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Aberrant Expression of Centractin and Capping Proteins, Integral Constituents of the Dynactin Complex, in Fetal Down Syndrome Brain

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Down syndrome (DS, trisomy 21) is the most frequent genetic cause of mental retardation. Although known for more than a hundred years the underlying pathomechanisms for the phenotype and impaired brain functions remain elusive. Performing protein hunting in fetal DS brain, we detected a series of cytoskeleton proteins with aberrant expression in fetal DS cortex. Fetal brain cortex samples of controls and DS of the early second trimenon of gestation were used for the experiments. We applied two-dimensional electrophoresis with in-gel digestion of protein spots, subsequent mass spectroscopical (MALDI) identification, and quantification of spots using specific software. Centractin α, F-actin capping protein α-1, α-2 and β subunits were significantly reduced in fetal DS cortex, whereas dynein intermediate clear 2, dynein intermediate chain 2, and kinesin light chain protein levels were unchanged. Centractins and F-actin capping proteins are major determinants of the cytoskeleton and are involved in pivotal functions including cellular, organelle, and nuclear motility. Deranged centractins and F-actin capping proteins may represent or induce deficient axonal transport and may well contribute to deterioration of the cytoskeleton's mitotic functions in trisomy 21.

Key Words: **centractin; capping protein; dynactin complex; axonal transport; Down syndrome; cytoskeleton.**

The functional architecture of neurons is composed of various cytoskeletal and membranous components. Each of them is dynamic, constantly changing and being renewed at a rate determined by the local environment and cellular metabolism. The directed motility of organelles along cytoskeletal microtubules is a pivotal process involved in cell functions as diverse as cell division and bidirectional vesicle transport of various organelles. The motor proteins, kinesin and dynein, are key molecules that provide the force for these cellular processes (1–3).

Cytoplasmic dynein possessing microtubule-activated ATPase activity can convey organelles from the "plus" ends to the "minus" ends of microtubules and participate in fast retrograde axonal transport of the nervous system for the return of trophic substances and growth factors to the cell body (4, 5). These factors assure the survival of the neuron and modulate neuronal gene expression. Changes in the return of trophic substances play a critical role during regeneration of neurites.

Recently, the dynactin complex has been identified as the activator of dynein-mediated organelle transport and is suggested to play a critical role in various cellular activities including retrograde axonal transport of the nervous system along with cytoplasmic dynein (6–8). Dynein–dynactin is also needed to keep microtubules associated with the centrosome and contributes to centrosome separation during cell division. In addition, the dynein–dynactin interaction is likely to be important in growth, development, and organization of neurites as well as Golgi dynamics (9–12). As shown in Fig. 1, the dynactin complex consists of at least seven different subunits varying in size from 150 to 22 kDa (13, 14). These include $p150^{Glued}$/p135, centractin (p45), capping protein (p37 and p32), dynamitin (p50), as well as uncharacterized polypeptides with molecular masses of 62, 27 and 22 kDa. Subsequent biochemical analyses have revealed that subunits of 150, 62, 50, 45, 37, 32, 27, and 22 kDa are found in a stoichiometry of 2:1:5:10:1:1:1:1 in the dynactin complex (7, 9, 15, 16).

The 45-kDa subunit of dynactin, centractin, has been defined as member of the actin-related protein 1 (Arp1) class, which shares ~50% sequence identity with con-

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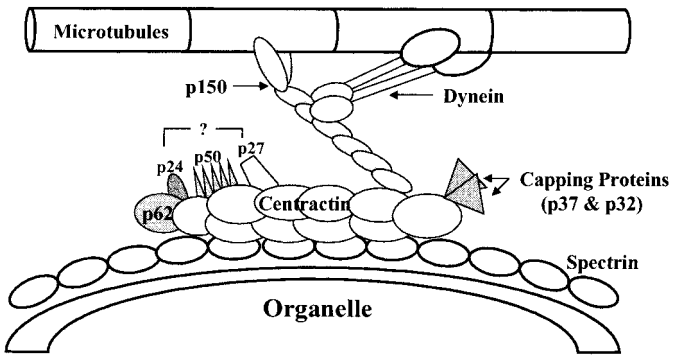

