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Mass-spectrometrical analysis of proteins encoded on chromosome 21 in human fetal brain

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Summary. Overexpression of chromosome 21 genes is directly or indirectly responsible for the Down syndrome phenotype. In order to analyse chromosome 21 gene products (Chr21Ps), we extracted proteins from fetal human brain cortex and applied an ultracentrifugal and chromatographic prefractionation principle followed by two-dimensional gel electrophoresis (2-DE) and mass-spectrometrical analysis using high-throughput automated MALDI-TOF/TOF. Nine Chr21Ps were identified: pyridoxal kinase; superoxide dismutase [Cu/Zn] 1; carbonyl reductase 1; ES1 protein homolog, mitochondrial [Precursor]; cystathionine-beta-synthetase; T-complex protein 1, theta subunit; cystatin B; 6-phosphofructokinase; glycinamide ribonucleotide synthetase. Mass-spectrometric characterisation of Chr21Ps following separation in 2-DE gels is a useful tool for the analysis of these structures in brain, independent of antibody availability and specificity.

Keywords: Human fetal brain – Human chromosome 21 – Ion-exchange chromatography – Two-dimensional gel electrophoresis – MALDI-TOF/TOF

Introduction

Genes encoded on chromosome 21 are a main focus in human genetics as trisomy 21 (Down syndrome [DS]) is present in one out of seven hundred life births and represents the major single genetic cause for dementia. Moreover, all patients with DS inevitably develop Alzheimer-like neuropathological changes from the fourth decade and DS can be therefore considered as a model for development of Alzheimer's disease (Engidawork et al., 2001).

Most studies on chromosome 21 structures have been carried out at the transcriptional level and overexpression of chromosome 21 genes forms a classical and current concept for the development of the DS phenotype (Kahlem et al., 2004; de Haan et al., 2003; Amano et al., 2004; Fuentes et al., 2000; Pucharcos et al., 1999). This hypothesis has been already challenged at the transcriptional and protein level (Ferrando-Miguel et al., 2003, 2004, 2005; Lubec et al., 2002, 2003; Shim et al., 2003; Cheon et al., 2003a–d; Engidawork et al., 2001, 2003) and is hampered by many confounding factors. There is a long and unpredictable way from nucleic acids to protein and this is obvious from theoretical and practical knowledge and experience. Apart from the fact that mRNA stability is different from protein stability, quantification of transcripts and proteins has to be taken with caution. In addition, most protein work in the past was carried out using immunochemical methods for determinations and only recently chromosome 21 products were studied by protein chemical methods, independent of antibody availability and specificity (Gulesserian et al., 2001; Shin et al., 2004). The advent of two-dimensional gel electrophoresis (2-DE) with in-gel digestion and subsequent mass-spectrometrical identification of protein spots along with high-throughput robot technology allows systematic analysis of large series of proteins with excellent sensitivity and specificity of identification, in particular when MS–MS is used, mainly in form of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and MALDI-TOF-TOF technology (Mann et al., 2001).

The shortcoming of the method is, however, that separation of protein extracts on a single gel does not lead to identification of more than a few hundred different proteins as many expression forms from the same proteins are detected on the same gel (Lubec et al., 2003; Fountoulakis et al., 2001). Methodologically there are several limitations to detect Chr21Ps and these obstacles go in gear with

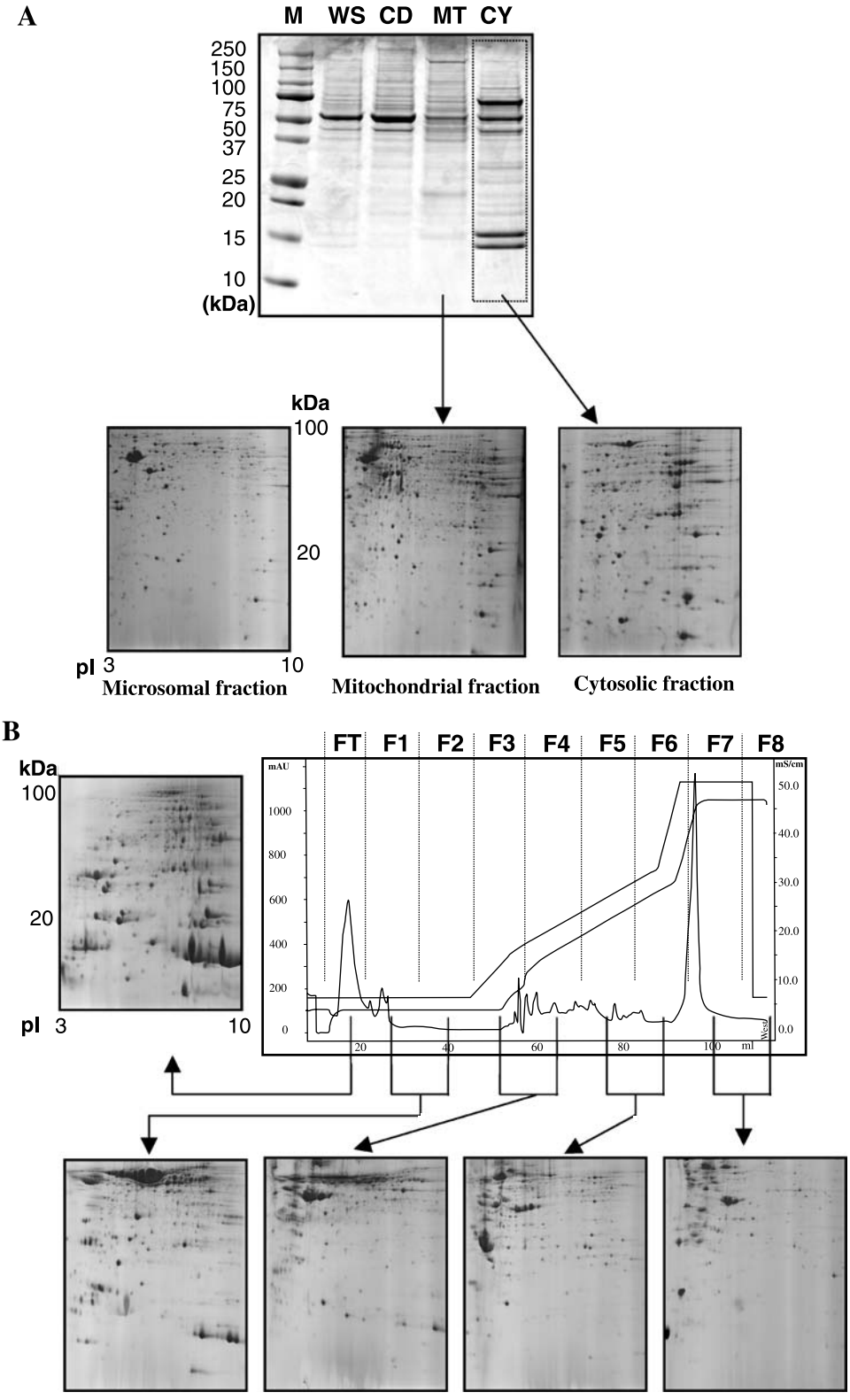


Fig. 1. **A** Centrifugal prefractionation of human fetal brain protein. Different centifugal forces lead to enrichment of cellular compartments. The cytosolic fraction was applied to further fractionation by IEC: M, molecular ladder; WS, washed solution; CD, cell debris. **B** Soluble proteins from human fetal brain (20 mg of protein) were separated by a linear salt gradient on TSK ion exchange chromatography and the absorbance was monitored at 280 nm. Each fraction was analysed by IEF (3–10 NL) in the first dimension and 12% 2D-PAGE in the second separation

characteristics of Chr21Ps (hydrophobic, extremely acidic or basic protein, low-copy proteins or uncharacterised proteins) and technical limitations in terms of mass-spectrometry and 2-DE. Therefore, novel techniques are required to detect the Chr21Ps and elucidate their expression pattern for understanding the phenotypic mechanism of DS.

