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Decreased guanine nucleotide exchange factor activity in eIF2B-mutated patients

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Mutations in each of the five eucaryotic initiation factor 2B (eIF2B) subunits have been found in leukodystrophies of various severity: Cree leukoencephalopathy, childhood ataxia with central hypomyelination/leukodystrophy with vanishing white matter and ovarioleukodystrophy. A continuum was observed from fatal infantile forms to adult forms without neurological deterioration. Disease severity was found to correlate with the age at disease onset and the specific amino-acid substitution. In order to analyze the functional consequences of eIF2B mutations, we measured the guanine nucleotide exchange factor (GEF) activity of eIF2B in transformed lymphocytes from 30 affected patients carrying mutations in eIF2B compared to 10 unaffected heterozygotes and 22 controls without eIF2B mutations. A significant decrease of 20–70% in GEF activity was observed in all mutated cells. The severity of this decrement of GEF activity correlated with age at onset of the disease. These results suggest that a deficiency in GEF activity underlies the encephalopathy associated eIF2B-related disease. Our study demonstrates that the evaluation of the GEF activity in transformed lymphocytes represents an interesting alternative test to the systematic screening of the five *EIF2B* genes. This relevant cellular model may also be used to test the functional impact of different molecules on the GEF activity for future therapeutic strategies.

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Introduction

Mutations in the eucaryotic initiation factor 2B (eIF2B) have been reported in patients with leukodystrophies of various severity. Mutations in each of the five subunits eIF2B α , β , γ , δ and ϵ have been initially found (genes

EIF2B1–EIF2B5 [MIM 606686], [MIM 606454]), [MIM 606272], [MIM 606687], and [MIM 603945]) in patients with leukoencephalopathy with vanishing white matter (VWM)^{1,2} also described as childhood ataxia with central hypomyelination (CACH) syndrome (CACH/VWM [MIM 603896]).³ This syndrome, with autosomal recessive inheritance, has an onset most often between 2 and 5 years of age. Affected individuals experience progressive neurological deterioration including ataxia, spasticity, exacerbated by episodes of fever or trauma of the head. Magnetic resonance imaging (MRI) shows a diffuse involvement of the cerebral hemispheric white matter, with CSF-like signal

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intensity related to rarefaction and cystic degeneration of the white matter.^{3–6} Subsequently, eIF2Bε mutations were found in fatal infantile forms described as Cree leukoencephalopathy (CLE),^{7,8} a severe variant of CACH/VWM^{9,10} and more recently in congenital forms.¹¹ At the opposite end of the clinical spectrum, eIF2B mutations were also found in adult-onset forms associated with ovarian failure (ovarioleukodystrophy), and a slow or absent neurological deterioration¹² leading to the concept of eIF2B-related disorders with a wide *continuum* of clinical severity. In a large cohort of 85 eIF2B-mutated patients, we demonstrated that disease severity is correlated to age at disease onset and to the specific amino-acid substitution.¹³

eIF2B converts the protein synthesis 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. This active complex plays an important role 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 is essential for continued protein synthesis, and eIF2B plays a key role in its regulation by promoting the release of GDP from eIF2.¹⁴

In order to analyze the functional consequences of eIF2B mutations, we measured for the first time the GEF activity of eIF2B in transformed lymphocytes from 30 affected patients carrying mutations in eIF2B, and exhibiting various types of disease severity, compared to 10 unaffected heterozygotes and 22 controls without eIF2B mutations. We hypothesized that although disruption of the ubiquitously expressed eIF2B causes mainly a brain disease, its dysfunction can be demonstrated in non-neural cells.

Materials and methods

Selection of patients

The 30 affected patients carrying mutations in eIF2B and representative of the clinical spectrum severity (Table 1) were selected from our series of 85 patients previously reported.¹³ The 10 healthy heterozygote patients were relatives (parents or siblings) of affected patients (Table 1).

The 22 controls were healthy, age- and sex-matched individuals. No mutations were found in the five patients completely screened for mutations by direct sequencing of the five eIF2B genes. In the other patients, sequences of all exons containing reported mutations^{2,13} were normal.

