The first ALS2 missense mutation associated with JPLS reveals new aspects of alsin biological function

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Primary lateral sclerosis (PLS) is a rare progressive paralytic disorder that results from dysfunction of the upper motoneurons. Although PLS is a sporadic disorder of adult middle age, it has also been described in children as juvenile PLS or JPLS. The causative gene for JPLS was found to be ALS2, which is also responsible for a recessive form of amyotrophic lateral sclerosis, for infantile onset ascending hereditary spastic paralysis (IAHSP) and for a form of complicated hereditary spastic paraplegia (cHSP). ALS2 gene encodes a protein termed alsin, containing multiple guanine nucleotide exchange factor domains, specifically binding to small GTPase Rab5 and acting as a GEF for Rab5. In vitro studies performed with full-length and truncating forms of alsin protein support its role in endosomal dynamics and trafficking of mitochondria. All ALS2 mutations so far reported generate alsin protein truncation. Here, we describe the first homozygous missense mutation in ALS2, p.G540E. The mutation, which falls within the RCC1 domain, was identified in a 34-year-old patient with typical signs of JPLS such as ascending generalized and severe spasticity involving the limbs and the bulbar region, dysphagia, limb atrophy, preserved cognition and sensation. The father and two proband’s sisters were found to be heterozygous carriers of the mutation with no signs of the disease. Studies in the neuronal cell line SK-N-BE indicated that the known subcellular localization of wild-type alsin with the early endosome antigen 1, in enlarged endosomal structures, and transferrin receptor is completely lost by the mutant protein, thus indicating that this mutation leads to protein delocalization. Mutant alsin induced neuronal death itself and also significantly enhanced the apoptotic effect of NMDA and staurosporine. This effect was associated with decreased Bcl-xL : Bax ratio. In contrast, wild-type alsin was neuroprotective and increased Bcl-xL : Bax ratio. Our results provide the first demonstration that a missense mutation in alsin is cytotoxic. In addition, the identification of Bcl-xL/Bax as target of protection by alsin and of cytotoxicity by the mutant form provides a new signalling event regulated by alsin protein that may be important to define its role in neuronal physiology and neurodegeneration. Finally, the phenotype–genotype correlation in our patient, in view of all other ALS2 mutant cases reported previously, suggests a functional interplay of long and short forms of alsin in relation to disease onset and progression.

Keywords: ALS2; alsin; missense mutation; cell death; apoptosis

Abbreviations: ALS = amyotrophic lateral sclerosis; EEA1 = early endosome antigen 1; EGFP = enhanced green fluorescent protein; GEF = guanine nucleotide exchange factor; GFP = green fluorescent protein; IAHSP = infantile onset ascending hereditary spastic paralysis; JPLS = juvenile primary lateral sclerosis; LMN = lower motoneuron; NMDA = N-methyl-D-aspartic acid; TFR = transferrin receptor; UMN = upper motoneuron

Received October 2, 2005. Revised February 23, 2006. Accepted March 27, 2006
Introduction

Amyotrophic lateral sclerosis (ALS) and primary lateral sclerosis (PLS) are closely related but clinically distinct neurodegenerative conditions, for which clear diagnostic criteria have been established (Charcot, 1965; Pringle et al., 1992). Both are progressive paralytic disorders that result from dysfunction of motor systems comprising the upper motoneurons (UMN) of the motor cortex and lower motoneurons (LMN) of the brainstem and spinal cord. In PLS, the degeneration is confined to the UMN (Pringle et al., 1992; Hudson et al., 1993), whereas in ALS, both sets of neurons are affected (El Escorial, 1994). ALS is a more severe disease and the phenotype is influenced by the relative ratio of UMN : LMN involvement. The PLS phenotype (Fisher, 1977; Beal and Richardson, 1981; Russo, 1982; Sotaniemi and Myllyla, 1982; Younger et al., 1988; Gascon et al., 1995) shows a slowly progressive spastic syndrome involving all four extremities and muscles of facial expression, impairing speech and swallowing, and sometimes eye movements and sphincter control. The diagnosis of PLS is essentially one of exclusion, as the UMN pathways can be involved in other disorders (pseudo-bulbar palsy, spastic paraparesis, multiple sclerosis, early ALS or other conditions). Although PLS is a sporadic disorder of adult middle age (Pringle et al., 1992), it has been described in children (juvenile PLS, JPLS), both in isolated (Grunnet et al., 1989) as well as in familial cases (Gascon et al., 1995; Lerman-Sagie et al., 1996).

