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Peptidomics and proteomics studies of transformed lymphocytes from patients mutated for the eukaryotic initiation factor 2B ☆

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Abstract

Mutations in the ubiquitous factor eIF2B involved in protein synthesis and its regulation have been reported in human brain genetic disorders. In order to analyse the functional consequences of the mutations and to find specific biomarkers of eIF2B-related disorders, proteomics and peptidomics studies were performed on lymphoblasts from eIF2B-mutated patients versus healthy patients. Curiously, following two-dimensional gel electrophoresis and mass fingerprints, mutations in the eIF2B complex did not significantly affect the proteome of the mutated lymphoblasts extracts. However, liquid chromatography based peptidomics studies revealed one apparently instable candidate compound in five out of the six mutated lymphoblastoid cell lines investigated.

Keywords: Lymphoblasts; eIF2B-related disorders; CACH/VWM leukodystrophy; eIF2B-pathies; Peptidomics; Proteomics; Differential analyses; Mass spectrometry

1. Introduction

Translation initiation in mammalian cells is a complex process involving a set of proteins, the eukaryotic initiation factors (eIFs). Interestingly, mutations in an eIF protein have been involved in a human genetic disorder. Indeed, mutations in each of the five genes *EIF2B1* to *5*, encoding the five subunits of the eukaryotic initiation factor 2B (eIF2B), have been reported in patients presenting with a degenerative disorder of the brain white matter, the childhood ataxia with central hypomyelination (CACH) syndrome, described also as leukoencephalopathy with vanishing white matter (VWM) [1–3]. The pentameric eIF2B complex is ubiquitously expressed and is implied in the first step of protein synthesis. It converts the initiation factor 2 (eIF2) from an inactive GDP-bound form to an active eIF2-GTP complex owing to its Guanine nucleotide exchange factor (GEF) activity and then catalyses the recycling of eIF2 between consecutive rounds of peptide-chain initiation [4,5]. This recycling step is a key regulatory point in the initiation of messenger RNA translation, allowing the formation of the 43S preinitiation complex, a precursor of the active 80S ribosome-mRNA complex. Regeneration of active eIF2 by eIF2B is essential for constant protein synthesis and this step is regulated under a variety of physiological conditions [4,5]. Indeed, the eIF2B GEF activity is decreased under cellular stresses, such as nutritional starvation, viral infection and/or hyperthermia. One consequence is the reduction of protein synthesis rate to avoid large intracellular accumulation of denatured/dysfunctional proteins. This mechanism is conserved from yeast to human and involves the activation of specific protein kinases that phosphorylate the alpha subunit of eIF2 [5]. This leads to the alteration of translation initiation rate [6]. Interestingly, under these conditions of general translation attenuation due to eIF2B dysfunction, the translation of certain mRNA molecules, such as mammalian activating transcription factor 4 (ATF4) is specifically induced leading to the activation of transcription factors and to the expression of numerous downstream genes. These genes encode series of proteins that allow

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cells to adapt appropriately to stress conditions [6]. Therefore, eIF2B is a key control complex for protein synthesis in response to cellular stresses and its regulation represents a major protective mechanism for the cell [7].

Curiously, disruption of the ubiquitously expressed eIF2B causes mainly a brain disease. The CACH/VWM leukodystrophy is an orphan disorder with worldwide 113 families described to date [1–3]. It is characterized by a cerebrospinal fluid-like white matter by cerebral magnetic resonance imaging (MRI). Classically, it is clinically characterised by a neurological deterioration between the age of 2–5 years with progressive gait difficulties and a relatively mild cognitive impairment. Additional episodes of acute deterioration after febrile infections or minor head trauma often occurred with subsequent partial recovery leading to death usually within 2–5 years of disease evolution [1,3]. Nevertheless, the use of MRI and *EIF2B* genes sequencing demonstrated the wider clinical spectrum of patients with eIF2B mutations: from rapidly fatal infantile forms to asymptomatic adult forms. This leads to the concept of eIF2B-related disorders (or eIF2B-pathies) with a wide *continuum* of clinical severity that is correlated to age at disease onset [1].

To date, diagnosis of eIF2B-pathies relies on clinical and MRI criteria and is confirmed by *EIF2B* mutations. Recently, the cerebrospinal fluid (CSF) from eIF2B-mutated patients was found to have a deficiency of the asialo form of transferrin usually present in CSF from healthy individuals [8].

eIF2B-mutated Epstein-Barr virus (EBV) transformed lymphocytes (or lymphoblasts) from affected patients exhibit a decrease in eIF2B GEF activity in correlation with disease severity, as well as for cells under a cellular stress [9]. Interestingly, eIF2B mutations were also found to reduce GEF activity in yeast [10] and in human embryonic kidney epithelial cell lines [11], as a consequence of changes that lead to complex instability and/or that prevent a particular subunit from binding to the others to form the holoenzyme. Furthermore, although disruption of eIF2B causes mainly a brain disease, its dysfunction can be measured in non-neural cells, such as transformed lymphocytes. These cells constitute suitable models for biochemical investigations and biomedical applications as they are stable, easy to amplify and to preserve on the long term [3,9]. Nevertheless, they must be regarded as a raw biological material of extreme complexity, particularly rich in proteins, polypeptides and peptides that are all present at different concentrations.