Prefractionation of protein mixtures is consequently needed to allow construction of maps with many different proteins (Righetti et al., 2003; Krapfenbauer et al., 2003; Shin et al., 2005; Karlsson et al., 1999; Lescuyer et al., 2004). It was the aim of this study to use an ultracentrifugal and chromatographic prefractionations principle followed by 2-DE and mass-spectrometrical analysis in order to identify and analyse chromosome 21 gene products, which forms the basis for further reliable protein chemical work on trisomy 21.

Materials and methods

Sample preparation

Human brain tissue of aborted control fetuses with no obvious abnormalities (three males, age of 18 ± 2.2 weeks of gestation and post-mortem interval of 5.5 ± 1 h) was used for the experiment. Brain samples were kindly obtained from Drs. M. Dierssen and J. C. Farreras of the Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain. All procedures were performed with the consent of the hospital's ethics committee, and following dissection, all samples were stored at $-70\,^{\circ}C$ and the freezing chain was never interrupted.

Each brain tissue (2.0 g) was suspended in sucrose buffer consisting of 20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM EDTA, 5 mM DTE, 1 protease inhibitor cocktail tablet (Boehringer Mannheim, 1697498) per 50 ml, 1 mM PMSF, 0.2 mM Na_3VO_3 and 1 mM NaF, using a glass-teflon potter for homogenisation. The suspension was centrifuged at $800 \times g$ for 10 min at $4\,^{\circ}C$ to sediment nonsuspended material. To obtain the microsomal, mitochondrial, and cytosolic proteins, the supernatant was further centrifuged at $10{,}000 \times g$ for 15 min at $4\,^{\circ}C$ and at $100{,}000 \times g$ for 1 h at $4\,^{\circ}C$ to collect the mitochondrial and

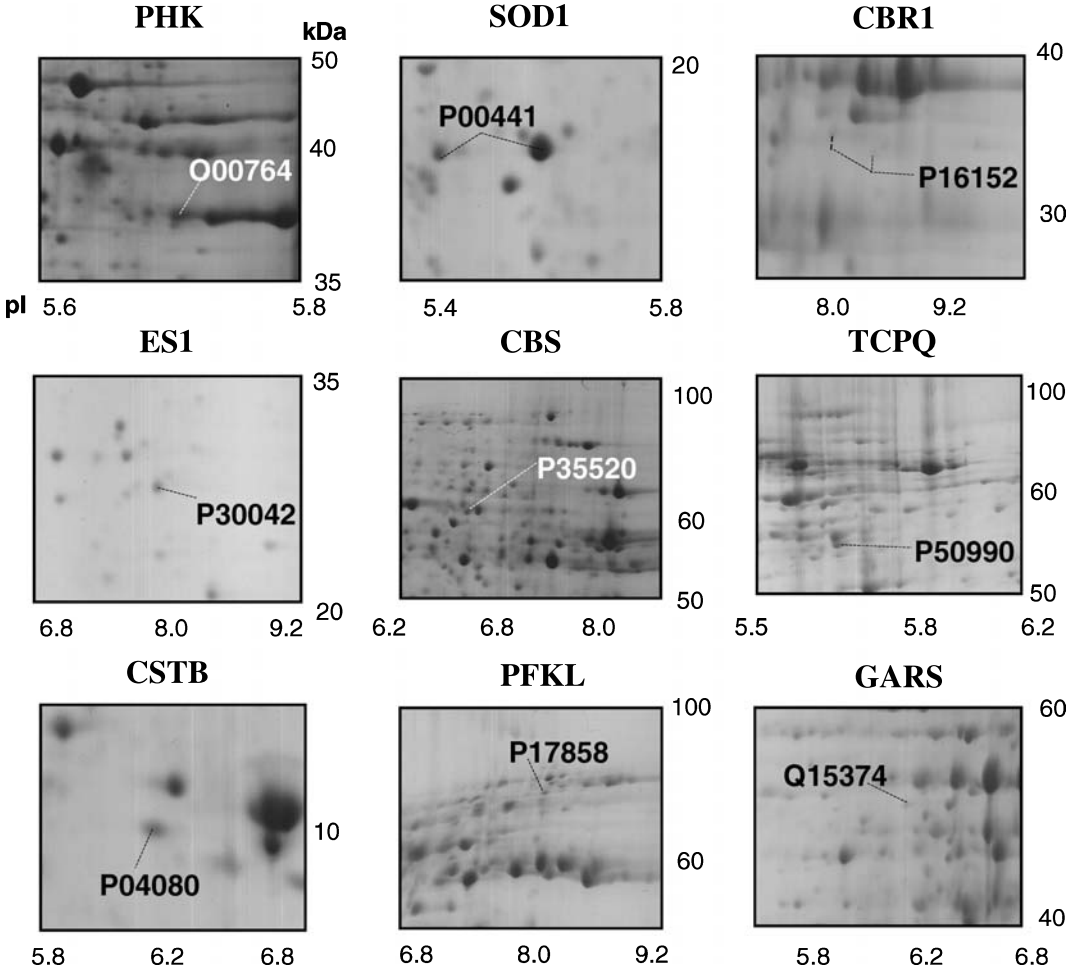


Fig. 2. Partial images of nine Chr21Ps in 2-DE gels. Enlarged images of PHK, SOD1, CBR1, ES1, CBS, TCPQ, CSTB, PFKL and GARS were taken from flow through, cytosolic fraction, cytosolic fraction, microsomal fraction, mitochondrial fraction, cytosolic fraction 3 and 4, cytosolic fraction, cytosolic fraction 3 and 4, and flows through, respectively. Proteins were fractionated and separated on an immobilised pH 3–10 NL gradient IPG strip, followed by separation on a 12% homogeneous polyacrylamide gel. The gel was stained with colloidal Coomassie blue and spots were analysed by MALDI-TOF/TOF. The chromosome 21 proteins identified are designated with their accession numbers and x- and y-axes indicate pI and M_r, respectively

cytosolic fraction. Material from the mitochondrial and microsomal fraction was suspended in 0.5 ml of a sample buffer consisting of 40 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM 1,4-dithioerythritol, 1 mM EDTA, 1 mM PMSF and 1 protease inhibitor cocktail tablet (Boehringer Mannheim, Art. No 1697498) per 50 ml. The supernatants were centrifuged at 100,000 × *g* for 1 h to sediment undissolved material. The protein concentration in the supernatant was determined by Bradford reaction (Bradford et al., 1976).