The causative gene for JPLS was found to be ALS2, which is also responsible for a recessive form of ALS (OMIM 205100) (Hadano et al., 2001; Yang et al., 2001). Later, homozygous mutations in the same gene were identified in families segregating an infantile onset ascending hereditary spastic paralysis (IAHSP; OMIM 607225) (Eymard-Pierre et al., 2002) and complicated hereditary spastic paraplegia (chSP) (Gros-Louis et al., 2003).

A total of ten mutations have been identified so far in the ALS2 gene. (Kress et al., 2005) Almost all of them result in a similar clinical phenotype of an infantile onset of limb and facial muscle weakness, accompanied by bulbar or pseudo-bulbar symptoms, which generally progresses to paraplegia during childhood. These features, taken together, suggest the involvement of UMN, although a family with LMN (i.e. muscular atrophy and EMG abnormalities) has also been reported (Ben Hamida et al., 1990). All the mutations of ALS2 described so far are spread widely across the entire coding sequence and are predicted to result in premature termination of translation, thus resulting in a complete loss of protein function (Hadano et al., 2001; Yang et al., 2001; Eymard-Pierre et al., 2002; Devon et al., 2003; Gros-Louis et al., 2003).

The protein encoded by the ALS2 gene, alsin (1657 aa residues), contains a number of cell signalling and protein trafficking domains. The structure of alsin predicts that it functions as a guanine nucleotide exchange factor (GEF). GEFs regulate the activity of members of the Ras superfamily of GTPases.

The protein contains three different GEF-like domains: (i) an RCC1 (regulator of chromatin condensation) like domain resembling GEF for the Ran GTPase at the N-terminus. Since this motif has also the potential to form a seven-bladed beta propeller structure, it may otherwise function as a protein–protein interaction domain (Topp et al., 2004); (ii) a middle Dbl-homology/pleckstrin-homology (DH/PH) domain, which is found in GEFs for Rho, Rac and Cdc42 (Topp et al., 2004; Kanekura et al., 2005). Indeed, it has been recently demonstrated that alsin is present in axons and dendrites and acts as a GEF for Rac1 to stimulate neurite outgrowth (Tudor et al., 2005); (iii) a C-terminal vacuolar-protein-sorting-9 (VPS9) domain with Rab5 GEF activity in conjunction with its upstream eight-membrane occupation and recognition nexus (MORN) motifs (Otomo et al., 2003; Topp et al., 2004).

The Rho and Rab5 family of proteins have been implicated in a multitude of cellular functions, with the best characterized being the regulation of the actin cytoskeleton (Rho) and protein trafficking through early endosomes (Rab5). Moreover, the alsin Rho GEF activity may be important in protecting cells against mutant SOD1-mediated toxicity through a Rac1/phosphatidylinositol-3 kinase/Akt3 pathway (Kanekura et al., 2005).

Alsin activity, although largely unknown, is likely to be modulated both by a short variant deriving from alternative splicing of the ALS2 gene at the level of exon 4 (encoding a 396 aa peptide) and by a second gene homologous to the C-terminus of ALS2 termed ALS2CL (Hadano et al., 2004). How alsin mutations lead to the pathology is still unclear. Indeed, preliminary genotype—phenotype correlations suggested that the truncation of full-length alsin, and therefore its loss of function, account for the UMN degeneration, whereas the short variant, and possibly loss of both full-length and short forms of ALS2, might be related to LMN defects. However, the finding that a mutation affecting both short and long variant caused no signs of LMN involvement (Eymard-Pierre et al., 2002) does not support this hypothesis.

