Stable Tubule Only Polypeptides (STOP) Proteins Co-Aggregate with Spheroid Neurofilaments in Amyotrophic Lateral Sclerosis

F. LETOURNEL, MD,* A. BOCQUET,* F. DUBAS, MD, A. BARTHELAIX, MD, PhD, AND J. EYER, PhD

Abstract. A major cytopathological hallmark of amyotrophic lateral sclerosis (ALS) is the presence of axonal spheroids containing abnormally accumulated neurofilaments. The mechanism of their formation, their contribution to the disease, and the possibility of other co-aggregated components are still enigmatic. Here we analyze the composition of such lesions with special reference to stable tubule only polypeptide (STOP), a protein responsible for microtubule cold stabilization. In normal human brain and spinal cord, the distribution of STOP proteins is uniform between the cytoplasm and neurites of neurons. However, all the neurofilament-rich spheroids present in the tissues of affected patients are intensely labeled with 3 different anti-STOP antibodies. Moreover, when neurofilaments and microtubules are isolated from spinal cord and brain, STOP proteins are systematically co-puriﬁed with neuroﬁlaments. By SDS-PAGE analysis, no alteration of the migration proﬁle of STOP proteins is observed in pathological samples. Other microtubular proteins, like tubulin or kinesin, are inconsistently present in spheroids, suggesting that a microtubule destabilizing process may be involved in the pathogenesis of ALS. These results indicate that the selective co-aggregation of neuroﬁlament and STOP proteins represent a new cytopathological marker for spheroids.

Key Words: Amyotrophic lateral sclerosis; Microtubules; Neuroﬁlaments; Spheroids; Stable tubule only polypeptides (STOP).

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an age-dependent neurodegenerative disease, starting in mid-adult life and leading to paralysis and death within 3 to 5 years. Pathologically, motor neurons are selectively affected and they present accumulations of neurofilaments in either their cell bodies or axons (spheroids). These aggregates represent a major hallmark also found in other human neurodegenerative diseases like Alzheimer and Parkinson diseases, as well as in toxin-induced neuropathies (1–4). Such lesions contain other proteins, like ubiquitin in “Skein-like” inclusions in ALS (5), α-synuclein in Lewy bodies (6), or tau in neurofibrillary tangles (NFTs) (7).

Genetic investigations have identiﬁed a mutated gene encoding the cytosolic copper-zinc superoxide dismutase protein 1 (SOD1) in several ALS families (8). However, fundamental questions remain unanswered regarding the localization of this mutated protein, its toxicity to cellular organelles such as mitochondria, neuroﬁlaments, or the Golgi apparatus, and why mutations in an ubiquitously expressed enzyme cause the selective death of motoneurons (9, 10). Compared to other neurons, motoneurons are very large cells, and therefore require a highly specialized cytoskeletal network to maintain their function. Neuroﬁlament mutations have been found in some sporadic forms of ALS (11), but the use of transgenic preparations, in which neuroﬁlament organization and expression is altered, has provided contradictory results. For example, the expression of a mutated form of the light neuroﬁlament subunit induced dramatic neurodegeneration (12), whereas massive perikaryal aggregations of neuroﬁlaments due to the expression of a neuroﬁlament-β-galactosidase fusion protein did not alter motoneuron viability (13). Using this last model, it was shown that neither initiation nor progression of axonopathies induced by mutated forms of SOD1 or dystonia muscularum require the aggregation of axonal neuroﬁlaments (14). While the disparity between humans and rodents limits a direct comparison, the use of transgenic models represents a powerful strategy to investigate the pathogenic mechanisms leading to these diseases.

Cytopathological markers that differentiate these lesions are crucial to understand their formation and their biological consequences. Therefore, it is fundamental to determine which molecules are abnormally sequestered in these inclusions. In such an effort, we have analyzed the distribution of stable tubule only polypeptide (STOP), a calmodulin-regulated molecule (15) shown to induce cold and drug stability both in vitro and in vivo (15–17). Three isoforms have been identiﬁed based on structure and tissue expression: an embryonic isoform (E-STOP), a ﬁbroblastic isoform (F-STOP), and a neuronal isoform (N-STOP) (18–20). This third isoform is abundant in neurons containing signiﬁcant amounts of stable microtubules and it associates preferentially with cold- and drug-stable microtubules (15, 19–21). Microinjection of...
### Clinical Data from Controls and Pathological Cases

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Abbreviations: Bulb: Bulbar signs; LMN: Lower motor neuron signs; RF: Respiratory failure; PE: Pulmonary embolism; SDH: Sub dural hemorrhage; GH: Gastrointestinal hemorrhage; N: normal; F: Frontal signs.