Ion-exchange chromatography (IEC)

The cytosolic protein homogenate from each fetal brain (10 ml) was concentrated to about 0.2 ml in an ultrafiltration device equipped with Biomax-10K NMWL membranes (Millipore). Equal protein amounts from the concentrated cytosolic proteins of three fetal brains were taken, containing 50 mg of total protein. pH was adjusted to 8.8 with 25 ml of 25 mM Tris-HCl. Proteins were applied onto TSK gel DEAE-5PW ion-exchanger column (article nr. 07164, 7.5 mm ID by 7.5 cm glass, 10 µm spherical particle; Tosoh Bioscience), equilibrated with buffer (25 mM $NaHPO_4$ [pH 7.8], 1 mM EDTA, 0.5 mM DTE). The column was washed twice with 20 ml of the same buffer and 10 mg of protein from the cytosol (dissolved in $NaHPO_4$ sample buffer, pH 7.8, described above) were eluted with a linear gradient of increasing salt concentration from 0 to 1 M NaCl in 25 mM $HaHPO_4$ buffer (pH 7.8) according to the elution profile. Fractions of 0.5 ml (1.0 mg) were collected and pooled according to the elution profile. Eight fractions (F I–VIII) were formed and pooled to 4 pools. The proteins were concentrated and desalted by using reversed phase chromatography (Poros R2; Perseptive Biosystems Inc., Framingham, MA) and the concentrates were resolved in urea sample buffer and analysed by IEF and 2-DE.

Two-dimensional gel electrophoresis

Samples of mitochondrial, microsomal, and cytosolic fractions from centrifugal fractionation were desalted by using membrane filter tubes (article nr. UFV4BGC25, 10.000 NMWL, Biomax-10 membrane; Millipore, Bedford, MA, USA) and 2.0 mg were applied on immobilised pH 3–10 nonlinear gradient strips (Amersham Pharmacia Biotechnology, Uppsala, Sweden) at both the basic and acidic ends of the strips. Proteins were focused at 200 V after which the voltage was gradually increased to 5000 V with 2 V/min (approximately 180,000 kVh). Focussing was continued at 5000 V for 24 h. The second-dimensional separation was performed on 12% homogeneous polyacrylamide gels (Serva, Heidelberg, Germany). The gels (180 by 200 by 1.5 mm) were run at 40 mA per gel, in an ISO-DALT apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) accommodating ten gels. After protein fixation with 50% (v/v) methanol containing 10% (v/v) phosphoric acid for 12 h, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 24 h. Molecular masses were determined by running standard protein markers (Gibco, Basel, Switzerland), covering the range of 10–200 kDa. pI values were used as given by supplier of the IPG strips. Gels were destained with H_2O and scanned with ImageScanner (Amersham Bioscience). Images were processed using Photoshop (Adobe) and PowerPoint (Microsoft) software.

MALDI-TOF/TOF

Spots were excised with spot picker (Proteineer sp™, Bruker Daltonics, Germany), placed into 96-well microtiter plates. In-gel digestion and MALDI sample preparation were performed by Digestion kit (Bruker Daltonics) and an automated procedure (Proteineer dp™, Bruker Daltonics). Briefly, spots were excised and washed with 10 mM ammonium

Table 1. Identification of nine proteins encoded on chromosome 21 in human fetal brain[a]

Accession nr.	Location[b]	Protein name	Matched peptides/ score[c]	Sequence coverage (%)	Gene locus (LocusLink)	Theoretical/observed	
						pI	M_r (kDa)
O00764	FT, Mt	Pyridoxal kinase	8/66	32	21q22.3	5.75/5.65	35.102/36.500
P00441	Cy	Superoxide dismutase [Cu–Zn] 1	4/67	31	21q22.1	5.70/5.40–5.60	15.805/17.000–17.455
P04080[d]	Cy	Cystatin B	5/72	66	21q22.3	6.96/6.18	11.140/10.504
P16152	Cy, FT, Mt	Carbonyl reductase [NADPH] 1	8/70	36	21q22.12	8.55/8.00–8.40	30.244/31.455
P17858[d]	FII	6-Phosphofructokinase, liver type	24/127	42	21q22.3	7.01/8.05	84.917/82.000
P30042	Mi	ES1 protein homolog, mitochondrial [Precursor]	5/66	23	21q22.3	8.50/7.98	28.142/28.298
P35520	Mt	Cystathione beta-synthase	9/76	22	21q22.3	6.22/6.60	60.455/60.197
P50990	FIII, FIV	T-complex protein 1, theta subunit	10/196	26	21q22.11 or 21q21.3 (Ensembl)	5.42/5.60	59.489/55.002
Q15374[d]	FT	GARS protein	10/76	43	21q22.11	6.34/6.18	45.987/50.495

[a] Proteins identified in fetal brain were separated by 2-DE and identified by MALDI-TOF/TOF, following in-gel digestion with trypsin. Spots representing the identified proteins are indicated in Fig. 2 and are designated with their accession numbers of SWISS-PROT. The number of peptides matched, probability of assignment (Score), theoretical molecular weight and pI values are given. For protein search, a mass tolerance of 25 ppm and oxidation of methionine residues was considered. Unmatched peptides or miscleavage sites were not allowed. The probability score (scores greater than 61 are significant; $p < 0.05$) calculated by the software was used as criterion for correct identification

[b] Cy, cytosolic fraction; Mt, mitochondrial fraction; Mi, microsomal fraction; FT, flow through; FI, cytosolic fraction 1 and 2; FII, cytosolic fraction 3 and 4; FIII, cytosolic fraction 5 and 6; FIV, cytosolic fraction 7 and 8

[c] Score is $-10 * \text{Log}(P)$, where P is the probability that the observed match is a random event (Mascot, http://www.matrixscience.com)

[d] Protein newly identified in this study

bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, the gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. Dried gel pieces were reswollen with 40 ng/ml trypsin 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.

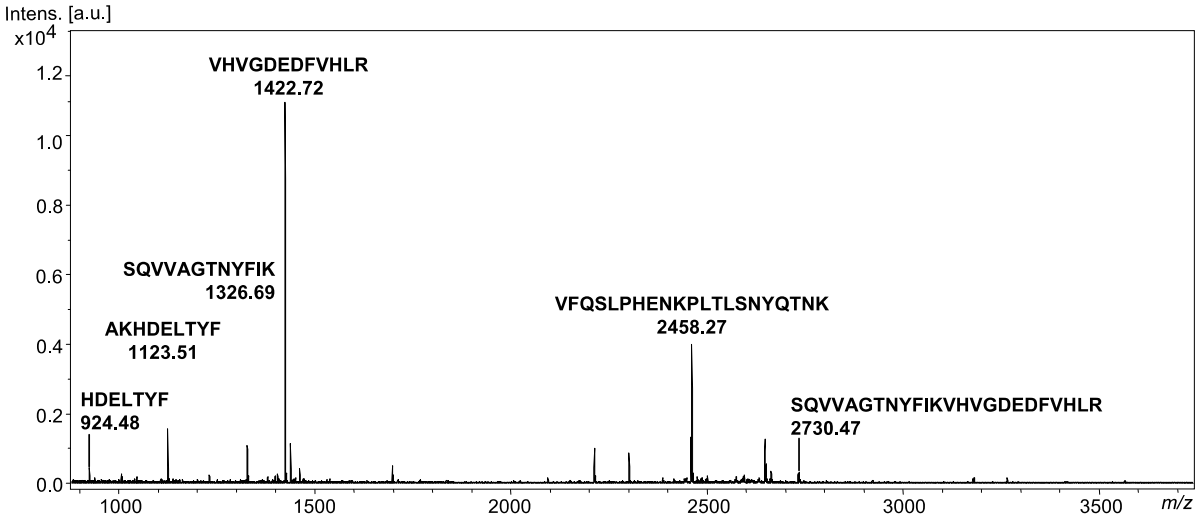
Peptide extraction was performed with 15 µl/spot of 1% TFA in 5 mM OGP. Extracted peptides were directly applied onto a target (AnchorChip™, Bruker Daltonics) loaded with 3.5 µl/spot of α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA) matrix (25 mg/ml solution in acetone–0.1% TFA [97:3, v/v]). MALDI-MS(/MS) data were obtained using an Ultraflex™ TOF/TOF (Bruker Daltonics) equipped with a LIFT-MS/MS facility controlled by the FlexControl™ 2.0 software package. An accelerating voltage of 25 kV was used for protein mass fingerprinting (PMF). For fragment ion analysis in the tandem TOF/TOF TOF mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed removal of precursor and metastable ion signals produced after extraction out of the second ion source. Masses were annotated and processed with FlexAnalysis™ 2.0. External calibration of MALDI-TOF mass spectra was performed using singly charged monoisotopic peaks of a mixture of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (ACTH 1–17 and 18–39) (Bruker Daltonics). For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of theses peptides. Each MS