Here, we report the first case of a homozygous missense mutation in the ALS2 gene, detected in a patient with juvenile primary lateral sclerosis (JPLS) and affecting only the long variant. This mutation shed light on alsin pathogenic role in disease onset and progression as shown by the results of subcellular localization studies, and biochemical and functional characterization of the mutant and wild-type proteins in a neuronal cellular model.

Patient and methods

Patient

This 34-year-old female was born fifth of five siblings from non-consanguineous parents. Pregnancy and delivery were uneventful. She developed normally till 2 years of age, when lower limb spasticity and equinism were first noted. A neurological examination at 2 years of age revealed pathological extensor pattern in the lower limbs, hyperactive deep tendon reflexes (DTRs), spontaneous bilateral Babinski, normal trophism and sensation. She was able to walk
unassisted, albeit the postural changes were clumsy. Manual ability and language were normal for the age. EEG, EMG and a brain CT scan were all normal. IQ was 100 (Stanford-Binet).

The initial diagnosis was cerebral palsy, diplegic type. Her neurological situation, however, progressed steadily, with generalized and more severe spasticity involving the upper limbs and the bulbar region. Dysarthria, noted at 6 years of age, led to anarthria by 20 years of age. Loss of ambulation occurred at the age of 19 years. The first electromyography at 10 years of age showed mild distal sufficiency of the explored nerves (left ulnar and common peroneal nerve) more evident in the lower limbs. The needle examination showed mild to moderate neurogenic patterns. Motor and sensory conduction velocities were within the normal range in the explored segments.

A re-evaluation of the clinical picture with more detailed investigations was carried out when the patient was 21 years old. At that time the patient showed spastic tetraplegia, with some active motility conserved only distally in the upper limbs, bilateral clonus and Babinski, anarthria and internuclear ophthalmoplegia. Electromyography showed a moderate chronic neurogenic change on both lower limbs. Normal somatosensory evoked potentials, increased central conduction times at motor evoked potentials, severely delayed auditory evoked responses, and mild pre-chiasmatic delay of visual evoked responses were observed. Brain MRI was normal. An extensive search for metabolic disorders (urinary amino acids and organic acids, acyl-carnitines, coenzyme Q, leucocyte exosaminidase) did not yield any significant result.

In the following years the clinical situation progressed even further, with more evident pseudobulbar signs (spastic crying and laughing), dysphagia now markedly interfering with feeding (a gastrosomy was proposed but refused by the patient), limb atrophy and extreme spasticity. Presently, the patient is wheelchair-bound, totally dependent, and is able to communicate only with uttered sounds, eye movements and a specially adapted PC. Her cognition seems still within the normal range.

Her 74-year-old father and her sisters (48, 46, 42, 40 years old) were carefully examined and no neurological abnormality could be seen.

### Mutation analysis
Blood specimens were obtained from the proband, all the available members of her family and from control subjects after informed consent. The study was approved by the ethical committee of IRCCS E. Medea. Genomic DNA from peripheral blood leucocytes were obtained from peripheral blood leucocytes from the proband, all the available members of her family and from control subjects after informed consent. Brain specimens were obtained from the proband, all the available members of her family and from control subjects after informed consent. Mutagenesis and RNA isolation

**Expression constructs, mutagenesis and RNA isolation**
Full-length ALS2 cDNA was synthesized from human brain cDNA prepared from total human brain RNA (BD-Biotech, San Jose, CA, USA), using the Superscript First Strand Synthesis System for reverse transcription–polymerase chain reaction (RT–PCR) kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and random exancers. Pfu polymerase and primers designed on the ATG (5'-ccgctcgaagATGGACCTCAGATGAGAAGAGT3') and on the stop codon (5'-ccgctcgaagGTATAGCTCTTCGAGGCTGATGAGAGT3') of ALS2 cDNA, both carrying an XhoI site at the 5' end were used in the following conditions: 94°C for 5 min and then 35 cycles as follows: 94°C for 30 s, 58°C for 1 min, 72°C for 6 min. The fragment of 5206 bp so obtained was subcloned in pcDNA3-Myc-EGFP XhoI linearized vector downstream the Myc/EGFP tags and the insert was sequence-verified with a Big Dye Terminator Sequencing Kit (version 3.1, Applied Biosystems), run on an Applied Biosystems ABI 3100 Avant Genetic Analyzer.