The measurement of protein/peptide expression is essential to analyse biological processes in normal and pathological conditions. In contrast with the genome, the proteome/peptidome is dynamic and constantly changing. Elucidating how the proteins/peptides complement changes in a cell type in diseases is crucial to understand how these processes occur at a molecular level. The key element of the classical proteome/peptidome research combines the multidimensional separation of polypeptides/proteins by two-dimensional gel electrophoresis (2-DE) [12] or by multidimensional gel electrophoresis [13] from a complex mixture and their identification by mass spectrometry (MS) [14].

Proteome studies using 2-DE have been recently performed on lymphoblast extracts. Joubert-Caron et al. constructed annotated reference 2-DE maps for human lymphoblast proteins (available at http://www-smbh.univ-paris13.fr/lbtp/Biochemistry/Biochimie/bquelympho.htm) and identified 40 different cellular soluble proteins by either mass fingerprinting or tandem MS [15,16]. Toda et al. also used 2-DE maps to identify proteins in human lymphoblast extracts and to analyse molecular mechanisms of immortalisation [17,18]. Whereas several proteomes have been investigated on extract from lymphoblasts, to date, no differential analyses were performed on specific peptidome (peptides and polypeptides <30 kDa) of these cells.

The peptides and/or proteins discriminating the eIF2B-mutated lymphoblasts from control ones may therefore represent potential biomarkers for the diagnosis and prognosis of the disease and may help to elucidate physio-pathological mechanisms of eIF2B-related disorders.

In order to analyse the functional consequences of eIF2B mutations and to find specific biomarkers of eIF2B-pathies, we investigated protein and peptide profile alterations in lymphoblasts from eIF2B-mutated patients in comparison to healthy patients. As eIF2B is a highly conserved ubiquitous factor involved in protein synthesis, and as its mutations lead to the decrease of its intrinsic GEF activity in lymphoblasts from affected patients, we hypothesized that quantitative and qualitative changes may occur at the protein and peptide levels in these cells.

2. Experimental

2.1. Chemicals

Acetonitrile, acetone and acetic acid (all HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and trifluoroacetic acid (TFA, HPLC-grade) from Pierce (Rockford, IL, USA). Deionised water was purified using a Milli-Q system (Millipore, Milford, MA, USA). All other solvents or reagents were of analytical grade or better.

Materials for the first and second dimension of 2-DE were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and from Bio-Rad (Richmond, CA, USA). Acrylamide, ammonium persulfate, SDS, Tris, urea, CHAPS, 1,4-dithioerythritol (DTE), iodoacetamide, ammonium hydroxide and glycine were from ICN (Eschwege, Germany); piperazine diacrylamide from Bio-Rad; TEMED and Pharmalyte™ pH 3–10 from Amersham Pharmacia Biotech; Servalyte® pH 4–7 from Serva (Heidelberg, Germany); formaldehyde, glutaraldehyde, silver nitrate, natrium sulfate, citric acid were from Fluka (Buchs, Switzerland); ammonia solution and sodium acetate from Merck, and 2,7-naphtalenedisulfonic acid from Brunschwig (Basel, Switzerland).

2.2. Biological materials

2.2.1. Culture of human lymphoblasts

A written consent was obtained from each of the 12 patients. Blood samples (10 mL) from six mutated and affected CACH/VWM patients and six age and sex-matched healthy patients were collected (Table 1). Lymphocytes were isolated

Table 1
Affected eIF2B-mutated patients and associated healthy patients codes and their GEF eIF2B activity measured in lymphoblasts [9]

eIF2B-mutated patients		Associated healthy control patients (age- and sex-matched to affected patients)	
Patients code	GEF eIF2B activity in lymphoblasts (%)	Patients code	GEF eIF2B activity in lymphoblasts
G359	54 ± 6	G786-2	Normal
G291	41.5 ± 6	G662-4	Normal
G76-2	67 ± 5.1	G978-4	Normal
G1036	30 ± 7.5	G812-1	Normal
G370-2	77 ± 2.5	G987-3	Normal
G571-2	50 ± 5	G396-1	Normal

according to standard procedures [19]. In order to have sufficient quantities of cells, lymphoblastoid cell lines were obtained by transforming lymphocytes with the EBV according to classical procedures [19]. Lymphoblast cultures were grown in RPMI 1640 medium supplemented with 10% heat-treated fetal calf serum, 100 UI/mL streptomycin B, 2.5 μg/mL amphotericin B, 0.1 UI/mL penicillin and 4 mM glutamine in humidified atmosphere of 5% CO_2 in air, at 37 °C in 75 cm^2 flasks. Cell culture reagents were from Invitrogen Gibco (Grand Island, NY, USA). All studies were performed in duplicate.

2.2.2. *Lymphoblasts extraction for proteomics and peptidomics*

For proteomics studies, the cellular proteins (1×10^6 cells, counted on Malassez cells) were solubilised in a test tube containing 80 μL of a solution composed of 8 M urea, 4% CHAPS, 40 mM Tris–HCl, 65 mM DTE and endonuclease (5 U, Sigma–Aldrich, Saint-Louis, MO, USA).