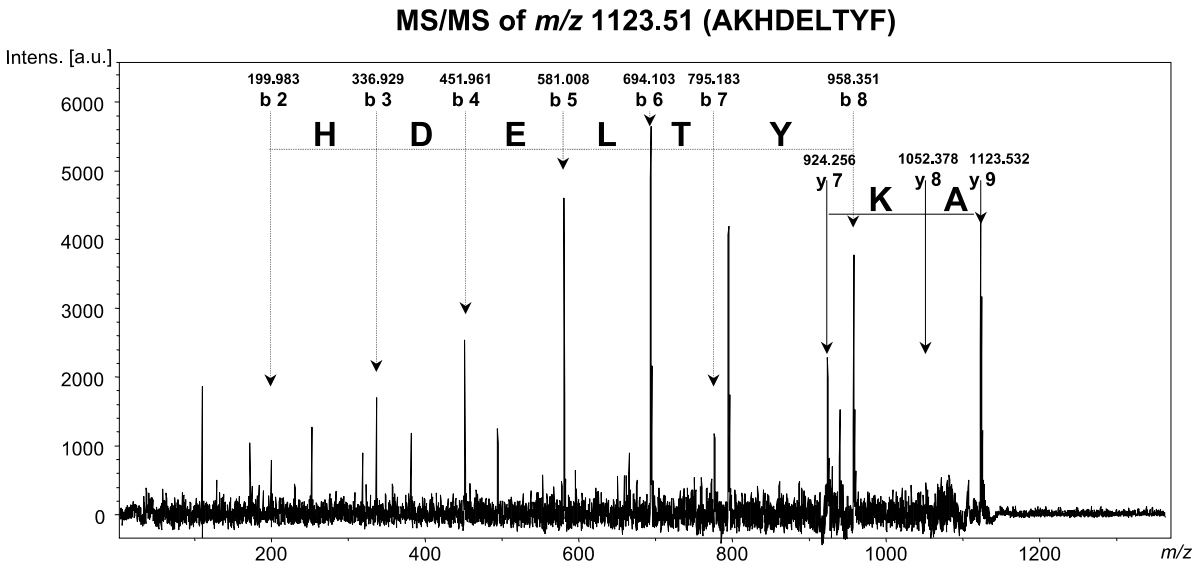
Table 2. Mass-spectrometical details of three Ch21Ps newly identified in human fetal brain

Protein name (accession nr.)	Matched peptides	Value for peptide for MS/MS (PMM)			
		M_r			Score[a]
		obsd	exptl	calcd	
Cystatin B (P04080)	MCGAPSATQPATAETQHIADQVR				
	SQVVAGTNYFIK				
	VHVGDEDFVHLR	1422.72	1421.71	1421.71	78
	VFQSLPHENKPLTLSNYQTNK	2458.28	2457.28	2457.27	54
6-Phosphofructokinase, liver type (P17858)	AIGVLTSGGDAQGMNAAVR				
	AIGVLTSGGDAQGMNAAVR (M)[b]				
	VFLIYEGYEGLVEGGENIK				
	AAAYNLVQHGITNLCVIGGDGSLTGANIFR				
	SEWGSLLEELVAEGK				
	TYSHLNIAGLVGSIDNDFCGTDMTIGTDSALHR (M)				
	IMEVIDAITTTAQSHQR				
	TFVLEVMGR				
	TFVLEVMGR (M)				
	LNIIIIAEGAIDR	1410.84	1409.83	1409.82	16
	MGMEAVMALLEATPDTPACVVTLSGNQSVR (3M)				
	GGSFENNWNIYK				
	SNFSLAILNVGAPAAGMNAAVR				
	SNFSLAILNVGAPAAGMNAAVR (M)				
	TGISHGHTVYVVHDGFEGLAK				
	GQLESIVENIR				
	TNVLGHLQQGGAPTPFDR	1907.97	1906.96	1906.96	59
	VFANAPDSACVIGLK				
	AAAFSPVTELK				
	EQWWLSLR				
	ISMAAYVSGELEHVTR				
	ISMAAYVSGELEHVTR (M)				
GARS protein (Q15374)	ISNTAISISDHTALAQFCK				
	IEFVVVGPEAPLAAGIVGNLR	2121.20	2120.19	2120.20	21
	SAGVQCFGPTAEAAQLESSK				
	AFTKPEEACSFILSADFPALVVK				
	AFGAAGETIVIEELLDGEEVSCLCFTDGK				
	LLEGDGGPNTGGMGAYCPAPQVSNDLLLK (M)				
	TVDGMQQEGTPYTGILYAGIMLTK (M)				
	TVDGMQQEGTPYTGILYAGIMLTK (2M)				
	VLEFNCR				
	FGDPECQVILPLLK				

[a] MS/MS score greater than 17 or 34 indicates that peptide is homolog or identity to MS results