Mutagenesis on the pcDNA3-ALS2 wild-type (wt) construct was performed using a QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the following primers: MutF5'-GAAGGCGACGTGAGCGAGATGTTCT-3' and MutR5'-GCAAATGCGGTGACCCAGCTCC-3'. The ALS2 mutant clone (mut alsn) was then completely sequence-verified.

Total RNA from SK-N- BE cells (~5-6 x 10^6 cells) was prepared by using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions.

### Cell culture and transfection
SK-N-BE cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) horse serum, 5% (v/v) foetal Clone III (Hyclone Cellbio, Milan, Italy), l-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) at 37°C under a humidified 95–5% (vol/vol) mixture of air and CO₂.

For cell death experiments cells were plated in 6-well dishes at 90% confluency 24 h before the transfection with pcDNA3-MycEGFP-ALS2wt and pcDNA3MycEGFP-ALS2mutG540E vectors, obtained using Lipofectamine 2000 (Invitrogen Life Science, Carlsbad, CA, USA) according to the manufacturer’s instructions. Transfection efficiency was >87% and did not vary significantly among the various preparations. Immunofluorescence experiments were carried out using cells plated at 60% confluency on polylysine-coated glass coverslips.

### Cell death induction and analysis
Cell death was induced by administration of NMDA (N-methyl-D-aspartic acid; 0.5–5 mM, 3 h) or staurosporine (1 µM, 4 h). Death was assessed 6 h later by counting the number of green fluorescence positive (i.e. GFP-positive) cells that incorporated propidium iodide
and by quantifying the percentage of propidium iodide staining using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Sunnyvale, CA, USA) (Sciorati et al., 1997). Quantification of apoptotic cells was obtained by monitoring internucleosomal fragmentation of genomic DNA, using an immunoassay kit (Cell Death Detection Elisa Plus Kit, Roche Diagnostic, CA, USA). Subcellular compartmentalization of Bax was assessed by cell fractionation experiments as described (Varadi et al., 2004). In brief, cells were scraped in 0.3 M sucrose, 10 mM Mes K⁺, 1 mM K⁺EGTA, 1 mM MgSO₄, 1 mM DTT, pH 6.5, supplemented with a protease inhibitor cocktail (Complete Roche Diagnostics, GmbH Mannheim, Germany) and homogenized in the same buffer with a ball-bearing homogenizer (EMBL, Heidelberg, Germany). Homogenates were centrifuged at 1200 r.p.m. for 5 min at 4°C. The post-nuclear supernatant was centrifuged at 10 000 g for 30 min at 4°C to separate the cytosolic (supernatant) and mitochondrial (pellet) fractions. Equal amounts of proteins for each fraction were then analysed by western blotting as described in the section below.

**Protein expression by western blot and flow cytometry analyses**

For western blot analyses cells were washed in phosphate-buffered saline (PBS), lysed in a buffer containing 150 mM NaCl, 15 mM MgCl₂, 1 mM EGTA, 50 mM HEPES-KOH, 10% glycerol, 1% Triton X-100, pH 7.5, supplemented with the protease inhibitor cocktail. Protein content in the lysates was assessed by the bicinchoninic acid procedure (Perbio, Bezons, France). Fifty micrograms of lysates was then subjected to either 6% (for alsin detection) or 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis as described (Bulotta et al., 2001). The separated proteins were electrophotroferently transferred to 0.2 μm pore nitrocellulose membrane at constant 250 mA for 2 h and the relevant antigens were revealed using polyclonal antibodies anti-alsin, glyceraldehyde phosphate dehydrogenase (Biogenesis, Poole, UK), cytochrome c oxidase (Molecular Probes, Eugene, OR, USA), Bax, Bcl-xL or β-actin (Cell Signalling Technology, MA, USA). After incubation with appropriate secondary Abs, blots were developed with the enhanced chemiluminescence procedure (ECL Amersham Bioscience, Little Chalfont, UK) (Bulotta et al., 2001).

