring and dysfunction of the brain in DS patients testing cytoskeleton elements.

Methods

Fetal brain tissue and brain preparation. Abortus fetal brain samples (cerebral cortex) of controls ($n = 7$; 1 female and 6 males with mean age of 18.8 ± 2.2 weeks) and DS ($n = 8$; 2 females and 6 males with mean age of 19.8 ± 2.0 weeks) were obtained from Drs. Mara Dierssen and J.C. Ferreres of the Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Fetal Brain Bank, Barcelona, Spain. Samples were taken in accordance with the local ethical committee. All

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Table 1
Effects of the eight investigated actin binding proteins on the actin cytoskeleton

Protein name	Function
Profilin l	Prevents or enhances actin polymerisation depending on concentration
Cofilin	Actin polymerization and depolymerization, binding and severing of actin filaments
Gelsolin	Actin nucleation of monomers and severing of actin filaments
Fascin	Bundling of actin filaments
Destrin	Actin depolymerization, severs actin filaments and binds to actin monomers
Lim + sh3 domain protein lasp-1 (mln 50)	Actin binding and co-localisation with actin at cell extensions
Coronin-like protein p57	Actin binding (by similarity)
Actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit	Linking together the Arp components of the Arp2/3 complex, which is involved in actin filament cross-linking, nucleation, and capping of actin filaments, induction of actin polymerization and Y-shaped actin branching

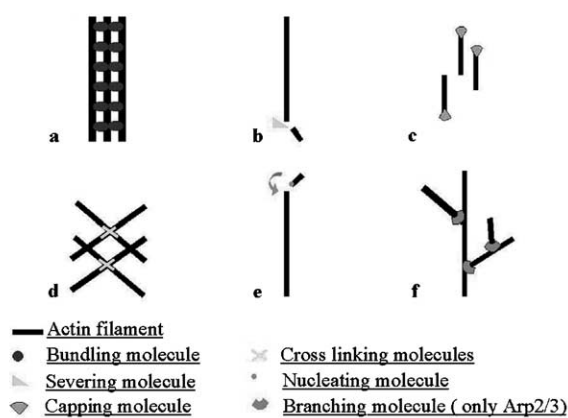

Fig. 1. Modulation of the actin system: (a) Binding of actin filaments into bundles (e.g., by fascin). (b) Severing of an actin filament into split products (e.g., by cofilin, gelsolin, destrin). (c) Cappissng of one end of an actin filament to provide further elongation at this side (e.g., by Arp2/3 complex). (d) Cross-linking actin filaments into a network (e.g., by Arp2/3 complex). (e) Elongation of an actin filament by nucleation (e.g., by Arp2/3 complex, gelsolin). This process is enhanced by, e.g., profilin l. (f) Actin branching as a combination of actin nucleation, capping, and cross-linking (only by Arp2/3 complex).

samples were stored at −70 °C and the freezing chain was never interrupted. The brain tissue was suspended in 0.5 ml sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (Sigma), 10 mM 1,4-dithioerythriol (Merck), 1 mM EDTA (Merck), 1 mM PMSF (Sigma), and 1 μL/mg of each pepstatin A, chymostatin, leupeptin, and antipain. The suspension was sonicated for approximately 30 s and centrifuged further at 150,000*g* for 45 min to sediment the undissolved material. Protein concentration of the supernatant was determined by the Coomassie blue method [14].

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed essentially as reported [15,16]. Samples of 2 mg protein were applied on immobilized pH 3–10 non-linear gradient strips in sample cups at their basic and acidic ends. Focusing started at 200 V and the voltage was gradually increased to 5000 V at 3 V/min and kept constant for a further 24 h (approximately 180,000 kV h totally). The second-dimensional separation was performed on 9–16% SDS gradient polyacrylamide gels. The gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. After protein fixation for 12 h in 40% methanol containing 5% phosphoric acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 48 h. Molecular masses were determined by running standard protein markers (Gibco, Basel, Switzerland), covering the range 10–200 kDa. p*I* values were used as given by the supplier of IPG strips. Excess of the dye was washed out from the gels with H_2O and the gels were scanned in an Agfa DUOSCAN densitometer (resolution 200). Electronic images of the gels were recorded using Photoshop (Adobe) and PowerPoint (Microsoft) software.

Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). MALDI-MS analysis was performed as described elsewhere [17] with some modifications. The spots were excised with a spot picker and placed into 96-well microtiter plates. Each spot was destained with 100 μL of 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a speedvac evaporator. Each dried gel piece was rehydrated with 4 μL of 3 mM Tris–HCl, pH 9.0, containing 50 ng trypsin (Promega, Madison, WI, USA). After 16 h at room temperature, 7 μL H_2O was added to each gel piece and the samples were shaken in for 10 min. Four μL of 50% acetonitrile, containing 0.3% trifluoroacetic acid, the standard peptide des-Arg-bradykinin (Sigma, 904.4681 Da), and adrenocorticotropic hormone fragments 18–39 (Sigma, 2465.1989 Da), in water was added to each gel piece and shaken for 10 min. Application of the samples was performed with a SymBiot I sample processor (PE Biosystems, Framingham, MA, USA). Peptide mixture (1.5 μL) was simultaneously applied with 1 μL matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% trifluoroacetic acid. The samples were analyzed in a time-of-flight mass spectrometer (Reflex 3, Bruker Analytics, Bremen, Germany). An accelerating voltage of 20 kV was used. Peptide matching and protein searches were performed automatically. Peptide masses were compared to the theoretical peptide masses of all available proteins from all species. Monoisotopic masses were used and a mass tolerance of 0.0025% was allowed. The algorithm used for determining the probability of a false positive match with a given MS-spectrum is described elsewhere [18].

Quantification of proteins. Protein spots were outlined (first automatically and then manually) and quantified using the ImageMaster 2D Elite software (Amersham Pharmacia Biotechnology). The percentage of volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-D gel.

Statistical analysis. Software calculated the percentage of spot volume in each particular gel after subtraction of the background values. Between-group differences were calculated by non-parametric Mann–Whitney U test. The level of significance was set at $P < 0.05$. If the spot of a protein was not detectable a value of 0.01 was inserted to make statistical analysis possible.

Results

Identification and quantification of Profilin l, Cofilin, Gelsolin, Fascin, Destrin, Lim + sh3 domain protein,

coronin-like protein p57, and actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit were carried out.

Human proteins obtained from the cerebral cortex of fetal brain were applied on a 2-D gel. Proteins were visualized by staining with colloidal Coomassie blue. A representative control gel with assignments of investigated proteins is shown in Fig. 2a. Protein spots were analyzed by MALDI-MS, following in-gel digestion. Peptide masses were matched with the theoretical peptide masses of all proteins of the SWISS-PROT database. We used internal standards to correct for the measured peptide mass thus reducing the windows of mass tolerance and increasing the confidence of identification. In this study eight proteins were quantified using the ImageMaster 2D Elite software. Expression levels of proteins were determined as a percentage of spot volume in the area of interest. SWISS-PROT accession numbers and names of quantified proteins with MALDI information as well as mean values and SD of the quantified protein levels are given in Table 2.

Protein expression levels of Profilin l were comparable between DS samples and controls.

Cofilin was represented by four spots but one spot could not be clearly separated and thus was not quantified. The levels of the other three spots were not significantly different between DS and control samples.

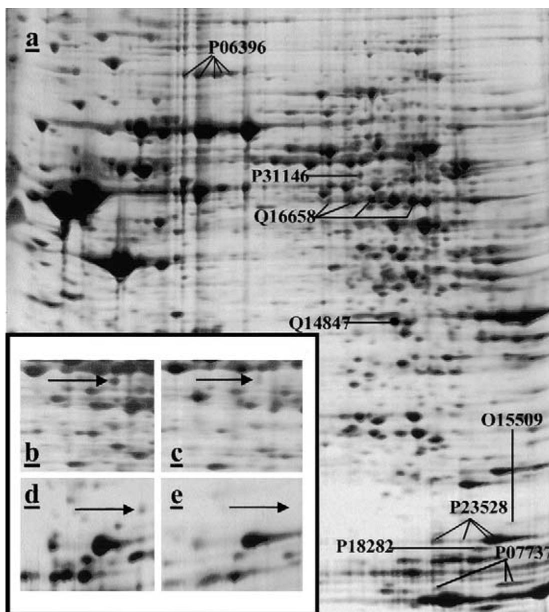

Fig. 2. Identification of all and comparison of the significant proteins investigated: (a) P07737—Profilin l; P23528—Cofilin; P06396—Gelsolin; Q16658—Fascin; P18282—Destrin, Q14847—Lim + sh3 domain protein lasp-1 (mln 50); P31146—Coronin-like protein p57; O15509—actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit. (b) Coronin-like protein p57 in control cortex (arrow). (c) Coronin-like protein p57 in fetal DS samples showed lower levels. (d) Arp2/3 complex 20 kDa subunit in fetal control brain (arrow). (e) In DS samples levels of Arp2/3 complex 20 kDa subunit were significantly reduced and in two out of seven DS patients the spot was not detected.