For peptidomics analyses, 5×10^7 lymphoblasts were centrifuged (5 min at 2500 rpm, room temperature) and washed five times with 10 mL PBS in order to avoid any contamination with components of the culture medium. Four different extraction buffers were tested on control patients: (i) homogenisation in 1 mL of buffer HB used to measure the eIF2B activity on lymphoblast extracts (HB: 45 mM HEPES, pH 7.4, 375 μM magnesium acetate, 75 μM EDTA, 95 mM potassium acetate, 2.5 mg/mL digitonin, microcystin and 10% (v/v) glycerol) [9]; (ii) homogenisation in 1 mL HB and sonication (three cycles of 20 s each); (iii) sonication (three cycles of 20 s each) alone in 1 mL PBS; (iv) homogenisation and sonication as before in 1 mL of 2 M acetic acid instead of PBS. Finally, the cell homogenates were extracted 10 min for (i)–(iii) and 45 min for (iv) at 4 °C under gentle stirring, and centrifuged 10 min at 4 °C and 13200 rpm. Total proteins were quantified (Hitachi clinical analyzer 7180, Tokyo, Japan; standard: human serum albumin, pyrogallol red technique) and supernatants were frozen at −80 °C until use.

2.3. *Differential proteomics analyses: sample preparation, 2-DE and image analyses*

The proteic extract was subjected to isoelectric focusing (IEF) that was performed under paraffin oil, using linear immobilised pH gradients (Immobiline Dry-Strip, pH range 4–7, 18 cm from Amersham Pharmacia). The strips were rehydrated overnight in 340 μL of a solution containing 8 M urea, 2% CHAPS, 10 mM DTE, 2% Pharmalyte™ pH 3–10, 1% Servalyte® pH 4–7 and traces of bromophenol blue. Sample (50 μg) was loaded on the cathodic side of the gels. The voltage was progressively increased from 300 to 3000 V during the first 3 h, followed by 1 h at 3500 V and finally stabilized at 5000 V, for a total of 100 kVh. Before the second dimension, strips were equilibrated in a solution containing 6 M urea, 50 mM Tris–HCl, 30% glycerol, 2% SDS, 2% DTE, for 12 min, and then in the same solution containing traces of bromophenol blue and 2.5% iodoacetamide instead of DTE, for 5 min. Strips were placed on the top of 9–16% gradient polyacrylamide second dimensional gels that were copolymerized with piperazine diacrylamide as a cross-linker. The migration was performed with a current of 40 mA per gel.

Silver staining was done according to standard protocols [20]. Briefly, at the end of the run, the gels were washed in deionised water, then soaked in a mixture of ethanol:acetic acid:water (40:10:50, v/v/v) for 1 h and in ethanol:acetic acid:water (5:5:90, v/v/v) overnight. After a wash with water, the gels were soaked 30 min in 1% glutaraldehyde buffered with 0.5 M sodium acetate and the glutaraldehyde was removed by deionised water washes. The gels were then soaked twice in a 2,7-naphtalenedisulfonic acid fresh solution (0.05%, w/v) for 30 min and rinsed again with deionised water. The gels were stained in a freshly made ammoniacal silver nitrate solution for 30 min and then rinsed with deionised water. The images were finally developed in a solution containing citric acid (0.01%, w/v) and formaldehyde (0.1%, w/v). Development was stopped with an acetic acid:water (5:95, v/v) solution. All incubations were performed on an orbital shaker.

Bioinformatic analyses of gel images have been described in details elsewhere [21,22]. The silver stained gel images were captured with high-resolution densitometry reading with a "Personal Laser Densitometer" (Amersham Pharmacia Biotech). All informatic analyses were performed using the software ImageMaster 2DPlatinum (Amersham Pharmacia Biotech) and spots were detected automatically. A manual spot editing (separation of two spots) or deleting (artifacts) was performed when necessary. Alignment and matching of the spots were performed by choosing one gel as a reference and manually selecting several common spots as landmarks. The landmarks were selected in order to be spread out through the studied areas. Heuristic clustering analysis was performed using a previously described algorithm [23]. For given sets of 2-DE images, a heuristic clus-

tering automatically forms classes and highlights the significant differences between the various classes. Finally, eventual statistical significant differences between the percentage volumes of each matched spots were tested using *t*-test.

2.4. Differential peptide profiling

2.4.1. Solid phase extraction (SPE)

Following acidification with 50 volumes of 0.1% TFA (acidified water); all samples were centrifuged at room temperature during 10 min at 13,200 rpm and supernatants were immediately subjected to SPE.

The acidified extracts were loaded onto two serially linked Sep-Pak C_{18} classic cartridges (Waters, Milford, MA, USA) equilibrated in acidified water. The proteins and peptides of interest were eluted from the cartridge with 60% acetonitrile in acidified water. The 60% Sep-Pak fraction (SPE extract) was freeze-dried in a Savant SC210A Speed-Vac Plus concentrator (GMI, Ramsey, MN, USA) and then reconstituted with 50 μL Milli-Q water and stored at −25 °C.

2.4.2. RP-HPLC profiling of lymphoblast extracts

The RP-HPLC runs were carried out at 30 °C on an Alliance 2690 system from Waters coupled to a 996 Photodiode array detector (Waters). The separation was performed on an analytical C_{18} reversed-phase column (Vydac™, 218TP54, 4.6 mm × 250 mm, protein–peptide C_{18}, 5 μm, Vydac Mojave, CA, USA). An equivalent of 530 μg of protein, diluted in acidified water, was injected. The SPE extract was purified using a linear gradient (0.6% per min increase) of acetonitrile in acidified water at a flow rate of 0.8 mL/min, where solvent A was acidified water and solvent B was 90% acetonitrile in acidified water. The column effluent was monitored for absorbance at 225 nm and the fractions were hand-collected following optical density, freeze-dried, re-suspended in 50 μL of Milli-Q water and stored at −25 °C until use.