[b] M, 2M, 3M in parentheses, methionine oxidation and its number in peptides





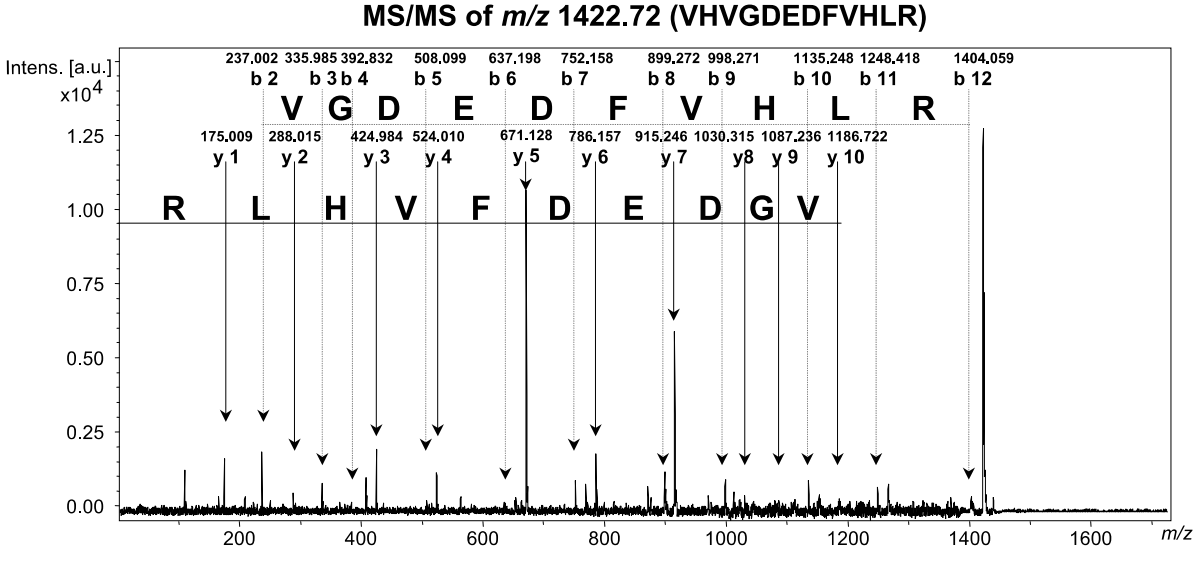


Fig. 3 (*continued*)

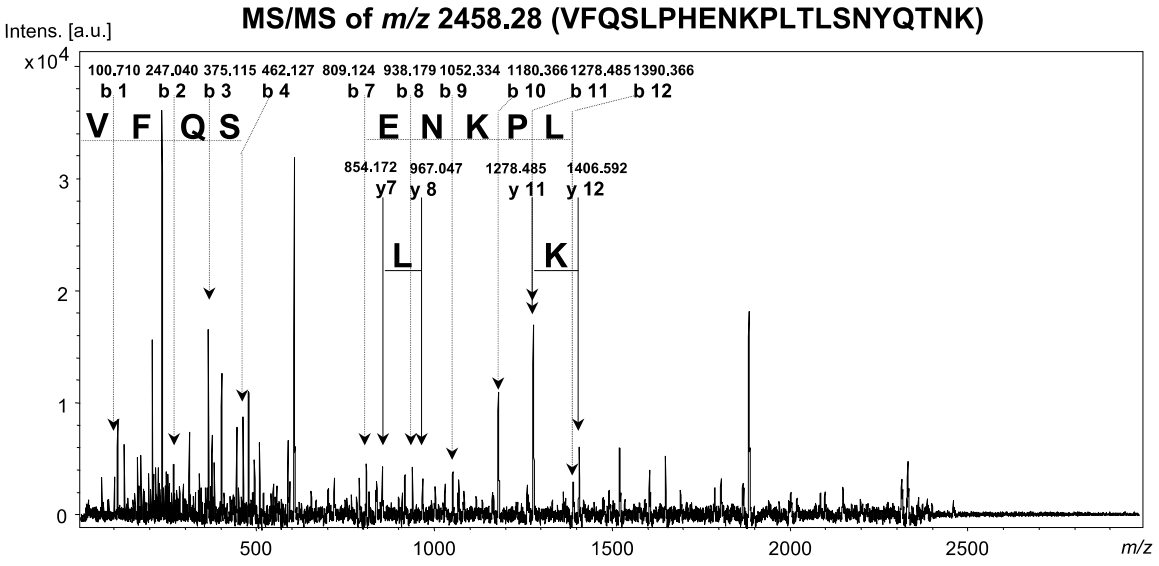


Fig. 3. MALDI-TOF MS and MS/MS analysis of CSTB (A), PFKL (B) and GARS (C). Two trypsin peaks $[M+H]^+$ (m/z 842.50 and 2211.10) were used for internal calibration. In the MS spectrum the methionine oxidation and its number in peptides is given in parenthesis and gray annotation indicates the peaks haboring 1 missing cleavage. MS/MS analysis of singly charged $[M+H]^+$ protonated molecules of precursor ions was performed

spectrum was produced by accumulating data from 200 consecutive laser shots and spectra were interpreted with the aid of the Mascot Software (Matrix Science Ltd, London, UK). For protein search, a mass tolerance (accuracy) of 25 ppm and oxidation of methionine residues was considered. Unmatched peptides or miscleavage sites were not allowed. The probability score (scores greater than 61 are significant; $p<0.05$) calculated by the software was used as criterion for correct identification. A maximum of three precursor ions per sample were chosen for MS/MS analysis. MS/MS tolerance of 0.5 Da and 0 missing cleavage site was allowed and oxidation of methionine residues was considered. Combination of PMF and MS/MS data was used to determine the protein identification with BioTools™ 2.2 software (Bruker).

Results

Human fetal brain proteins on 2-DE gels

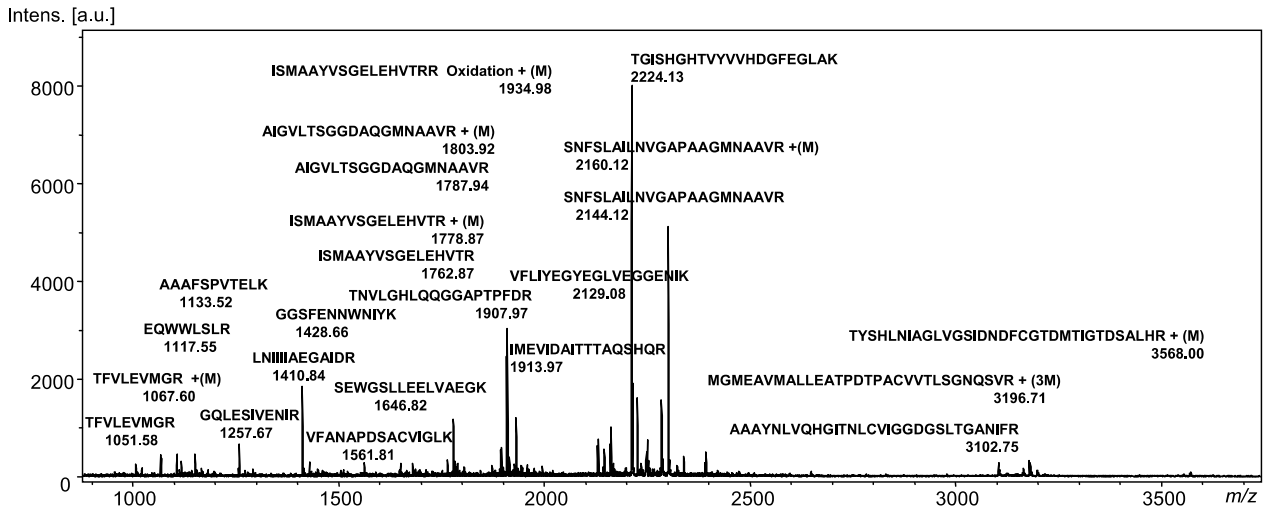
Human fetal brain proteins were extracted and fractionated by centrifugal force and IEC (Fig. 1). Following chromatography, fractions were desalted and applied on 2-DE gels using IEF-compatible reagents urea, thiourea and CHAPS. 2-DE separation was performed on broad pH range 3–10 NL IPG strips and protein spots were visualised following staining with colloidal Coomassie blue. Nine Chr21Ps were successfully identified and represented in Fig. 2.