Establishment of transformed lymphocytes

In order to have large quantity of lymphocytes, lymphoblastoid cell lines were obtained by immortalizing patient's peripheral blood lymphocytes with Epstein–Barr virus according to classical procedures.¹⁵

Measurement of eIF2B GEF activity

To determine the GEF activity of eIF2B, we adapted the GDP dissociation assay described by Kimball *et al.*¹⁶ This assay is a direct measurement of eIF2B GEF activity since, in the presence of Mg^{2+} , the rate-limiting step of the nucleotide exchange reaction is GDP dissociation from eIF2. One million cells from patient transformed lymphocytes were lysed in 100 μ l of ice-cold extraction buffer (45 mM HEPES, pH 7.4, 0.375 mM magnesium acetate, 0.075 mM EDTA, 95 mM potassium acetate, 2.5 mg/ml digitonin, microcystine and 10% (v/v) glycerol). The lysates were centrifuged at 10 000 g for 10 min at 4°C and the resulting supernatants were assayed immediately for the guanine nucleotide exchange activity of eIF2B. Volumes of 60 μ l of the lysate supernatants were added with 2 mM Mg^{2+} to 100 μ l assay buffer (62.5 mM MOPS, pH 7.4, 125 mM KCl, 1.25 mM dithiothreitol, and 0.2 mg/ml bovine serum albumin) containing a 100-fold excess of GDP, subsequently followed by 1–2 pmol of radiolabeled binary eIF2- $[^3H]$ GDP complex (eIF2 purified from rat liver: 1.38 μ g/assay). The mixture was then incubated at 30°C for 0, 2, 4 or 6 min. The exchange reaction was measured as a linear decrease in the eIF2-mediated binding of $[^3H]$ GDP to nitrocellulose filters with time. The slope of each reaction was used to represent GEF activity. Each assay was carried out in triplicate.

Measurement of eIF2alpha phosphorylation

Proteins (60 μ g) extracted from transformed lymphocytes cells with RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 2 mM $NaVO_4$, 0.1 μ M okadaic acid, 25 mM beta-glycerophosphate, 1 mM PMSF, protease inhibitors (Sigma-Aldrich, Protease inhibitor cocktail for general use)) were loaded per well. The relative amount of eIF2 α in the phosphorylated form was quantitated by protein immunoblot analysis using an affinity-purified antibody that specifically recognizes eIF2 phosphorylated at Serine 51 (Phospho-eIF2alpha (Ser51), Cell Signaling Technology). The total amount of eIF2 α in the samples was determined by reprobing the blot with a monoclonal that recognizes equally the phosphorylated and unphosphorylated forms of eIF2alpha (adapted from Kimball *et al.*¹⁷).

Statistics

Data are expressed as mean $\pm$ SEM. Analysis of variances in GEF activities were performed by one-way ANOVA. Correlation tests and regression were evaluated using the statistical software STATA® (StataCorp LP, Version 6.0).

Results

The eIF2B GEF activity was measured in transformed lymphocytes from 30 affected patients exhibiting various types of eIF2B mutations and different ages at disease onset

Table 1 eIF2B GEF activity in lymphocytes from 30 *EIF2B*-mutated patients and 10 heterozygote individuals

Patient ^a	Sex ^b	Age at disease onset (year) ^c	Mutated gene	Amino-acid change ^d	State ^e	GEF eIF2B activity (%) ^f
1036	M	0.8	<i>EIF2B5</i>	P323S/P427L	het/het	30 ± 7.5
*571-1	F	0.8	<i>EIF2B5</i>	F56V/R315H	het/het	40 ± 3
*571-2	M	1.2	<i>EIF2B5</i>	F56V/R315H	het/het	50 ± 5
995	M	1	<i>EIF2B5</i>	R113H/L425R	het/het	48 ± 2
291	F	1.5	<i>EIF2B5</i>	R113H/D387G	het/het	41.5 ± 6
*570-2	M	1.5	<i>EIF2B4</i>	R209Q/R209Q	hom	52 ± 3
357	M	2	<i>EIF2B5</i>	R136C/R339W	het/het	44.5 ± 4.5
357m	F	NA	<i>EIF2B5</i>	R339W	het/wt	94.3 ± 7
904	M	2	<i>EIF2B5</i>	R113H/G481fs493stop	het/het	70 ± 1.2
442	M	2.5	<i>EIF2B5</i>	E81K/R113H	het/het	67 ± 7
435	F	3	<i>EIF2B5</i>	R113H/R422stop	het/het	67 ± 3
928	F	3	<i>EIF2B5</i>	R113H/R113H	hom	49 ± 3
928b	M	NA	<i>EIF2B5</i>	R113H	het/wt	100 ± 0.3
522	M	3.5	<i>EIF2B5</i>	A16D/R113H	het/het	54 ± 6
569	F	3.5	<i>EIF2B5</i>	R113H/?	het/het	40 ± 2
*370-1	F	3.5	<i>EIF2B5</i>	R113H/R195H	het/het	71.5 ± 9
*370-2	F	4.5	<i>EIF2B5</i>	R113H/R195H	het/het	77 ± 2.5
823	M	4	<i>EIF2B3</i>	H341Q/H341Q	hom	61 ± 6
630	M	4.5	<i>EIF2B5</i>	R113H/R113H	hom	77.5 ± 2.5
984	F	5	<i>EIF2B2</i>	E213G/E213G	hom	51.5 ± 0.5
359	M	6	<i>EIF2B4</i>	P243L/P243L	hom	54 ± 6
359m	F	NA	<i>EIF2B4</i>	P243L	het/wt	95.3 ± 7.5
359f	M	NA	<i>EIF2B4</i>	P243L	het/wt	88.5 ± 1.5
*648-1	M	7	<i>EIF2B2</i>	E213G/E213G	hom	59 ± 1
*648-2	M	7	<i>EIF2B2</i>	E213G/E213G	hom	64 ± 4
997	M	7	<i>EIF2B5</i>	R113H/R113H	hom	61.3 ± 0.3
*576-2	M	7	<i>EIF2B5</i>	V73G/R113H	het/het	61 ± 0.2
*576-1	M	8	<i>EIF2B5</i>	V73G/R113H	het/het	56 ± 4
*76-2	M	7	<i>EIF2B2</i>	E213G/K273R	het/het	67 ± 5.1
*76-1	F	17	<i>EIF2B2</i>	E213G/K273R	het/het	75.5 ± 10
*76m	F	NA	<i>EIF2B2</i>	E213G	het/wt	97.5 ± 2.5
*76f	M	NA	<i>EIF2B2</i>	K273G	het/wt	99.5 ± 7
736	F	8	<i>EIF2B4</i>	R374C/R374C	hom	80 ± 0
338	F	10	<i>EIF2B5</i>	R113H/R113H	hom	75.2 ± 1.5
1012	M	14	<i>EIF2B4</i>	R209Q/R209Q	hom	60 ± 2
1014	F	16	<i>EIF2B4</i>	E213G/E213G	hom	68 ± 4
*393m	F	NA	<i>EIF2B5</i>	V309L	het/wt	99.3 ± 6.1
*393f	M	NA	<i>EIF2B5</i>	V309L	het/wt	93.5 ± 0.5
*894m	F	NA	<i>EIF2B5</i>	R195H	het/wt	96.5 ± 0.5
*894f	M	NA	<i>EIF2B5</i>	R195H	het/wt	102 ± 2