Analysis of alsin expression was carried out also by flow cytometry essentially as described (Nisoli et al., 2004). Briefly, cell suspensions (1 × 10⁶ cells/sample) were fixed in 4% paraformaldehyde for 10 min and incubated for a further 1 h at room temperature in a permeabilization buffer containing 1% bovine serum albumin (BSA), 0.1% saponin and the anti-alsin Ab. Expression of alsin was analysed by flow cytometry after staining with appropriate tetrahydrodichromidine (TRITC) labelled Ab, and quantified in the GFP-positive cells.

**Immunofluorescence**

SK-N-BE cells grown on coverslips pre-coated with polylysine were washed free of the medium with PBS, fixed and permeabilized in PBS containing 1% BSA and 0.1% saponin for 30 min at room temperature. Samples were incubated with the primary antibodies against the following antigens: early endosome antigen 1 (EEA1; Affinity Bioreagents, IL, USA), transferrin receptor (TFR; US Biological Swampscott, MA, USA), green fluorescent protein (GFP; Roche Diagnostic, CA, USA), Cathepsin D (Cat D; Calbiochem International, CA, USA), in the saponin-containing solution for 6 h at 37°C, washed and incubated for 90 min at 22°C with the appropriate secondary, TRITC- or fluorescein isothiocyanate (FITC) conjugated antibodies. Subcellular antigen distribution was analysed under a BioRad MRC 1024 microscope confocal system. Images were acquired with ×63 or ×100 magnification oil immersion lenses at 1024 × 1024 pixel resolution.

**Statistical analysis**

The results are expressed as means ± standard error of the mean (SEM); n represents the number of individual experiments. Statistical analysis was carried out using the Student’s t-test for unpaired variables (two-tailed). Single, double or triple asterisks and crosses in Fig. 3 refer to statistical probabilities (P < 0.05, < 0.01 and < 0.001, respectively), measured in the various experimental conditions as detailed in the legend to the figure. P-values of <0.05 were considered significant.

**Results**

**Mutation analysis**

We first ruled out the possibility of mutations in some spastic paraparesis-related genes, testing the patient’s DNA for mutation in SPG7 and SPG4 with negative results. Then, the ALS2 gene was considered and all exons from the patient were sequenced. A homozygous nucleotide change, c.1619 G > A, was found in exon 6 leading to the replacement of a glycine with a glutamic acid residue in position 540 of alsin protein (p.G540E) (Fig. 1A). As shown in Fig. 1B, the amino acid substitution falls within the RCC1 domain, in a region highly conserved among different species. The father and two of the four healthy sisters of the proband are heterozygous carriers of the mutation (Fig. 1A). The mother (deceased) could not be analysed. The nucleotide change was not found in a panel of 600 control chromosomes from the Italian population.

**Expression constructs and in vitro mutagenesis**

To evaluate whether this newly identified mutation in alsin affects protein function, and thus is responsible for the observed clinical phenotype, full-length cDNA from ALS2 was amplified from human brain cDNA, and subcloned in pcDNA3MycEGFP expression vector for subcellular localization studies. The construct was then mutagenized in vitro to introduce the mutation identified.

**The intracellular localization of the mutant alsin differs from that of wt alsin**

Previous studies showed that wt alsin is predominantly cytosolic, however, with a strong association with endosomes, as demonstrated by immunoprecipitation studies and co-localization with early and recycling endosome markers (Otomo et al., 2003; Yamanaka et al., 2003; Kunita et al., 2004; Topp et al., 2004; Devon et al., 2005). To study whether the missense mutation of alsin affected its intracellular localization, we transiently transfected wt and mutant alsins, tagged at their N-terminus with GFP, into the SK-N-BE human neuroblastoma cell line, a widely employed neuronal cell model
(Lievremont et al., 1999; Perini et al., 2002). Figure 2A illustrates a representative western blot showing the expression levels of the transfected wt and mut proteins, which were compared with those of endogenous alsin using a specific Ab (Tudor et al., 2005). At the concentration of polyacrylamide used (6%) the GFP tagged and endogenous alsins are expected to co-migrate. Densitometric analyses revealed that the expression levels of alsin in cells transfected with the wt and mut alsin constructs were increased by 89.29 ± 31.2 and 101.88 ± 49.6% with respect to those observed in cells transfected with the empty pcDNA3MycEGFP vector (n = 3). Since the Ab used recognizes both the endogenous and
transfected alsins, it appears that the transfected wt and mut alsins accounted for ~50% of the protein in the transfected cell population. These results were confirmed by flow cytometry quantitative analyses that, in addition, allowed us to exclude the small percentage of cells in the population that were not transfected with the various constructs (i.e. GFP-negative cells) (Fig. 2B).