Identification of nine proteins whose genes are encoded on chromosome 21

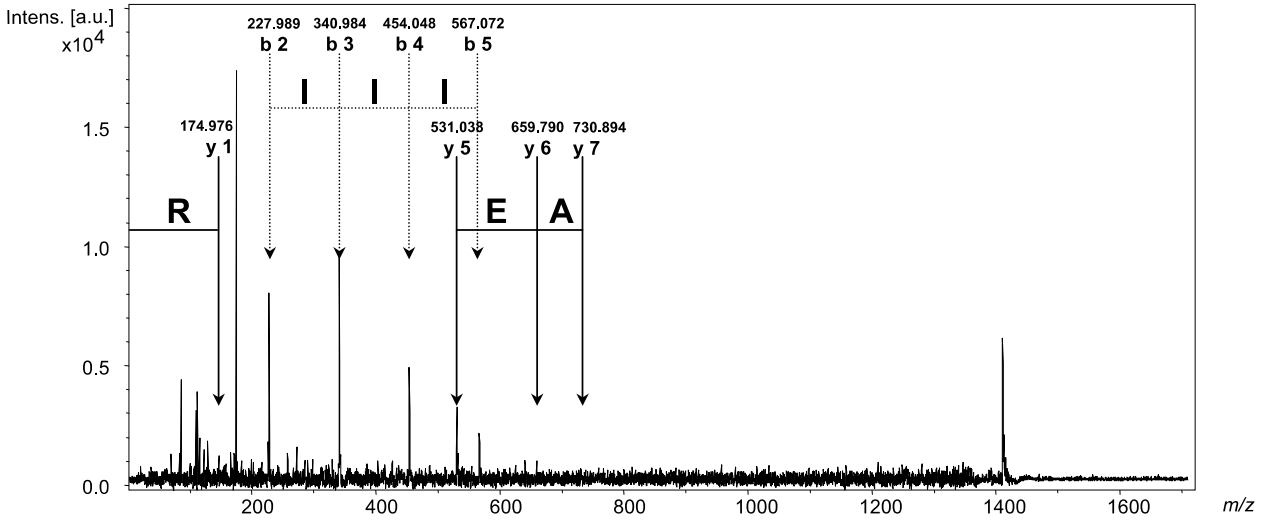
The spots in subcellular and fractionated 2-DE gels were selected randomly with the goal to detect as many new Chr21Ps as possible. Totally about 3264 spots from 8 gels were analysed and resulted in the identification of about 2200 proteins which were the products of 543 different genes (Shin et al., 2005). We successfully identified six Chr21Ps (PHK, pyridoxal kinase; SOD1, superoxide dismutase [Cu/Zn] 1; CBR1, carbonyl reductase 1; ES1, ES1 protein homolog, mitochondrial [Precursor]; CBS, cystathionine-beta-synthetase; TCPQ, T-complex protein 1, theta subunit) which were already observed in human cerebral cortex with traditional 2-DE and MALDI-MS (Gulesserian et al., 2001; Shin et al., 2004; Fountoulakis et al., 2002) (Table 1). In addition, cystatin B (CSTB), 6-phosphofructokinase, liver type (PFKL), and glycinamide ribonucleotide synthetase (GARS) proteins encoded on chromosome 21 were newly identified in human fetal brain by a chromatographic prefractionation step prior to 2-DE and MALDI-TOF/TOF (Tables 1 and 2). All proteins were presented by one spot except SOD1 and CBR1 showing two spots. We attribute heterogeneity to the presence of posttranslational modifications or splicing variants.

Mass-spectrometrical analysis of CSTB, PFKL, and GARS proteins

For MS analysis of CSTB, PFKL, and GARS proteins, we excised spots from fractionated gels stained with colloidal Coomassie blue. Tryptic digests were analysed by MALDI-TOF or MALDI-TOF/TOF. On the basis of its MALDI-TOF spectrum, each spots was identified as CSTB, PFKL, and GARS protein by fingerprinting.



MS/MS spectrum of m/z 1410.84 (LNIIIIAEGAIDR)



MS/MS spectrum of m/z 1907.97 (TNVLGHLQQGGAPTPFDR)

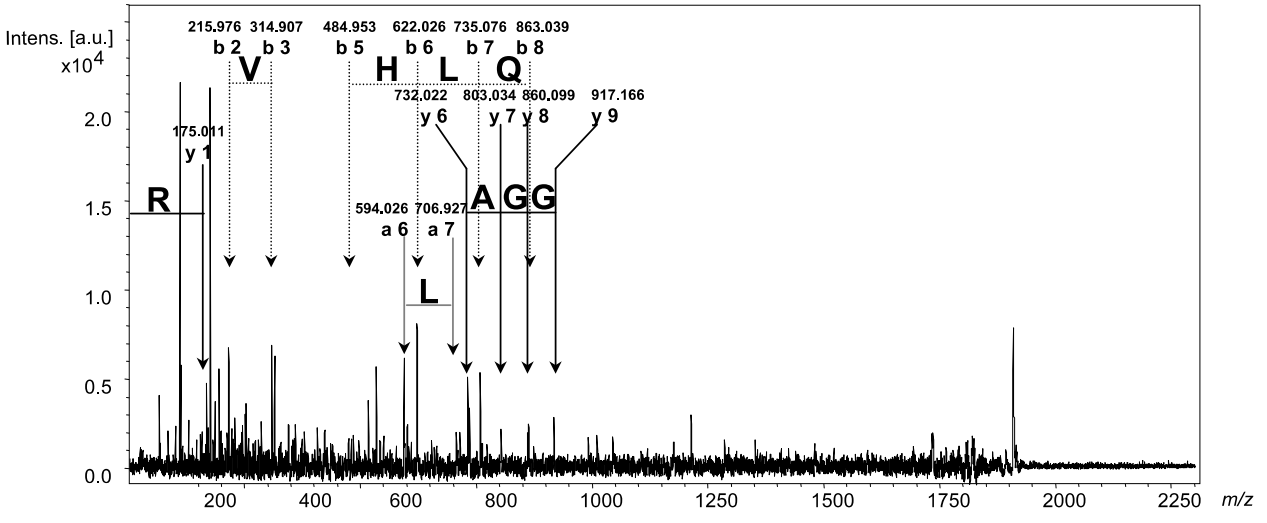
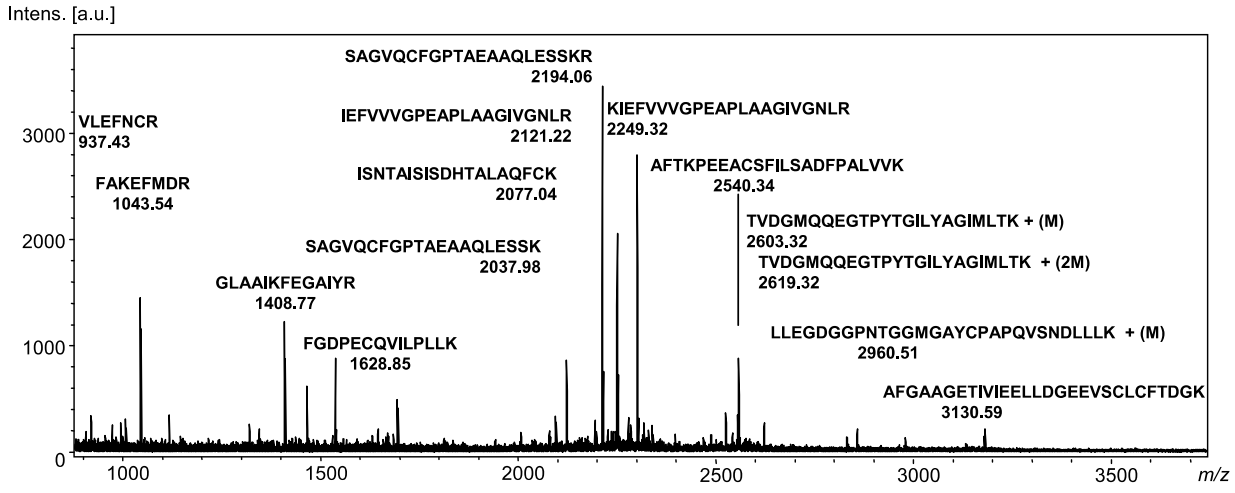
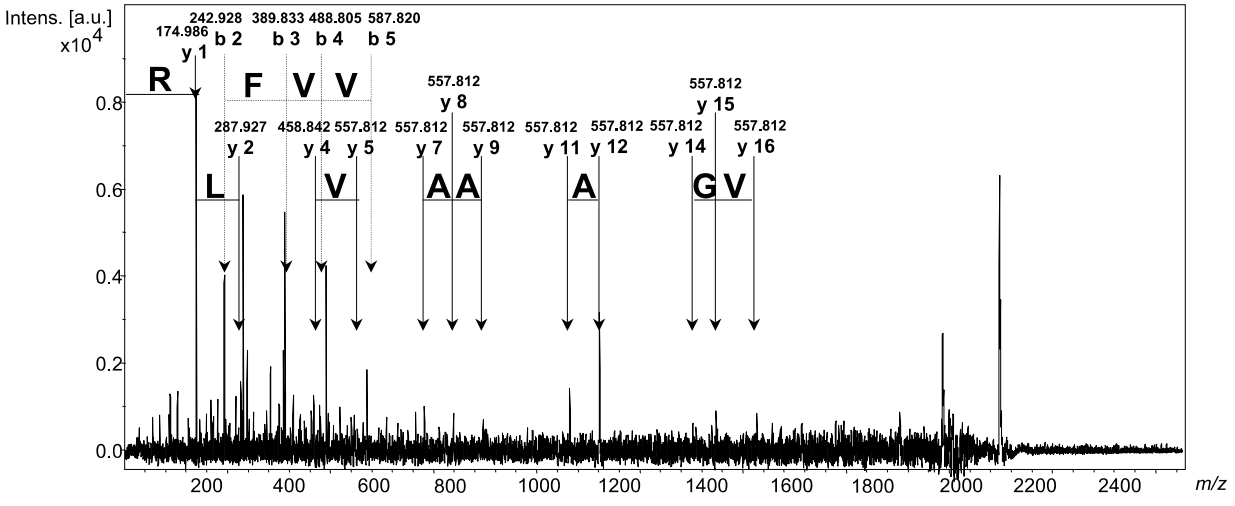