Quantification of gelsolin, represented by five spots, did not reveal a difference between DS and control individuals.

Fascin was represented by four spots. Quantification of the four clearly separated spots showed comparable levels between the two groups.

Expression levels of Destrin and Lim + sh3 domain protein lasp-1, each represented by a single spot, were comparable in DS and control cortex.

The levels of actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit, which was represented by a single spot, were significantly reduced in fetal DS cortex as compared to controls (P value = 0.008). In two out of the seven quantified samples the spot was even absent.

Quantification of the single spot representing the coronin-like protein p57 revealed statistically significant decreased levels in DS individuals as compared to controls (P value = 0.03) (see Fig. 2b–e).

Discussion

The main findings in the present study were decreased levels of the actin-related protein complex 2/3 20 kDa subunit as well as the coronin-like protein p57 in fetal DS cortex as compared to control individuals. In contrast, levels of other actin binding proteins investigated were comparable, indicating specificity of these results. Reduction of these two proteins due to neuronal or glial loss could be ruled out as levels of neuron-specific enolase (NSE), a marker for neuronal density, and levels of glial fibrillary acidic protein (GFAP), a marker for astrocytic density were comparable between groups (data not shown).

The Arp family consists of 10 members of multisubunit protein complexes involved in a variety of cell functions. The Arp 2/3 complex has seven subunits named Arp3, Arp2, p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc [19], is ubiquitous in eukaryotic cells [20], was shown to be localized at the leading edge of motile cells and in the actin 'tail' of the intracellular pathogen listeria monocytogenes, and was also used for motility of vaccinia virus and *Shigella flexneri* [21]. As seen in 3-D structure the 20 kDa subunit of the complex, quantified in the present study, is holding the Arps together [22].

Different roles of the actin system have been assigned to the complex such as filament cross-linking, nucleation, and capping of actin filaments as well as induction of actin polymerization [23]. Moreover, at the leading edge of motile cells Arp2/3 is so far the only complex, leading to a branched growth of actin in a Y shape with a constant angle of 70° between mother and daughter filaments [24–26].

One essential mechanism during development of the nervous system is the organization of cell processes for

Table 2
Identification and levels of proteins

Protein name	Accession number	p*I*	kDa	Matches	Probability	Means ± SD Control (*n*)	Means ± SD DS (*n*)
Profilin 1	P07737	8.36	15.084	7	1.00E − 10	1.07 ± 0.48 (5)	1.11 ± 0.39 (7)
Cofilin (spot 1)	P23528	8.16	18.718	6	1.74E − 05	0.56 ± 0.21 (6)	0.39 ± 0.16 (7)
Cofilin (spot 3+4)	P23528	8.16	18.718	6	1.74E − 05	9.08 ± 3.17 (6)	6.79 ± 2.02 (7)
Gelsolin (spot 1)	P06396	6.21	86.043	8	1.00E − 08	2.87 ± 2.80 (7)	3.62 ± 1.98 (8)
Gelsolin (spot 2)	P06396	6.21	86.043	8	1.00E − 08	7.06 ± 2.49 (6)	8.58 ± 5.34 (8)
Gelsolin (spot 3)	P06396	6.21	86.043	8	1.00E − 08	2.45 ± 1.67 (7)	3.63 ± 2.51 (8)
Gelsolin (spot 4)	P06396	6.21	86.043	8	1.00E − 08	3.45 ± 3.19 (7)	2.81 ± 2.07 (8)
Gelsolin (spot 5)	P06396	6.21	86.043	8	1.00E − 08	1.88 ± 2.05 (7)	3.15 ± 1.67 (8)
Fascin (spot 1)	Q16658	7.21	55.123	10	1.61E − 15	0.70 ± 0.44 (7)	0.34 ± 0.18 (8)
Fascin (spot 2)	Q16658	7.21	55.123	10	1.61E − 15	2.56 ± 1.35 (7)	2.14 ± 0.45 (8)
Fascin (spot 3)	Q16658	7.21	55.123	10	1.61E − 15	5.25 ± 2.11 (7)	3.13 ± 2.42 (8)
Fascin (spot 4)	Q16658	7.21	55.123	10	1.61E − 15	6.94 ± 2.88 (7)	3.69 ± 2.55 (8)
Destrin	P18282	7.81	18.949	4	7.56E − 06	0.44 ± 0.14 (6)	0.38 ± 0.17 (7)
Lim + sh3 domain protein lasp-1 (mln 50)	Q14847	7.04	30.097	7	1.00E − 09	1.87 ± 0.75 (5)	1.20 ± 0.26 (5)
Coronin-like protein p57	P31146	6.66	51.678	4	2.27E − 06	0.68 ± 0.24* (6)	0.34 ± 0.15* (5)
Actin-related protein complex 2/3 (Arp2/3) 20 kDa subunit	O15509	8.65	19.768	5	2.59E − 06	0.15 ± 0.005** (6)	0.05 ± 0.04** (7)