STOP antibodies abolishes microtubule cold and drug stability of neurites in neuronal and NIH/3T3 cells (18, 19, 22).

In this study, STOP proteins are found to co-purify with neurofilaments and to associate with neurofilaments in the cold-stable fraction during a typical microtubule preparation. Moreover, we show that STOP proteins are systematically retained in neurofilament-labeled spheroids present in ALS, together with peripherin, another intermediate filament. However, Western blot analysis has shown no major modification of the migration profile of STOP proteins between pathological and control samples. Finally, as it has been previously shown, tubulin and kinesin epitopes are not systematically sequestered in spheroids with neurofilaments (23, 24). Taken together, these data show that the precipitation of STOP proteins together with neurofilaments is a new cytopathological hallmark of spheroids present in ALS. Such an abnormal distribution of neuronal STOP proteins, as well as the frequent lack of tubulin and kinesin in spheroids, suggests that microtubule stability is altered in these lesions, which could contribute to the pathogenesis of ALS.

### MATERIALS AND METHODS

#### Human Tissues

Spinal cords and brains from autopsy cases (Neurology Department of the Hospital of Angers in agreement with our institutional committees, PHRC no. R2108) were fixed for 1 month in formalin 10% and then embedded in paraffin. Thirteen cases with a clinical ALS diagnosis were analyzed. Tissues from 1 Steinert’s case, 2 chronic neuropathy cases, and 1 carcinomatous meningitis case were studied as controls. Tissues from 2 other controls (Table, cases 5 and 6) were used for biochemical investigations (microtubule and neurofilament isolation) and Western blot analysis. Spinal cord tissues from pathological cases were also frozen as described below for Western blot analysis, immunohistochemistry, and confocal microscopy. All these cases were characterized neuropathologically as indicated in the Table.

#### Immunohistochemistry

Paraffin-embedded or frozen tissue (7-μm-thick) sections from lumbar and cervical regions, brainstem, hippocampus, frontal, parietal, and temporal cortices were used alternatively for Luxol-hematein-phloxine staining and immunohistochemistry. After removal of paraffin, sections were incubated with
the diluted primary antibodies overnight at 4°C, then washed with phosphate buffer (PBS), and incubated with a biotinylated secondary antibody (1:100) for 1 hour. Localization of primary antibody was visualized by the avidin-peroxidase complex following manufacturer’s recommendations (ABC kit, Dako, Trappes, France).

Frozen sections used for double immunofluorescence labeling were first incubated for 1 hour in the blocking buffer (PBS, newborn goat serum 10%, bovine serum albumin 3%). Primary and secondary antibodies were incubated sequentially for 90 min each, with 3 intervening PBS washes. Slides were mounted using glycerol in PBS (90%) and analyzed by confocal microscopy (Olympus BX 60, with Fluoview software).

Primary antibodies were diluted in blocking buffer as follows: anti-NFM (1:100, N5264, Sigma, Saint-Quentin Fallavier, France), anti-Stop (1:100, Polyclonal AB1530 from Chemicon, Montlucon, France), anti-Stop polyclonal 23C (1:100), anti-Stop polyclonal 23N (1:500), and anti-N-Stop monoclonal (1:1,000). STOP antibodies were kindly provided by Dr. D. Job (INSERM, CEA, Grenoble, France). Secondary antibodies for fluorescence were used at 1:200 each: Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 568 (goat anti-rabbit) (Molecular Probes, Montlucon, France).

### Neurofilament and Microtubule Isolation

Neurofilaments were isolated according to the procedure described by Eyer and Leterrier (25), modified as follows. Spinal cord samples were homogenized in RB buffer (EGTA 1 mM, MgCl2 1 mM, MES 0.1 M, pH 6.8) (chemicals were from Sigma). Following centrifugation at 100,000 g for 1 hour at 4°C, the first pellet (P1) was discarded and 4 M glycerol was added to the supernatant (S1), which was incubated for 1 hour at 4°C to prevent microtubule assembly while allowing neurofilaments to form reticulated networks. This suspension was centrifuged at 100,000 g for 2 hours at 4°C to recover tubulin in the supernatant (S2) and neurofilaments in the pellet (P2).