Fig. 4. MALDI-TOF MS and MS/MS analysis of PFKL. For details see legend of Fig. 3



MS/MS spectrum of m/z 2121.20 (IEFVVVGPEAPLAAGIVGNLR)



MS/MS spectrum of m/z 2249.32 (KIEFVVVGPEAPLAAGIVGNLR)

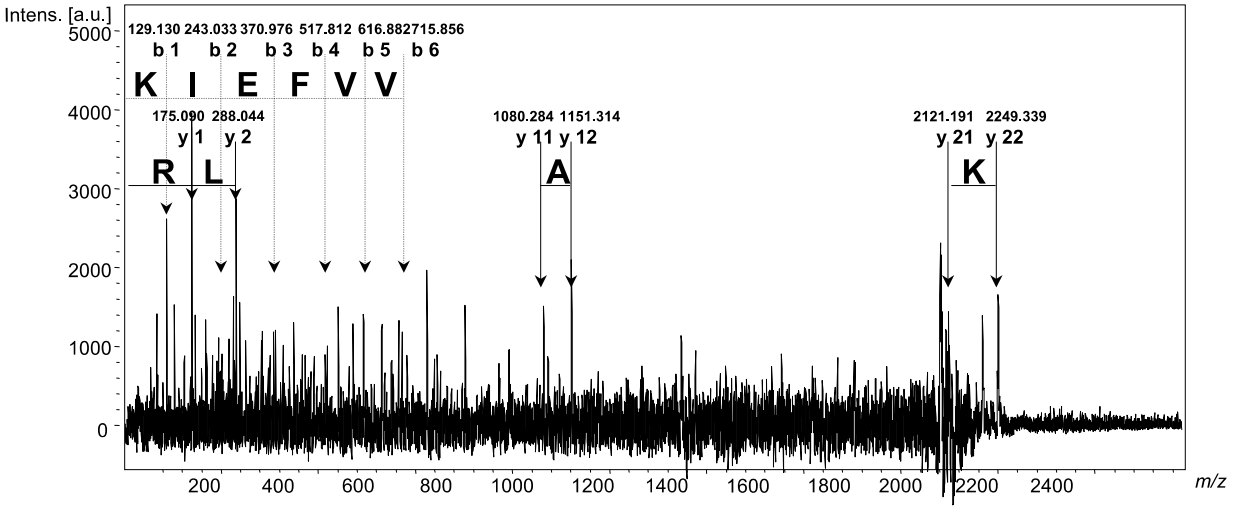


Fig. 5. MALDI-TOF MS and MS/MS analysis of GARS. For details see legend of Fig. 3

The three most abundant peaks were automatically selected for MS/MS acquisition using data-dependent switching, which excludes known background peaks. For example, we obtained three CSTB MS/MS spectra, where the singly charged $[M+H]^+$ protonated molecules with m/z 1123.514, 1422.708, and 2458.273 were selected (Fig. 3). Identification of PFKL and GARS was unambiguously re-evaluated by two MS/MS spectra, respectively (Figs. 4 and 5). MS/MS spectra were obtained with a mass accuracy of 25 ppm and 0 missing cleavage.

The observed fragmentation pathways can be interpreted as y series and b series ions, immonium ions, and amine and water losses. To ensure fair identification, results from both MS and MS/MS spectra were used in the database search. Protein identification was accepted when the score reported by the Mascot search was higher than 61 and confirmed with theoretical and observed MW and pI values.

Discussion

Chromosome 21 is the smallest of the human autosomes constituting approximately 1–1.5% of the haploid genome. The major part of chromosome 21 is the long arm (21q), essential for normal development and function, and harbouring almost all genes of known function, except ribosomal RNA (Epstein et al., 1995). The current human chromosome 21 gene catalogue (Hattori et al., 2000) (http://chr21.molgen.mpg.de) contains 238 entries and 168 cognate mouse orthologues were identified. Among 168 genes, the expression of 158 unique genes (referred to as mmu21 genes) was explored by systematic in situ hybridization in neonatal brain (at postnatal day 2), the most important organ affected in DS. Sixty percentages of the mmu21 genes were expressed in discrete or whole brain sections (Gitton et al., 2002). In another study (Reymond et al., 2002), expression of 161 mouse orthologues was investigated by RT-PCR and 85% of 161 genes were detectable in mouse brain. Although these studies have indicated the expression pattern of mmu21 genes in mouse brain, it was predicted that about 41% of Chr21 genes have no functional attributes (Hattori et al., 2000) and expression was evaluated at RNA level. Therefore, investigation of Chr21Ps expressed in human fetal brain at protein level is a major challenge for DS research.