* $P < 0.05$.
** $P < 0.01$.

cell motility and outgrowth of axons and dendrites. This procedure depends on the exact arrangement of the actin filament network through actin depolymerization and polymerization. Evidence for the involvement of the Arp2/3 complex in the stimulation of actin polymerization in growth cones caused by nerve growth factor (NGF) has been reported [27]. A growth cone is a structure on the tip of the outgrowing axon, which is receiving and dynamically responding to different guidance cues. Candidates for targeting the Arp2/3 complex are proteins of the Wiskott–Aldrich-syndrome protein (wasp)/scar family, while the absence of normal wasp causes cytoskeletal defects, leading to severe immunodeficiencies [28]. The veroproline, cofilin homology and acidic (VCA) region of the neuronal wasp (N-wasp) is required to activate an Arp2/3 complex induced actin polymerization [29]. Mutation in this region was shown to lead to suppression of neurite extension [30]. In addition, lateral binding of the Arp2/3 complex to actin filaments stimulates actin nucleation, a process essential for lamellipodium extension at the leading edge of the cell during migration [31]. These observations suggest a key role of the Arp2/3 complex in the elongation process of axons and dendrites. Thus our findings may contribute to a morphological hallmark in DS children, i.e., the degeneration of the dendritic tree and arborization [1,2]. Pathfinding of the growth cone was recently suggested to be impaired in fetal DS cortex and these recent observations are now extended by the present results, suggesting that in addition to impaired levels of guidance molecules [32], the machinery of the cytoskeletal response to these cues may also be disturbed.

Furthermore, abnormalities of the actin system were reported in the lymphocytes of DS patients presenting an abnormal posttranslational modification of actin [33] and investigations on peripheral polymorphonuclear leukocytes (PMLN) of DS children and controls revealed a chemotactic defect of these cells in DS children [34]. The Arp2/3 complex was shown to play an important role in the chemotaxis of immune cells as dislocation of the Arp2/3 complex leads to polarisation defects in Wiskott–Aldrich syndrome macrophages, resulting in dysfunction of the chemotactic procedure [35]. To elucidate the relevance of the Arp2/3 complex and the coronin-like protein p57 for additional symptoms of DS patients, such as immunodeficiency, further investigations on these proteins in different cell types/tissues of DS patients are needed.

The tentative functional consequences of our findings are that reduction of the Arp2/3 complex 20 kDa subunit in fetal DS cortex may lead to abnormal neuronal and glial migration as well as impaired neurite extension and may, at least partially, explain the aforementioned morphological changes [36].

Coronin-like protein p57 is a member of the coronin-like protein (Clipin) family. It is mainly expressed in immune tissues (thymus, spleen, bone marrow, lymph nodes) but only weakly in brain [37] whereas coronin-1, the mouse ortholog of p57, demonstrates a restricted expression to hematopoietic cells [38]. The protein

shares a sequence similarity of 40% with coronin, an actin binding protein in *Dictyostelium discoideum*, necessary for chemotaxis, cell motility, and cytokinesis [39]. Coronin-like protein Human p57 was shown to interact with the p47 component of phox proteins, which are playing a role in phagocytosis in neutrophils [40]. Although its biochemical function in brain has not been described yet, it is tempting to speculate that it plays a role in the migration of cells and/or neuronal outgrowth and thus deficiency of the protein during brain development might contribute to the abnormal wiring of the brain in DS.

We conclude that the modulation of the actin system is impaired in DS brain development in the early second trimester and propose that reduction of actin-related protein complex 2/3 20 kDa subunit and coronin-like protein p57 is—at least in part—responsible for dysgenesis of the brain and the associated mental disabilities in DS patients by derangement of cytoskeletal elements.

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