^am = mother; f = father; b = brother; * = familial form: two affected children in the same family.^bM = male; F = female.^cNA = nonaffected.^dAmino-acid numbers refer to the eIF2B peptide corresponding sequences. R113H = mutation of histidine to arginine, stop = stop codon, fs = frameshift mutation, ? = mutation not identified (probably a mutation in intron).^ehet = heterozygous mutated allele; hom = homozygous mutated allele; wt = normal allele.^fExpressed as % control value ± standard deviation (assays performed in triplicate).

(mean age = 5.39 ± 4.29 years, range 0.8–17). These activities were compared to those of 22 age-matched wild-type controls (mean age = 8.6 ± 6.5 years, range 0.5–25), and to 10 age-matched unaffected heterozygotes (Table 1). GEF activities measured in transformed lymphocytes from the 22 nonmutated controls were not statistically different (99.6 ± 3.5%). A significant decrease in the GEF activity ($P < 0.01$) was observed in cells from affected mutated patients compared to nonmutated cells (mean decrements = 41%, range 20–70% of the control value) (Figure 1). No statistical significant difference was observed between control and heterozygote cells (mean 96.7 ± 3%).

In addition, the GEF activity measured on transformed lymphocytes from affected patients correlated with the age at disease onset ($P < 0.01$, correlation coefficient $r = 0.51$) (Figure 2). This correlation remained highly significant when the 18 patients with a disease onset before 5 years of age were considered ($P < 0.01$, $r = 0.59$), but correlation was not significant for the 12 patients with disease onset after 5 years of age ($r = 0.37$). To rule out the role of phosphorylated eIF2 α on GEF eIF2B activity decrease, we determined the amount of phosphorylated and unphosphorylated forms of eIF2 α in eight representative cells from patients with eIF2B mutations (patients 1036, 76-1, 370-1, 435, 823,

Figure 1 Decrement of GEF activity in mutated cells, independent of eIF2 α phosphorylation. **(a)** Significant decrement of GEF activity in cells from affected patients compared to nonmutated or heterozygote ones. eIF2B GEF activity in extracts of transformed lymphocytes from 30 eIF2B-mutated and affected patients (Mut), 10 heterozygote nonaffected patients (Het) and 22 nonmutated patients (non-mut). The guanine nucleotide exchange activity was measured as the exchange of [3 H]GDP bound to eIF2 for nonradiolabeled GDP over time. The slope corresponding to the linear decrease in [3 H]GDP bound to eIF2 over time was used for comparisons. The results for mutant cells are expressed as a percentage of GEF activity of control cells and represent three independent experiments. *Significantly different ($P < 0.001$) from the heterozygous and nonmutated response. **(b)** Examples of nucleotide exchange activity shown as picomoles of GDP exchanged. GEF activity was measured in cells from three representative patients with eIF2B mutations: patients 736 (squares), 928 (triangles), 1036 (circles) and compared to control cells (rhombuses). **(c)** Amounts of total eIF2 α and phosphorylated eIF2 α in cells from patients with mutations in eIF2B in comparison to age-matched controls are identical.