Microtubules were isolated from brain. Tissues were homogenized in RB buffer then centrifuged at 100,000 g for 1 hour at 4°C to recover soluble proteins in the first supernatant. Four M glycerol and GTP (1 mM) were added to this supernatant and incubated for 1 hour at 37°C to allow microtubule polymerization. Microtubules were sedimented by centrifugation at 100,000 g for 1 hour at 37°C. Microtubules present in the resulting pellet (P2) were resuspended and incubated at 4°C to allow their depolymerization. Following centrifugation at 100,000 g for 1 hour at 4°C, the resulting supernatant (S3) contained free tubulin and the pellet (P3) contained cold-stable microtubules. All fractions were stored at −20°C prior to analysis. The amount of protein present in each fraction was measured using the BCA Protein assay (Pierce, Montlucon, France).

### SDS-PAGE and Immunoblotting

Proteins (45 μg/well) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Strasbourg, France). Membranes were washed with TBS (NaCl 0.9%, Tris 20 mM pH 7.5) and incubated overnight in the blocking solution (10% dry milk in TBS). After washing with TBS, membranes were incubated for 3 hours with the primary antibody diluted in TBS, BSA 3% w/v (Sigma), PVP 0.5% w/v (Prolabo, Strasbourg, France), and thimerosal 0.2 μg/ml (Sigma). They were then washed in TBS and TBS-Tween 0.05% and incubated for 90 min with peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, 1:2,000, goat anti-rabbit IgG, 1:2,000; Dako). Primary antibodies and dilutions employed were as follows: polyclonal rabbit anti-STOP antibodies 23C or 23N (1:4,000), monoclonal anti-NFH (1:2,000) (clone N52 from Sigma), monoclonal anti-NFM (1:1,000) (clone NN18 from Sigma), monoclonal anti-NF-L (1:1,000) (clone NR4 from Sigma), monoclonal anti-α-tubulin (1:1,000) (clone DM1A from Sigma). The enhanced chemiluminescence detection system (ECL, Amersham Life Sciences, Orsay, France) was used to detect immunoreactive polypeptides.

### RESULTS

#### Composition of Human STOP Proteins in Neuronal Tissues and their Co-Purification with Neurofilaments

Protein composition of human brain and spinal cord crude extracts was analyzed by Western blotting using a monoclonal anti-STOP antibody specific to the neuronal N-STOP isoform. Mouse brain and spinal cord crude extracts were used as positive controls. A major band migrating at 145 kDa was detected in the human sample (Fig. 1A), similar to the N-STOP isoform of mouse tissue. Migrating below, another band was also revealed at 120 kDa, which could correspond to a modified form of STOP proteins or a proteolytic product. Western blot analysis of crude extracts isolated from spinal cord and brain of 3 ALS cases and 2 normal cases revealed no detectable differences in the migration profile of STOP and neurofilament proteins (Fig. 1B).