FIG. 1. Schematic diagram of the dynactin complex. Modified from Ref. 14. It consists of at least seven different polypeptides that cosediment at 20S. These include $p150^{Glued}$, centractin (p45), capping protein (p32, p37), dynamitin (p50), as well as uncharacterized polypeptides p62, p24, p22. Centractin could link between organelles, the dynactin complex and capping protein localize to a single end of the centractin filament.

ventional actin (17). Centractin, possessing three isoforms (α, β, and γ), appears to be ubiquitously expressed in vertebrate tissues (18) and requires a multisubunit heteromeric chaperonin to fold correctly (19). Analysis of cells transfected with centractin revealed an association between centractin and spectrin. Antibodies to the Golgi-specific isoform of spectrin colocalized with the centractin filaments (20). These data suggest that dynactin associates with intracellular organelles through an association of centractin filament with organelle-associated spectrin.

Capping protein (also named CapZ) (15) consists of two isoforms α (37-kDa) and β (32-kDa) and is ubiquitous among eukaryotes. Capping protein nucleates actin filaments from monomeric actin, thus inducing polymerization (21–24). In structural studies, antibodies to capping protein have been localized to a single end of the centractin filament of native dynactin (15), suggesting that capping protein associates with one end of the centractin polymer. This localization indicates the possibility that capping protein polymerizes with the centractin filament.

Although recent studies have identified a potential link of dynactin to neurodegenerative diseases such as Huntington's disease (25, 26), connections between dynein–dynactin and other neurodegenerative diseases such as Alzheimer's disease (AD) and Down syndrome (DS) remain to be explored (27). We therefore studied protein levels of centractin and capping protein in fetal brain cortex of DS patients as compared to controls. For this purpose we applied the proteomic approach using two-dimensional (2-D) gel electrophoresis with subsequent matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) identification and specific software for high-resolution quantification of proteins.

MATERIAL AND METHODS

Brain samples preparation. Brain tissues of fetuses with DS and controls were obtained from M. Dierssen and J. Ferreres (Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain). Cerebral cortex of controls (n = 7, male/female = 6/1, 18.79 ± 2.23 weeks) and fetuses with DS (n = 8, male/female = 6/2, 19.81 ± 2.00 weeks) were used. Brain tissues were suspended in 0.5 ml of sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Sigma), 10 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetetraacetic acid, Merck), 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma) and 1 μg/ml of each pepstatin A, chymostatin, leupeptin and antipain. The suspension was sonicated for approximately 30 s and centrifuged at 10,000*g* for 10 min and the supernatant was centrifuged further at 150,000*g* for 45 min to sediment undissolved material. The protein concentration of the supernatant was determined by the Coomassie blue method (28).

Two-dimensional (2-D) gel electrophoresis. The 2-D gel electrophoresis was performed essentially as reported (29). Samples of 2 mg were applied on immobilized pH 3–10 nonlinear gradient strips (IPG, immobilized pH-gradient strips, Pharmacia Biotechnology, Uppsala, Sweden) in sample cups at their basic and acidic ends. Focusing stated 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 kVh 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 with 40% methanol containing 5% phosphoric acid for 12 h, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA) for 48 h. Molecular masses were determined by running standard protein markers (Gibco, Basel, Switzerland), covering the range 10–200 kDa. Isoelectric point (p*I*) values were used as given by the supplier of the IPG strips. Excess of 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 with some modifications (30). 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). After 16 h at room temperature, 7 μl of H_2O were 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 peptides des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da), in H_2O were added to each gel piece and shaken for 10 min. The application of the samples was performed with a SymBiot I sample processor (PE Biosystems, Framingham, MA). 1.5 μl of the peptide mixture was simultaneously applied with 1 μl of matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, containing 0.1% trifluoroacetic acid. 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. The 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 (31).