2.4.3. Purification of the potential biomarker (MK1)

A second purification step was performed with a biphasic linear gradient from 2% to 20% acetonitrile in acidified water over 10 min and from 20% to 32% in 70 min (0.17% increase per min). RP-HPLC runs were carried as above. The separation was performed on an analytical C_{18} reversed-phase column (Symmetry™, 4.6 mm × 250 mm, 3.5 μm, Waters, Milford, USA). The column effluent was monitored for absorbance at 214 nm and the fractions were hand-collected following optical density, freeze-dried under vacuum in a Savant SC210A Speed Vac Plus (GMI, Ramsey, MN, USA) and stored at −25 °C until use.

2.5. MS analyses

2.5.1. MALDI-TOF-MS

Two sample preparations were tested on 100-well stainless steel plates (Applied Biosystems, Foster City, CA, USA): the dried-droplet technique and the sandwich method. The dried-droplet technique requires the mixture of the sample within the matrix solution: a 10-fold dilution of the pre-purified sample in saturated matrix (α-cyano-4-hydroxycinnamic acid (4-HCCA) or sinapinic acid (SA) at the concentration of 10 mg/mL in 27% acetonitrile in acidified water) was performed and 1 μL of the diluted sample was deposited on the sample plate. The sandwich method is derived from the fast-evaporation and overlayer methods [24]. It consists of the deposition of 0.5 μL of a saturated solution of 4-HCCA in acetone (Sigma–Aldrich) allowed to dry on stainless steel target plate, when dried 0.5 μL 0.1% TFA was deposited on the crystallised matrix bed, followed by the deposition of 0.5 μL of sample, and of 0.5 μL of a saturated solution of 4-HCCA in 50% acetonitrile prepared in acidified water.

Analyses were performed on a Voyager-DE™ STR biospectrometer (Applied Biosystems) with delayed extraction technology. All analyses were processed using the positive and linear mode coupled to the delayed extraction method. External calibration of the mass scale was performed with a mixture of bovine pancreas insulin (Sigma–Aldrich) with signals at *m/z* 5734.57 (M^+) and *m/z* 2867.78 (M^{2+}) and horse heart myoglobin (Sigma–Aldrich) with signals at *m/z* 16952.5 (M^+) and *m/z* 8476.7 (M^{2+}). Data were processed with Data Explorer™ Version 3.5 software (Applied Biosystems).

2.5.2. ESI-MS

Analyses were performed on a Platform LCZ single quadrupole electrospray instrument (Micromass Ltd., Manchester, UK). The samples were infused in 50% acetonitrile in 0.2% formic acid at a flow rate of 20 μL/min. The cone voltage was set to 20 V and analyses were performed by cumulating 10–15 scans in positive and negative ionisation modes with a scan range of *m/z* 50–1250 over 4 s. Data were acquired and analysed using the MassLynx version 3.5 software (Micromass Ltd.). External calibration of the mass analyser was performed with a mixture of sodium and caesium iodide salts covering the mass range considered, namely 50–1250 amu.

3. Results/discussion

Our aim was to develop a methodology adapted to the detection of peptides/proteins in order to identify putative biomarkers of eIF2B-pathies. As these diseases are linked to mutations in eIF2B, a ubiquitous initiating factor involved in the first step of protein synthesis, we favoured a differential display analysis to identify proteins or peptides that are up- or down-regulated in a disease-specific manner. Selected biological material had to be available in sufficient quantities for such studies; we therefore chose transformed lymphocytes collected from affected patients and healthy matched individuals. This strategy was operated by 2-DE for the identification of large and medium size proteins and by a more sensitive peptidomic technology based on liquid chromatography to analyse peptides and polypeptides. For peptides and polypeptides below 30 kDa, we performed the differential analysis by mass spectrometry and off-line HPLC. Four human eIF2B-mutated lymphoblast extracts and four age- and sex-matched selected control patients were investigated in duplicate for proteome versus six couples for peptidome.

3.1. Differential proteomic analysis

Sixteen 2-DE gels were analysed, corresponding to four affected patients and four healthy (control) individuals, each sample being analysed in duplicates.

The global protein pattern of controls (Fig. 1A and B) as well as that of affected patients (Fig. 1C and D) did not differ from one another, and was similar to the 2-DE map of cultured lymphoblasts, available on the Internet (http://www-smbh.univ-paris13.fr/lbtp/Biochemistry/Biochimie/bquelympho.htm), or to the 2-DE map of human lymphocytes from blood donors [25]. These negative results probably reflect that the electrophoretic patterns of lymphoblats as well as of freshly collected lymphocytes are mainly related to the most abundant and soluble proteins. A systematic comparison, by heuristic clustering analysis, of 2-DE gels of cultured lymphoblasts and of normal lymphocytes was not done, because cell culture readily influence the protein pattern [22].