We examined the localization of both wt and mut alsins along the pathway of endosomal trafficking, by comparing their localization with the early and recycling endosome markers EEA1 and TFR, and with Cat D, which recognizes the lysosomes. Figure 2C shows the localization of each specific antigen, the wt and mut alsins, and the merge pictures. Consistent with previous results in other types of transfected cells, wt alsin was predominantly cytosolic; however, it localized also with EEA1 in enlarged endosomal structures, and with TFR (Otomo et al., 2003; Yamanaka et al., 2003; Kunita et al., 2004; Topp et al., 2004). In contrast, no association with the endosomes could be detected with mut alsin. Neither mut nor wt alsin co-localized with lysosomes. These results indicate that the newly identified missense mutation in alsin leads to protein delocalization.

**Alsin is endowed with antiapoptotic activity that is lost by the missense mutant protein**

A prominent feature of neurodegenerative diseases, including ALS, is death of motoneurons, essentially via apoptosis (Nixon et al., 2005). We investigated the role of wt and mut alsin in neuronal cell death induced by two known, unrelated neuronal damaging agents, NMDA (0.5–5 mM), which activates specific glutamate receptors involved in excitotoxicity (Hardingham and Bading, 2003), and the protein kinase C inhibitor staurosporine (1 μM) (Swannie and Kaye, 2002). We studied the effects of these compounds on SK-N-BE cells transfected with MycGFP-tagged mut and wt alsin, as well as on control SK-N-BE, that is, transfected with the vector carrying only MycGFP. As shown in Fig. 3A and B, NMDA and staurosporine induced cell death in control SK-N-BE cells, measured by counting the number of GFP-positive cells incorporating propidium iodide, and by flow cytometry analyses of percentage propidium iodide staining. Of importance, both the NMDA- and staurosporine-induced...
Fig. 3  Wt alsin is endowed with antiapoptotic activity that is lost by its missense mutant. SK-N-BE human neuroblastoma cells were transiently transfected with wt alsin (Alsin-WT-GFP), mut alsin (Alsin-MT-GFP), both GFP-tagged at their N-terminus, or the vector carrying only GFP (pcDNA3-GFP), used as control. Apoptosis was triggered by administration of either NMDA (5 mM) or staurosporine (1 μM) and compared with that of the control treated with vehicle only (NT). (A) Number of GFP-expressing dead cells (in 10^5 cell samples), assessed by their propidium iodide incorporation. NMDA increased death by 207.41 ± 18.1, 225.32 ± 11.6 and 68.7 ± 8.0% in cells transfected with pcDNA3-GFP, Alsin-WT-GFP and Alsin-MT-GFP, respectively. Staurosporine increased death by 383.33 ± 19.3, 412 ± 27.1 and 168.75 ± 11.5% in cells transfected with pcDNA3-GFP, Alsin-WT-GFP and Alsin-MT-GFP, respectively. All these increases were significant (P < 0.01; n = 4). (B) Propidium iodide staining of GFP-expressing, non-permeabilized cells expressed as the mean fluorescence intensity value (RFI) ± SEM (n = 4). (C) Formation of histone-conjugated DNA fragments. Shown are absorbance values ± SEM at 405 nm/mg protein (n = 3). (D and E) Western blot analysis of Bax and Bcl-xL expression and of the NMDA-induced Bax translocation from the cytosol (C) to the mitochondria (M). On the same nitrocellulose filters were routinely carried out the evaluation of β-actin expression, an internal control of accurateness of sample loading (D), or of glyceraldehyde phosphate dehydrogenase (GAPDH) and cytochrome c oxidase subunit IV (COX IV), used as controls of purity of the cytosolic and mitochondrial fractions, respectively (E). Shown are both representative images and graphs, the latter reporting the ratio of densitometric values ± SEM of Bax and Bcl-xL versus those of β-actin (D; n = 4) and Bax in the cytosol and mitochondria versus those of GAPDH (E; n = 3), both expressed as %. In all experiments, the expression level of the wt and mut alsin in each sample was routinely assessed by staining with an anti-GFP Ab, and found not to differ significantly among the various preparations (see example in D). In all panels, asterisks indicate statistical significance versus NT, while crosses indicate those observed with NT, NMDA or staurosporine in Alsin-WT-GFP or Alsin-MT-GFP versus the respective treatments in pcDNA3, calculated as described in the Patient and methods section.
The fact that a missense mutation in the RCC1 domain is associated with a phenotype similar to the one observed with small deletions suggests that alteration of this domain is specifically related to motor neuron degeneration observed in JPLS and related diseases (Millecamps et al., 2005).