Quantification of proteins. In partial 2D gel images including either each protein (α/β isoforms of centractin, α/β-capping protein,

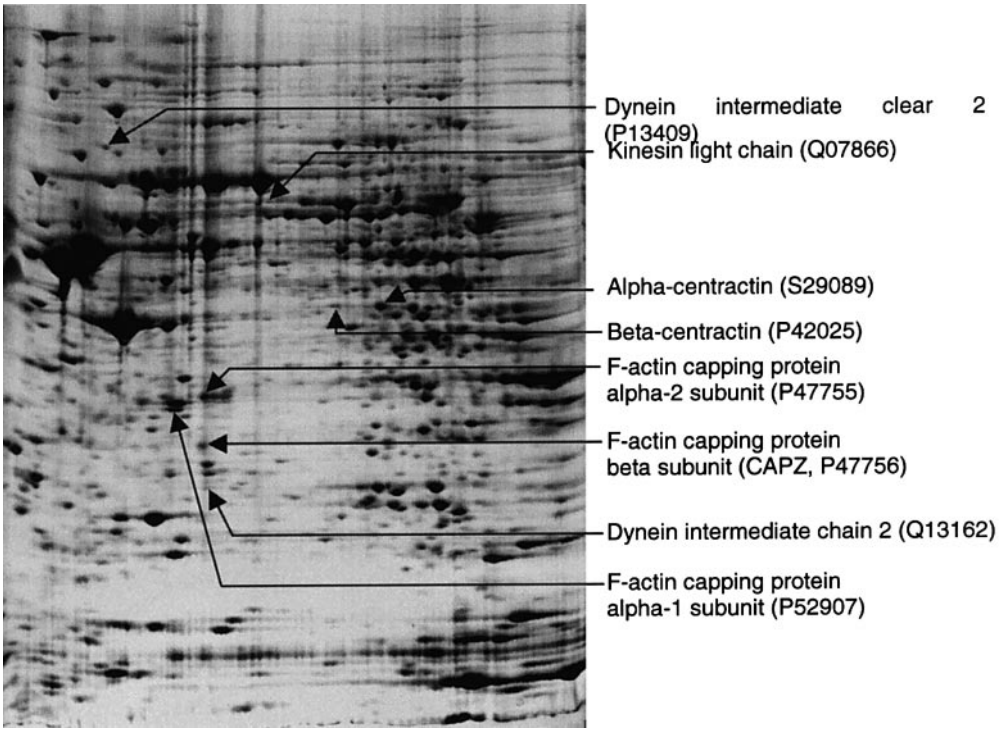

FIG. 2. 2-D gel images of centractin and capping protein in the cortex of control. The human brain proteins were extracted and separated on an immobilized pH 3–10 nonlinear strip, followed by a 9–16% linear gradient polyacrylamide gel, and separated proteins were detected by colloidal Coomassie blue staining and the spots were identified by MALDI-MS. The spots represent α-(S29089), β-(P42025) centractin, α-1-(P52907), α-2-(P47755), β-(P47756) capping protein, dynein intermediate clear 2 cytosolic (P13409), dynein intermediate chain 2 cytosolic (Q13162), and kinesin light chain (Q07866).

kinesin light chain, dynein intermediate clear 2 cytosolic, dynein intermediate chain 2 cytosolic) and the neighboring proteins, the volume-percentage of the spot representing each protein was quantified. These values were compared to the one that represents the volume-percentage of all present proteins by using the ImageMaster 2D Elite software (Amersham Pharmacia Biotechnology) referring to the previously constructed 2D map of human brain proteins (32).

Statistics. Results are expressed as means ± standard deviation (SD). Comparison of groups was calculated using the nonparametric Mann–Whitney *U* test. Correlations of age or postmortem time and the chemical parameters as well as the relation amongst the density of individual proteins was examined with linear regression analysis. Statistical significance was considered at the $P < 0.05$ level. All statistical analysis was performed with the GraphPad Instat2 software, version 2.05.

RESULTS

Identification and Quantification of Human Brain Proteins

The two groups studied had no significant differences in sex, age and postmortem interval (data not shown). Protein extracts from cortex of patients with DS and age-matched controls were separated by 2-DE. Identification of human brain proteins was based upon molecular weight, p*I* value and peptide matches (33). The proteins were identified as α-centractin (SWISS-PROT Accession No. S29089), β-centractin (P42025), F-actin capping protein α-1 subunit (P52907), F-actin capping protein α-2 subunit (P47755), F-actin capping protein β subunit, dynein intermediate chain 2 cytosolic (Q13162), dynein intermediate clear 2 cytosolic