The resolution is good meaning that the solubilisation of proteins was efficient and that the correct amount was loaded. The optimisation of sample preparation led to 16 well-resolved silver stained 2-DE gels showing an average of 2793 ± 311 spots (mean $\pm$ S.D.) with molecular masses between 10 and 200 kDa and within a 4–7 pH range. No statistically significant difference was observed between the number of spots detected in controls (2964 ± 270; mean $\pm$ S.D.) with the one in patients with eIF2B-pathies (2623 ± 261; mean $\pm$ S.D.). From all these spots, 1611 ± 187 (mean $\pm$ S.D.) matched together. The synthetic gel of the controls (1301 spots) was matched with the one of the affected patients (1339 spots), giving rise to the master gel (1710 spots). The matching of the master gel (1355 ± 108; mean $\pm$ S.D.) with all the gels did not reveal sig-

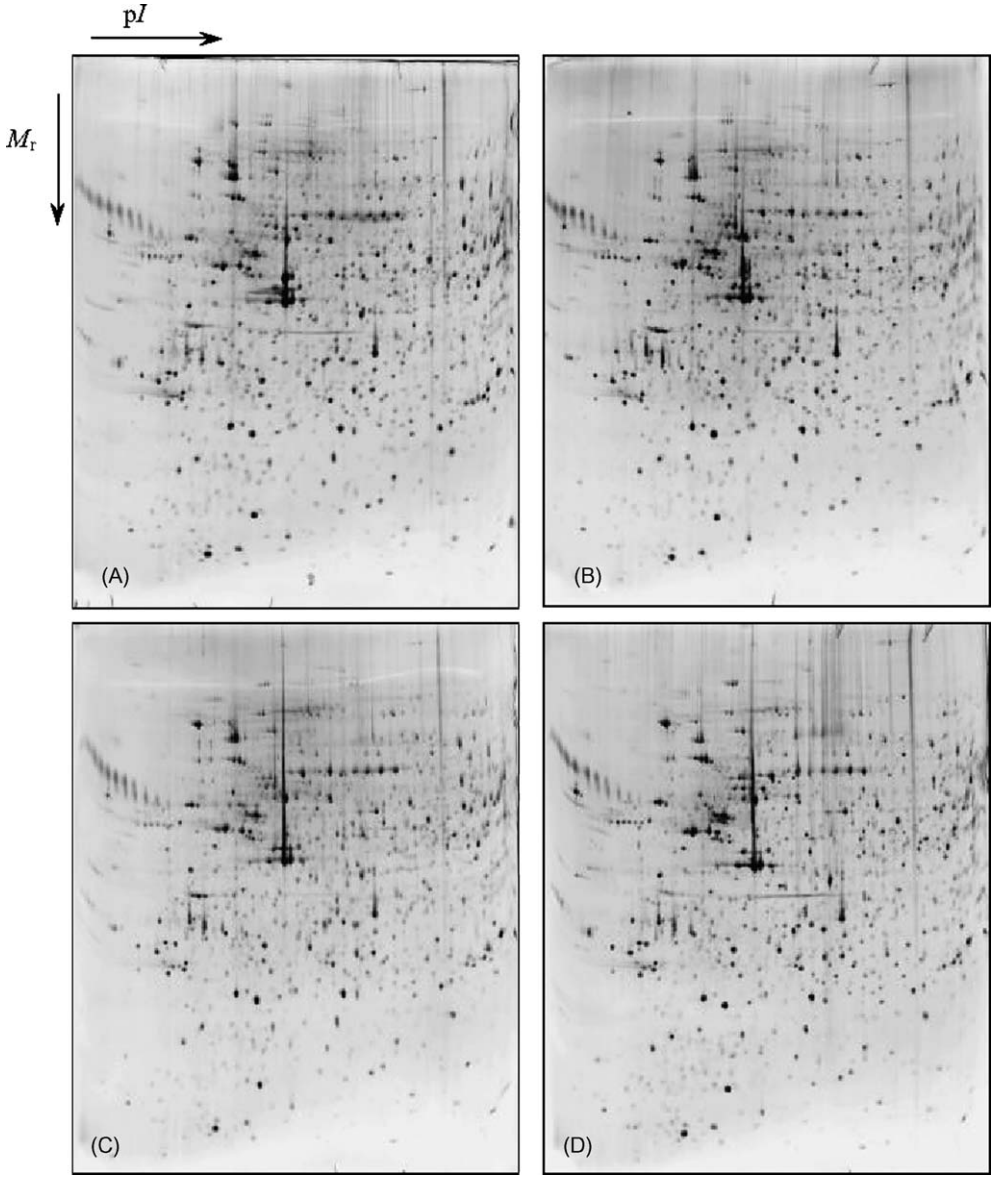

Fig. 1. High-resolution silver stained 2-DE gels of lymphoblast extracts from two healthy patients (panels A and B), and from two individuals with eIF2B-pathies (panels C and D). First dimension, isoelectric focusing (pH 4–7 gradient); second dimension, SDS-PAGE (9–16% polyacrylamide gels, resolved range: 10–200 kDa).

nificant differences between the two types of patients. Based on these results, it was not possible to differentiate gels from controls to those of patients with eIF2B-pathies by heuristic analyses. Taken together, bioinformatic analysis of the sets of 2-DE gels showed no significant differences between eIF2B-mutated lymphoblasts from affected patients and lymphoblasts from corresponding controls. The negative results obtained by 2-DE analysis of control lymphoblasts as well as in cultured lymphoblasts from affected individuals certainly reinforce the impression that eIF2B deficiency is not responsible for major alteration of soluble protein synthesis (at least in cells after culture).