Unlike the overexpression studies of truncating forms of alsin, analysis of the p.G540E mutant alsin provides for the first time an opportunity for direct testing of molecular consequences of both full-length protein alteration and RCC1 domain functional specificity.

The newly identified missense mutation affects only the long form of alsin protein, while the function of the short form is preserved, and this may provide some clues on the role of the protein in generating the phenotype. In particular, the associated JPLS phenotype supports the hypothesis of genotype-phenotype correlation whereby the functional impairment of the long variant only, and preservation of the short one, leads to UMN degeneration sparing motor-neurons from the spinal cord and brainstem (Yang et al., 2001). In addition to that, in our patient we observe an early age of onset (18–24 months of age), as shown by all cases carrying mutations affecting only the long form of alsin, and a slow progression as displayed by the ALS2 family with deletion c.261delA (Hadano et al., 2001; Devon et al., 2003) affecting both the long and short forms of alsin (Eymard-Pierre et al., 2002; Devon et al., 2003). Apparently, an exception to that would be the deletion c.1130delTA, which affects both alsin forms and is associated with an early onset phenotype such as IAHSP (Eymard-Pierre et al., 2002), but in this case the mutation interrupts the short form at the residue 335, thus maintaining the major portion (335/396 aa) of the short ALS2 protein, which perhaps acts like a wt one. Therefore, the previously unreported association of slow progression and early disease onset we detect in our patient raises the hypothesis of a direct involvement of the dysfunctional but complete long form in generating the phenotype. Overall, although additional missense mutations in the ALS2 gene need to be identified and characterized to confirm this interesting association, these observations are consistent with the hypothesis of a functional and regulatory interplay of both forms as postulated previously (Hadano et al., 2001; Yang et al., 2001).

In vitro studies performed with full-length and truncating forms (some of which have been identified in ALS patients) of alsin protein support its role in endosomal dynamics (Otomo et al., 2003; Yamanaka et al., 2003) and trafficking of mitochondria (Millecamps et al., 2005). In this regard the RCC1 domain was found to be, in part, responsible for endosomal localization (Yamanaka et al., 2003).

Expression of the p.G540E mutant protein in neuronal cells and localization studies now clearly demonstrate the pathological effect of the newly identified mutation. This is supported by morphological and functional evidence. Indeed, consistent with previous results in other types of transfected cells, wt alsin is predominantly cytosolic, partly localizing also with EEA1, in enlarged endosomal structures, and TFR.
ALS2 missense mutation in JPLS

(Fig. 2) (Otomo et al., 2003; Yamanaka et al., 2003; Kunita et al., 2004; Topp et al., 2004). On the contrary, p.G540E alsin fails to associate with endosomes, while neither mut nor wt alsin co-localized with lysosomes. These results indicate that the newly identified missense mutation in alsin leads to protein mislocalization.