TABLE 1

Means and SD of Controls and DS

	Control	Fetal Down	*P* value
α-Centractin	8.65 ± 1.90	4.79 ± 1.43**	0.0012
β-Centractin	2.60 ± 0.91	2.09 ± 1.18	0.0939
F-Actin capping protein, α-1 subunit	1.41 ± 0.63	0.84 ± 0.52*	0.0418
F-Actin capping protein, α-2 subunit	0.82 ± 0.16	0.44 ± 0.25**	0.0093
F-Actin capping protein, β subunit	0.77 ± 0.20	0.51 ± 0.33*	0.0311
Dynein intermediate clear 2, cytosolic	0.10 ± 0.05	0.05 ± 0.03	0.0727
Dynein intermediate chain 2, cytosolic	0.16 ± 0.05	0.1 ± 0.05	0.1714
Kinesin light chain	0.04 ± 0.01	0.03 ± 0.03	0.1419

Note. Means and standard deviations of the expression levels of α-(S29089), β-(P42025) centractin, α-1-(P52907), α-2-(P47755), β-(P47756) capping protein are represented. In comparison with controls, $*P < 0.05$ and $**P < 0.01$ are considered significantly decreased.

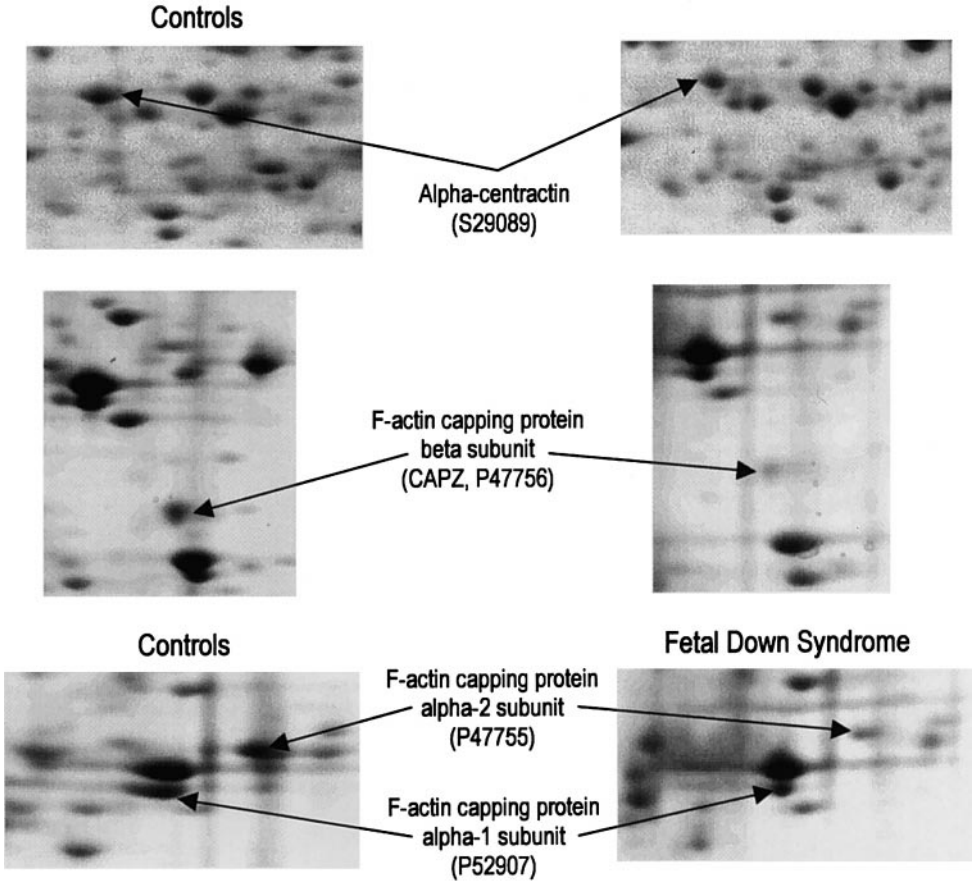

FIG. 3. Typical electrophoresis pattern of α-centractin, α-1-(S29089), α-2-(P47755), β-(P47756) capping proteins in controls and DS patients. α-centractin, α-1-(S29089), α-2-(P47755) and β-(P47756) capping proteins on the 2-DE gels from age-matched controls and patients with DS were quantified using the ImageMaster 2D Elite software and compared to each other employing the non parametric Mann–Whitney *U* test in order to detect different expressional levels of those proteins. Protein expressional levels were determined as the percentage volume of the total proteins present in the gel part considered. Quantitative analysis was performed in brains of patients with DS compared with controls; α-centractin ($P = 0.0012$), α-1-($P = 0.0418$), α-2-($P = 0.0093$), β-($P = 0.0311$) capping proteins in controls and DS patients revealed significantly decreased expression.