3.2. Differential peptidomic analysis

3.2.1. Optimisation of the lymphoblast extraction

To get the most representative and exhaustive lymphoblast peptidome, we tested four different conditions of extraction by changing the extracting buffer (HB, PBS and 2 M acetic acid) and by inserting or not a sonication step. The lymphoblast pellets were subjected to the four different extraction procedures in parallel. The optimal conditions for the extraction of the peptides/polypeptides were determined through a three-step procedure. First mass fingerprints of the crude extracts were performed by MALDI-TOF-MS analysis using the two matrixes 4-HCCA that favoured the detection of peptides below 10 kDa and SA for larger molecules with the two deposition methods reported in Section 2. Second, the peptidic extracts were pre-purified by SPE and the SPE extracts subjected to RP-HPLC. Finally, molecular mass spectra of some of the individual collected fractions were recorded by MALDI-TOF-MS. Analyses of 10-time serial dilutions (10, 100 and 1000 times) were performed on the crude fractions of control G786-2 (see Table 1) by MALDI-TOF-MS. The best mass spectra, in term of peptidome complexity, were acquired on lymphoblast extracted with 2 M acetic acid at a 10-fold dilution and with 4-HCCA as matrix when using the sandwich method (data not shown). Indeed, when analysing the HB extracts, sonication appeared to be essential but most of the masses recorded were found to correspond to components of the buffer that deeply interfered with the MS analyses. Following this observation, we tested more simple media, such as PBS and 2 M acetic acid for the extraction of peptides with sonication. Indubitably, the most complex mass spectra were acquired with extracts performed in the acidic condition (data not shown). In fact, the direct analysis of the peptidic extracts from the lymphoblasts by MALDI-TOF-MS using these optimal conditions outlined the complexity of the extracts (more than 100 compounds detected). However, at this stage no clear difference was evidenced between control and mutated cells. This was confirmed through analysing each of the four pre-purified extracts by RP-HPLC (Fig. 2). The comparison of the four chromatograms obtained evidenced that among the four extraction methods, the most efficient procedure (i.e. the one having the most complex peptidome) is acetic acid with sonication. Finally, this observation was confirmed by analysing some of the isolated RP-fractions by MALDI-TOF-MS using the two MALDI sample preparations tested on the four crude extracts

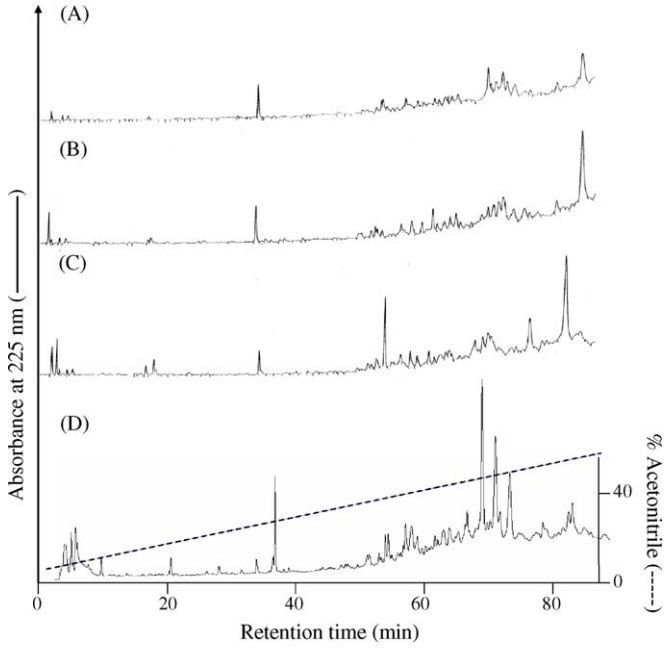

Fig. 2. Differential analysis by RP-HPLC of lymphoblast extracts from the control patient G786-2. Four extraction procedures were performed: (A) homogenisation in PBS plus sonication, (B) homogenisation in HB, (C) homogenisation in HB plus sonication and (D) homogenisation in 2 M acetic acid plus sonication. Only the 60% acetonitrile fractions obtained after SPE were analysed by RP-HPLC. Absorbance was measured at 225 nm and the broken line stands for the acetonitrile gradient in % used for RP-HPLC.

(data not shown). As already observed for the crude extracts, for a further selection by mass fingerprinting of a peptidic marker of eIF2B-pathies, the sandwich sample preparation was found to be the one resulting in the highest number of interesting masses detected in lymphoblast extracts subjected to RP-HPLC profiling. In order to have the most resolved peptidome for marker identification, RP-HPLC was performed on each lymphoblast extract in duplicate after SPE (a total of 24 samples) using a linear gradient of acetonitrile.

3.2.2. Differential analysis by RP-HPLC and MALDI-TOF-MS mass fingerpring

The chromatograms obtained for each couple were compared to detect differences between mutated and control lymphoblasts. From the six selected couples, qualitative and quantitative differences were observed but in most of the cases the differences visualised did not discriminate the mutated cells from the control lymphoblasts. Detailed analyses of such differences evidenced that a large part of them are likely to result from variations between individuals (a same mass variation is generally not found in two different couples) as exemplified in Fig. 3 (inserts C and D). Moreover, analysis of duplicates from identical cell cultures extracted at different times minimised certain differences, suggesting that the culture duration could induce some variations on the lymphoblast peptidome. Surprisingly, as already observed for the proteome performed by 2-DE differential display, very few differences were noticed in the peptidome between control and mutated cells for each couple and also between the group of control and the group of eIF2B-mutated cells.