In the present study, nine Chr21Ps were detected at the protein level by advanced proteomics techniques, IEC and automated MALDI-TOF/TOF with high sensitivity (Fig. 6). Expression of three Chr21Ps (CSTB, PFKL and GARS proteins) was newly observed and those proteins may play an important role in fetal brain development.

CSTB, which was described as a cystein protease inhibitor, is a nonglycosylated protein comprising a single polypeptide chain of 98 amino acids (Jarvinen et al., 1982; Ritonja et al., 1985; Turk et al., 1991). Mutation in the CSTB gene has been shown to cause progressive myoclonus epilepsy (Koskiniemi et al., 1974; Norio et al., 1979; Eldridge et al., 1983). Although CSTB binds tightly to cathepsins B, H, and L, and mice with a gene deletion of CSTB exhibit increased apoptosis of specific neurons (Brannvall et al., 2003), the exact functions of CSTB in the nervous system and the physiological role of CSTB in brain cells are not fully understood. CSTB is present in embryonic and adult neural stem cells (NSC) and in the neuroepithelium (Brannvall et al., 2003). CSTB was expressed in both neurons and glial cells differentiated from NSC and in hippocampal cultures. CSTB localised mainly to the nucleus in NSC and in neurons, whilst in astrocytes and glial cells, CSTB was in the cytoplasm and lysosomes, respectively (Brannvall et al., 2003). The results demonstrate a nuclear expression of CSTB in NSC and in neurons, suggesting a novel function for this molecule. Increased levels of cystatin B protein and the presence of cathepsins in senile plaques have been observed in brains of patients with adult DS and Alzheimer's disease (Lemere et al., 1995). A recent in vitro study has demonstrated that cathepsin B, which is inhibited by cystatin B, induced neuronal apoptosis and caspase 3 activation (Kingham et al., 2001), indicating an important role of cystatin B in blocking apoptosis. In our previous study, protein levels of cystatin B were comparable between control and fetal DS brain (Cheon et al., 2003c). However this result was obtained by an immunochemical method

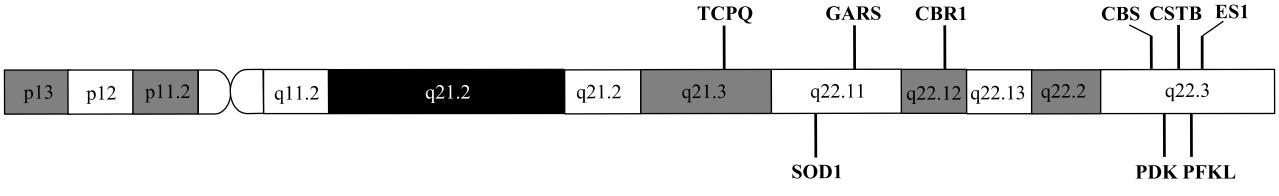


Fig. 6. Gene locus of nine proteins identified on chromosome 21. Gene locus was searched at www.ncbi.nlm.nih.gov/LocusLink/

and is thus reflecting immunochemical reactivity rather than a protein chemical result. Recently, Vitorino et al. (2004) reported uncommon cystatin A and B in saliva by 2-DE and MALDI-TOF and CSTB was represented by one spot.

Increased PFKL activity, a result of gene dosage, was observed in erythrocytes and fibroblasts from Down syndrome patients. In transgenic mice overexpressing murine PFKL (Tg-PFKL mice), PFK specific activity was found to have been almost doubled in embryonic brains but not in adult brains. This pattern of overexpression of PFKL in brains of Tg-PFKL mice suggests that the extra copies of the PFKL gene are expressed during the developmental period and gene-dosage effects may be temporally separated from some of their consequences, adding an additional layer of complexity to the analysis of gene dosage in trisomy 21 (Elson et al., 1994). In addition, Tg-PFKL mice had an abnormal glucose metabolism with reduced clearance rate from blood and enhanced metabolic rate in brain. Tg-PFKL mice exhibited elevated activity of phosphofructokinase in both blood and brain, as compared to control nontransgenic mice. Following glucose infusion, the rate of glucose clearance from the blood of Tg-PFKL mice was significantly slower than that of control nontransgenic mice, although basal blood glucose levels were similar. However, unlike the slower rate of glucose metabolism in blood, the initial rate of glucose utilisation in the brain of the transgenic mice, was 58% faster than in control nontransgenic mice. Faster utilization of glucose in Tg-PFKL brain is similar to the increased rate of cerebral glucose metabolism found in the brains of young adult DS patients, which may play a role in the etiology of their cognitive disabilities (Peled-Kamar et al., 1998).

Purines are critical for energy metabolism, cell signalling, and cell reproduction. In humans, the second, third, and fifth steps of de novo purine biosynthesis are catalysed by a trifunctional protein with GARS, aminoimidazole ribonucleotide synthetase (AIRS), and glycinamide ribonucleotide formyltransferase (GART) enzymatic activities. The gene encoding this trifunctional protein is located on chromosome 21. The human GARS-AIRS-GART gene encodes not only the trifunctional protein with a molecular mass of 110 kDa but also a monofunctional GARS protein with a molecular mass of 50 kDa (Brodsky et al., 1997). In this study, we detected GARS protein showing 50 kDa in 2-DE gel of human fetal brain, while GART showing 110 kDa was observed in a previous study (Fountoulakis et al., 2002). This carboxy-truncated human GARS protein is produced by alternative splicing resulting in the use of a polyadenylation site in the intron between the terminal GARS and the first AIRS exons (Brodsky et al., 1997). The expression of both the GARS and GARS-AIRS-GART proteins are expressed at high levels during normal prenatal cerebellar development, while the GARS and GARS-AIRS-GART proteins become undetectable in this tissue shortly after birth. In contrast, the GARS and GARS-AIRS-GART proteins continue to be expressed during the postnatal development of the cerebellum in individuals with Down syndrome (Brodsky et al., 1997). In our previous study, both GARS and GARS-AIRS-GART proteins were detected by western blot in cerebral cortex of fetal brain and expression levels of both proteins were unchanged in fetal DS brain relative to controls (Cheon et al., 2003b). These results demonstrate that both GARS and GARS-AIRS-GART proteins may play a normal role in the regulation of purine biosynthesis in prenatal stage of fetal DS brain. However, these data need to be evaluated by 2-DE approach for ruling out the bias of immunodetection.

In conclusion, the application of MALDI-TOF/TOF for identification of the Chr21Ps expressed in human fetal brain is a major step forwards. The ability to acquire MS and MS/MS spectra in one run improves the quality of identifications and economises time and sample. With the robotic high-throughput spot picking, digesting, automatic acquisition system and data handling, three Chr21Ps were newly identified and characterised and MS(/MS) analyses of six Chr21Ps were confirmed. Mass spectrometric characterisation of Chr21Ps from 2-DE gels following chromatographic prefractionation may be a useful principle to analyse more Chr21Ps, thus enabling qualitative and quantitative studies on the gene dosage hypothesis for the development of DS brain phenotype at the protein level.

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