Disruption of the endosomal–endocytic pathway arising from delocalization of alsin from endosomes and/or impaired Rab5 activity may contribute to neurotoxicity (Roberts et al., 1999; Otomo et al., 2003; Topp et al., 2004; Nixon et al., 2005). Consistent with this, the missense mutant protein induced apoptosis.

Recent experimental evidence indicates that loss of Als2 function in Als2−/− mice is not sufficient to trigger motoneuron degeneration but can predispose neurons to oxidative stress (Cai et al., 2005). In addition, alsin may exert a protective effect by interacting with SOD1 mutant protein (Kanekura et al., 2004, 2005). How modifications in alsin structure and function lead to neuronal cell damage, however, is still unclear.

Our results shed light on the mechanism of action of the mutant alsin by providing the first demonstration of an active role of a mutant full-length alsin protein in both inducing neuronal death per se, and significantly enhancing the apoptogenic effect of two unrelated stimuli, NMDA and staurosporine. In particular, we found that the effect on apoptosis was accompanied by a significant change in the ratio of Bax and Bcl-xL and by translocation of Bax to mitochondria. An altered ratio and localization of these proteins contributes to apoptosis induction in a variety of pathophysiological conditions, including neuronal damage associated with neurodegenerative diseases (Kirkland and Franklin, 2003; Tatton et al., 2003). In particular, decreases in Bcl-xL and increases in Bax have been found to occur in mouse models of ALS (Mu et al., 1996; Kostic et al., 1997; Gonzalez de Aguilera et al., 2000). It is conceivable, therefore, that the alterations in Bax and Bcl-xL ratios and Bax localization reported here contribute to death induction by the p.G540E alsin protein. Moreover, our experiments suggest a direct effect of the protein itself on the pathway of apoptosis, since the missense alsin induced significant, even if little, basal apoptosis, and wt alsin prevented apoptosis induction and changes in Bcl-xL and Bax induced by both NMDA and staurosporine.

SK-N-BE cells express wt alsin; the fact that the p.G540E mutant alsin was apopotic even in the presence of the wt protein indicates that the missense mutation confers on alsin the ability to inhibit, or overcome, the antiapoptotic function of the endogenous wt protein. This suggests a partial gain of function of the overexpressed mutant protein as the pathogenic mechanism underlying cell death, at least in vitro. Alsin is known to be active when in a homo-oligomerized form, generated by its ability to bind through specific carboxyl-terminal domains (Kunita et al., 2004). Whether the dominant negative function of the p.G540E mutant protein arises from inhibition of this oligomerization, or generation of non-functional oligomers, remains to be studied. In spite of the in vitro evidence, individuals carrying the heterozygous mutation show no signs of motoneuron dysfunction. Whether the apparent lack of clinical signs results from a compensation of mutant alsin gain of function in vivo remains to be investigated.

The studies in the SOD1 model of ALS mentioned above showed an antiapoptotic action by alsin, occurring because of a direct interaction of the protein with SOD1, with ensuing activation of a PI3K/Akt-dependent pathway (Kanekura et al., 2005). However, the mechanism of that protection was specific for the SOD1 mutant, since alsin did not interact with the wild-type SOD1 protein (Kanekura et al., 2004). Our results now unravel a new mechanism for the antiapoptotic role of wt alsin, independent of interaction with SOD1, and show that this mechanism is lost in an ALS2 mutant in vitro model, thus substantiating and further expanding those previous observations. In addition, the fact that protection by alsin occurs through a signalling event impinging on a pathway commonly used by most neurotoxic stimuli contributes to defining the importance of this protein in neuronal physiology, and of its pathogenic role, when altered, in neurodegenerative processes.

Acknowledgements

We express our gratitude to the family for their participation in the study. We also thank D. Galbiati and C. Sciorati for technical assistance. The work was supported by grants from the Italian Ministry of Health, RC2005 (M.T.B.), the Italian Association of Cancer Research and Fondazione Telethon (E.C.) MRC, MNDA and EU framework 6 NeuroNE (C.C.M.).

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