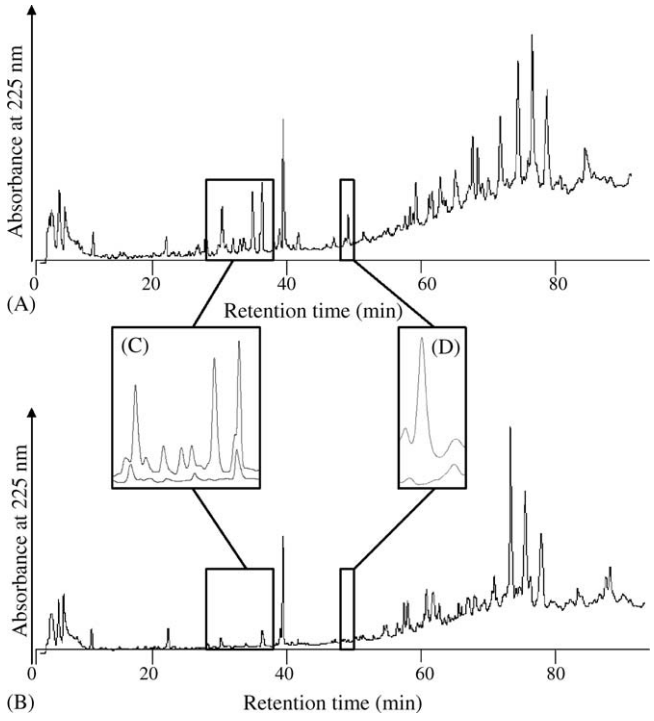

Fig. 3. Differential analysis by RP-HPLC of lymphoblast extracts from patients (A) G359 (affected) and (B) G786-2 (healthy). (C and D) Some of the inter-individual variations observed between these two patients.

To improve the resolution of our analysis, all the hand-collected fractions were subjected to MALDI-TOF-MS measurement. Using this approach, more than 300 compounds per extract were identified by their molecular masses.

We performed for each couple a differential analysis, comparing all the masses found in RP-HPLC fractions from mutated lymphoblasts to their associated controls. This study revealed the complexity of these extracts with an average of 338.3 ± 11.5 (average $\pm$ S.D.) compounds identified per sample by their molecular masses. When comparing the molecular masses within a couple, some differences were observed while extending our comparison from couple to couple these differences appeared non-specific to the pathology. None of these variations were recovered in common in two different couples. This suggests that the variations observed within a couple are associated to individual variations and not to the pathology. Similar results had been reported recently on the presence of many inter-individual variations in eIF2B-mutated lymphoblasts [26]. Summarising these results for each group of six healthy patients versus affected individuals, the different compounds could be tentatively classified according to their molecular masses in three classes. The majority of the compounds (92%) has molecular masses below 20 kDa with 41% masses detected below 10 kDa and 51% between 10 and 20 kDa (Fig. 4). Nevertheless, one area of the chromatograms revealing a potential profile (marked MK1) in correlation with the physio-pathology process involved in eIF2B-pathies was found in the lymphoblast extracts of five affected patients out of the six investigated compared to the control ones (Fig. 5, insert C, MK1). Lymphoblast extract from patient G1036, who exhibits the lowest eIF2B GEF activity (Table 1) and the most severe phenotype, is the only one for which MK1 was not detected on the RP-HPLC profiles. Its eIF2B residual activity is of $30 \pm 7.5\%$, whereas the other lymphoblasts extracts exhibits an average eIF2B activity between 77% and 41.5%. This could suggest that MK1 is involved in a mechanism depending on disease severity, which is correlated to the decrease of eIF2B activity.

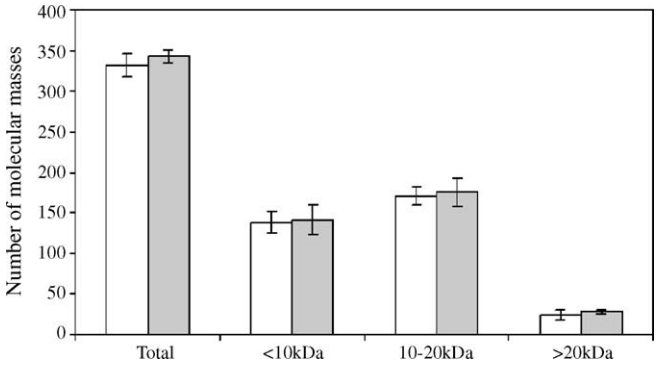

Fig. 4. Number of molecular masses detected during the peptidomics study of lymphoblast extracts of the six patients with eIF2B-pathies (in grey) in comparison to the six associated healthy patients (in white). Total mass numbers and three ranges of masses (<10 kDa, 10–20 kDa and >20 kDa) with S.D. are presented here.