(P13409), kinesin light chain (Q07866) (Fig. 2). Density of spots representing the identified proteins was determined as a percentage of total proteins present in the gel part considered using the ImageMaster 2D Elite software. This percentage value was used in statistical analyses.

The Levels of Identified Proteins in Fetal DS Brain

The means and standard deviations derived from percentage values are shown in Table 1.

α-Centractin ($P = 0.0012$), F-actin capping protein α-1 ($P = 0.0418$), α-2 ($P = 0.0093$), and β subunits ($P = 0.0311$) were significantly decreased in cortex of fetal DS (Table 1 and Fig. 3). However, β-centractin, kinesin light chain, dynein intermediate clear 2 cytosolic and dynein intermediate chain 2 cytosolic exhibited comparable levels in controls and the DS group (data shown in Table 1).

DISCUSSION

A series of studies has suggested that deranged centractin and F-capping protein levels may have various detrimental effects on axonal and organelle transport as well as cell division. Dynactin, a macromolecular multisubunit complex that includes centractin and capping proteins, is involved in retrograde axonal transport (minus-end) by mediating cytoplasmic dynein binding to selected cargos (33–35). More recent studies have examined the role for dynactin in neuronal transport and proposed the possibility that dynein and dynactin may be involved in the pathogenesis of neurological disorders (26, 27). Our findings of deranged α-centractin, F-actin-capping protein α-1, α-2 subunits and β subunit (CAPZ) in fetal DS cerebral cortex may well represent anomalies of axonal and/or neuronal transport in the early second trimenon of gestation of DS (Table 1 and Fig. 3). However, no

aberrant levels of dynein and kinesin proteins in fetal DS cerebral cortex were found. Dynein, unchanged in fetal DS brain, mediates the retrograde axonal transport of neurotrophins *in vivo* such as nerve growth factor (NGF) that plays a crucial role in the maintenance, survival and selective vulnerability of various neuronal populations (4, 36). Neuronal or glial loss in the fetal brain was not found in the identical cohort as represented by comparable neuron specific enolase, a marker for neuronal density and comparable glial fibrillary acidic protein, a marker for astrocytic density (data not shown). Comparable levels of dynein and kinesin proteins may indicate the specificity of the reduction of centractin and capping proteins, ruling out simply overall reduction of motor or cytoskeleton proteins in the fetal DS brain or an effect of generally impaired protein expression or protein instability in DS.

Recent studies on centractin also provided evidence for its involvement not only in axonal transport but also in cell division (9, 13, 37–39). Centractin acts as a regulator for various dynein-mediated functions during mitosis, i.e., mitotic spindle dynamics, nuclear migration and general cellular trafficking. A perturbation of this process, a delay in separation or a basic defect in a mechanism controlling microtubular polymerization and/or function might be a factor for nondisjunction in DS (37, 38). Another effect caused by overexpressed centractin is the formation of supernumerary spindles leading to imbalance of tension forces acting on the spindle, thereby preventing the onset of anaphase.

Centractin and the capping proteins play an important role in cell division. As a component of the dynactin complex they bind to microtubules during prometaphase. Therefore, one could suggest that the disturbed microtubular network affects the process of cell division and our findings of deranged α-centractin, F-actin-capping protein α-1, α-2 subunits and β subunit (CAPZ) in fetal DS cerebral cortex provide evidence for this tentative mechanism.

Taken together, we conclude that reduced centractin and F-actin capping proteins may well lead to impaired stoichiometry of dynactin complex proteins with the biological consequence of deranged cytoskeleton function including axonal transport and cell division. Chromosomal imbalance by trisomy 21 may be the underlying mechanism for the decrease of centractin and F-actin capping proteins rather than neuronal or glial loss in the fetal brain with DS. Reduction of actin-related proteins in this study are in line with a series of significantly reduced other related actin-bundling proteins including drebrin and moesin (40, 41). Alternatively, we may propose that the transcription deficits found in DS brain (42, 43) may have been causing downregulation of centractin and F-actin capping proteins at the transcriptional level.

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