571-1, 291, 359) in comparison to controls (Figure 1c comparing three controls with patients 1036, 291 and 359). We found no significant difference in the amount of

Figure 2 Correlation between GEF activity and age at disease onset. eIF2B GEF activity, in % control value, of the 30 eIF2B-mutated patients, correlates with age at disease onset (r (correlation coefficient) = 0.516, $P < 0.01$). This correlation could be modeled with a logarithmic curve whose equation is: $y = 9.6 \ln(x) + 45.9$.

total eIF2 α and eIF2 α phosphorylated forms between controls and patients with eIF2B mutations.

Discussion

We demonstrated for the first time that measurement of the GEF activity of eIF2B is possible on human transformed lymphocytes, and that this activity is decreased in cells from patients homozygous or compound heterozygotes for eIF2B mutations. No abnormalities were found in heterozygotes, in agreement with their normal clinical and MRI presentations. These immortalized mutated lymphocytes are stable, easy to amplify and to preserve, even after patients' death, and offer a good model to study functional consequences of eIF2B mutations. The amount of reduced GEF activity correlated with the age at disease onset, particularly in patients with disease onset before 5 years of age and GEF activity <50%. Absence of correlation for patients with later disease onset and higher GEF activity, suggests that other factors can modulate the effect of mutations on the phenotype.¹³

The catalytic subunit of eIF2B is the epsilon subunit where the majority of the mutations were found.^{18,19} A decrease in GEF activity according to the type of eIF2B mutations has been also reported in yeast.¹⁴ In this report, mutations in the other subunits were found to reduce eIF2B activity as well. Mutations in one of the five eIF2B subunits would decrease the rate of GTP/GDP exchange due to conformational changes or by preventing a particular subunit from binding to the others to form the holoenzyme. Although the epsilon subunit of eIF2B is the only one that exhibits catalytic activity, alone it only has approximately 5% as much activity as the holocomplex. In particular, the beta, gamma, and delta subunits are required for maximal activity in mammals.¹⁴ Thus, mutations in any

of the subunits except the alpha could have dramatic effects on eIF2B activity if it affects the interaction of the epsilon subunit with any of the three regulatory subunits.

The GEF activity of eIF2B is a key control point for eukaryotic protein synthesis particularly in response to cellular stresses.¹⁸ In response to stress, the phosphorylated form of eIF2, which has a much higher affinity for eIF2B than the unphosphorylated form, acts as a competitive inhibitor of eIF2B activity and results in a rapid reduction in the rate of protein synthesis, which avoids large intracellular accumulation of denatured proteins.^{19,14,20} In patients with eIF2B mutations, a high susceptibility to cellular stress is suggested by the acute neurological deterioration observed after minor head trauma or banal viral infections. However, we found no difference in eIF2B activity nor in the amount of eIF2 α phosphorylation in mutated heat-shock stressed cells (1 h at 42°C) *versus* normal ones (personal communication). Further analyses are needed to study the effect of this decrement in eIF2B activity in response to different stress conditions.

The predominant susceptibility of the cerebral white matter to eIF2B dysfunction remains to be elucidated. eIF2B dysfunction in humans may cause abnormal glial cells development, as suggested by the recent description of congenital forms of eIF2B-related disorders,¹¹ and the similar findings in a mouse knockout model for a transcriptional regulator of heat-shock gene expression (Hsf2).²¹ This abnormal development of the white matter would increase susceptibility of cells containing mutated eIF2B to cellular stress with subsequent progressive neurological deterioration and cavitation. For patients with a severe decrease in eIF2B activity (age at disease onset <5 years), eIF2B mutations may supersede environmental or other genetic factors to precipitate white-matter consequences of cellular stress leading to a rather homogenous clinical presentation. On the other hand, in milder forms with a disease onset after 5 years, environmental or other genetic factors would play a role that could explain the variability observed in the onset of the neurological deterioration even within individuals of the same family. For example, the two siblings (patients 76-1 and 76-2) who had similar decrement in GEF activity, had a disease onset at, 17 and 7 years of age respectively (Table 1).

In conclusion, all eIF2B-mutated patients exhibited a decreased GEF activity of eIF2B. Accordingly, this assay could be developed as a biochemical screening test to select patients eligible for sequencing of the five *EIF2B* genes. This eIF2B assay may also be used to test the functional impact of different molecules on the GEF activity for future therapeutic strategies.

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