3.2.3. *Delineation of MK1, an eIF2B-mutated profile*

The fractions constituting the area eluting at approximately 25% acetonitrile in lymphoblast extracts from patients with eIF2B-pathies and absent or with a weak intensity in control cells (Fig. 5, insert C) were pooled and subjected to further anal-

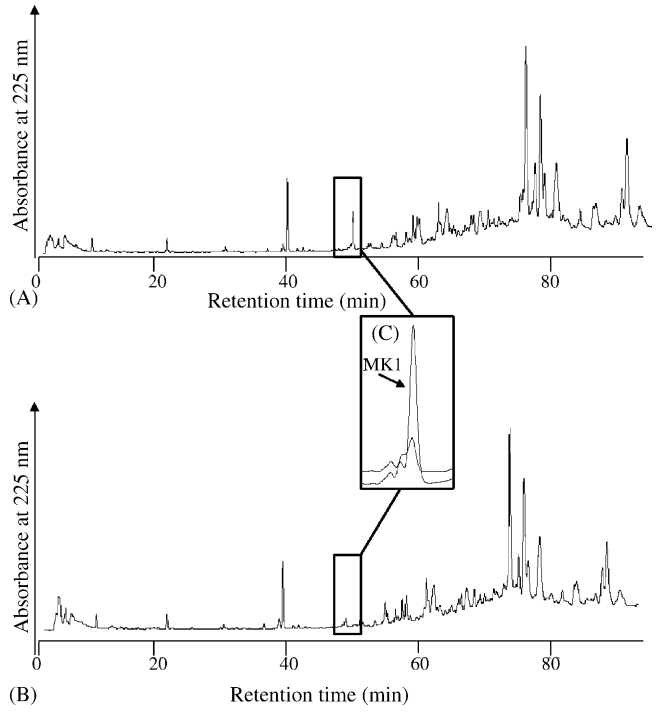

Fig. 5. Differential analysis by RP-HPLC of lymphoblast extracts from patients (A) G370-2 (affected) and (B) G987-3 (healthy). (C) Enlarged zone containing peak MK1, present in mutated lymphoblasts and absent in control ones.

ysis. In order to isolate the component(s) of interest, this fraction was sub-fractioned using a more resolutive gradient (see Section 2) and the corresponding areas from healthy patients were fractionated using the same procedure. Following purification (data not shown), the fraction MK1, found to be present only in the samples originating from patients, was subjected to MALDI-TOF-MS and ESI-MS analyses. By MALDI-TOF-MS, no clear *m/z* signal could be attributed to the peak induced in affected patients and detected at 214 and 225 nm. When MK1 was subjected to ESI-MS analysis in positive mode, only three minor signals at *m/z* 377.32, 461.31 and 529.20 could be attributed to components detectable only in affected patients (data not shown). No clear signal could be detected using negative ionisation mode, whatever the fraction considered. In addition, we measured the absorbance of MK1 between 200 and 390 nm and evidenced high absorbance values at 227.9 and 276.5 nm (data not shown), suggesting a non-peptidic or an unusual peptidic structure for MK1. Finally, when studying duplicates, we observed that MK1was not systematically found on RP-HPLC profiles at the expected intensity when compared to our initial studies. This was specially the case when the acidic extracts were stored for more than one month at −80 °C. These observations strongly suggest that MK1 may be an unstable modified peptide or a non-peptidic component sensitive to acidic conditions.

The structural elucidation of these molecules will require other strategies. Nevertheless, to perform such experiments, a new preparation of MK1 in satisfactory conditions for preserving its integrity will have to be performed.

In addition, as this variation was not observed in all mutated cell lines, lymphoblasts from 30 other eIF2B-mutated patients will be tested for the presence of MK1 profile in comparison with healthy patients and patients affected with other neurodegenerative disorders. This should help us in validating this (these) putative marker(s) as specific biomarker(s) of eIF2B-pathies.

4. Conclusion

The aim of this study was to better understand the functional consequences of eIF2B mutations and to search a proteomic and/or peptidomic biomarker in lymphoblast cell lines for eIF2B-pathies. This is the first time that such analyses were performed to study eIF2B-related disorders. Our results confirm the feasibility of the differential analyses on lymphoblast extracts in a peptidomics and proteomics point of view in parallel. Curiously, the mutations in the eIF2B complex, which is involved in the protein synthesis, did not strongly affect the peptidome and the proteome of the mutated lymphoblast extracts. Only one apparently instable differential compound was found by the peptidomics study, in five out of the six mutated lymphoblastoid cell lines considered, and it may even not be of peptidic structure. This has to be confirmed through elucidating the structure of this molecule. In addition, we will have to increase the sampling to minimise the false positive linked to inter-individual variations in the peptidic and proteic lymphoblast content, as already suggested [26]. Following this first global study, we will evaluate the presence of alternative changes, such as minute quantitative variations or tiny changes in post-translational modifications (phosphorylation, glycosylation, etc.), differences that would have been difficult to detect in our first approach. For reasons that remain unknown, the central white matter appears to be selectively affected in eIF2B-related disorders, although it has been shown recently that involvement of ovaries, liver, kidney or pancreas may accompany the main symptoms [3]. We suppose that the eIF2B defect must involve compensatory mechanisms in non-affected cells including the lymphoblast model.

Furthermore, Elroy-Stein and co-workers recently reported that eIF2B-mutated fibroblasts developed a heightened stress response following an endoplasmic reticulum stress induction in comparison with control fibroblasts treated in a same manner [27]. We also intend to take advantage of this observation on fibroblasts to extend our peptidomics study on at least 12 mutated and 12 non-mutated stressed lymphoblasts, as stress is an important factor involved in deterioration in CACH/VWM patients.

To date, diagnosis of eIF2B-pathies is performed based on clinical and MRI criteria and validated by molecular studies (detection of eIF2B mutations). As five different genes are concerned, identification of a proteic or peptidic marker, as for the decreased eIF2B GEF activity in lymphoblasts, would be a benefit in order to restrict eIF2B sequencing to positive screened samples. Moreover, such a specific biomarker would give access to functional information on the patho-physiological mechanism of this severe neurodegenerative disease.

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