To investigate the association of STOP proteins with cytoskeletal structures we isolated microtubules and neurofilaments from human spinal cord and brain. Using a standard microtubule preparation, soluble tubulin in the first supernatant (S1) was induced to polymerize into microtubules for 1 hour at 37°C by adding GTP (1 mM) and glycerol (4 M). Such polymerized microtubules were recovered by sedimentation in the second pellet (P2) and then resuspended and incubated at 4°C for 1 hour to depolymerize them into tubulin. Following a third centrifugation, depolymerized tubulin was recovered in the third supernatant (S3), while the pellet (P3) contained cold-stable microtubules. Western blot analysis of each fraction showed that STOP proteins and neurofilaments were predominantly enriched in P3, suggesting the interaction between these molecules (Fig. 1C). To further confirm this possibility, we also isolated neurofilaments from human spinal cord. Western blot analysis revealed that neurofilaments were present in the second pellet (P2) together with STOP proteins, while tubulin was equally distributed between the different fractions (Fig. 1D). Due to the small size of the samples, it was not possible to perform a third cycle of purification. Note that the migration profile of NFH is similar in these different fractions, suggesting a similar phosphorylation level (Fig. 1C, D).
Fig. 1. Western blot analysis of STOP proteins in normal human and mouse nervous tissues (A), in ALS patients (B), and their association with neurofilaments during isolation of either microtubules (C) or neurofilaments (D). A: Analysis of crude extracts of spinal cord (1 and 2) and brain (3 and 4) with a monoclonal antibody against N-STOP reveals a major 145-kDa band migrating slightly above the mouse N-STOP. Mouse crude extracts (2 and 4) were used as internal controls for the presence of human N-STOP (1 and 3). Lighter bands were also seen at 120 and 55 kDa, which could correspond to either proteolytic products or modified forms of N-STOP. B: Typical Western blot analysis of STOP and neurofilament proteins (NFH and NFM) present in the crude extracts from affected (Lane 1: spinal cord from ALS case 1. Lane 3: brain from ALS case 1) and control samples (Lane 2: spinal cord from control case 6. Lane 4: brain from control case 5). Analysis with a monoclonal antibody against N-STOP reveals a 145-kDa band. The migration profiles of STOP and neurofilament proteins are similar between ALS and control samples. C: Following a typical microtubule preparation from human brain (Table, control case 6), STOP proteins are found predominantly in the third pellet (P3), which contains the cold-stable microtubule fraction and is also enriched for neurofilaments. D: During the isolation of neurofilaments from human spinal cord (Table, control case 6), STOP proteins together with neurofilaments are found mainly in the second pellet (P2).
STOP CO-AGGREGATE WITH NEUROFILAMENTs INALS

Distribution of STOP Proteins in Normal Human Brain and Spinal Cord

The cellular distribution of neuronal N-STOP in control human brain and spinal cord was analyzed by immunohistochemistry using the monoclonal anti-N-STOP, which recognizes a repeated epitope present only on the C-terminal domain of N-STOP. Neurons in the hippocampus were intensely labeled with the monoclonal anti-STOP antibody (Fig. 2A). Staining was regularly seen in the cytoplasm and neurites and was similar to that found using an anti-neurofilament antibody (Fig. 2B). In the spinal cord, ventral horn motoneurons together with interneurons were highly labeled with anti-STOP antibodies. In the white matter, axons were also labeled but no staining was observed in the myelin sheath (Fig. 2C). Here too, the distribution of STOP epitopes was similar to that seen using an anti-neurofilament antibody (Fig. 2D). One special feature was the labeling of corpora-amylacea with the anti-N-STOP antibody (Fig. 2E). These immuno-labeled structures had a round shape with diffuse staining and were located under the pia matter. This typical staining pattern and localization was also observed when a standard stain such as hematein-phloxine was used (Fig. 2F).

STOP Proteins Co-Aggregate Specifically with Neuronal Intermediate Filaments in Spheroids Present in ALS Tissues

To test whether STOP distribution could be perturbed by neurofilament aggregation in human neurodegenerative diseases we analyzed spinal cords from ALS patients by immunohistochemistry. Thirteen patients were selected from our database according to the El Escorial criteria (Table). Eight patients were females and 5 were males, with a median age of 61.5 years. The mean duration of the illness was 31 months.

The general pattern of anti-STOP staining in both white and gray matter was similar to controls. However, fewer gray matter motoneurons were present in pathologic samples due to the disease. In 7 cases, spheroids were clearly detectable by standard staining (Fig. 3A) and anti-neurofilament immunostaining (Fig. 3B). They had a classical round shape and were primarily located in the gray matter. All the detected spheroids were also intensely labeled with anti-STOP antibodies (Fig. 3C). To test directly the colocalization of neuronal intermediate filaments with STOP proteins in these lesions, we performed a double labeling immunofluorescence study on frozen tissue. Using confocal microscopy, we routinely observed in the spheroids a co-localization of peripherin and NFM (Fig. 4A–C), as well as peripherin and STOP proteins using the monoclonal anti-STOP antibody (Fig. 4D–F). Colocalization of neurofilaments with STOP proteins was also observed using the polyclonal anti-STOP antibody (Fig. 4G–I). However, numerous spheroids were unstained by anti-tubulin (Fig. 4J–L) or anti-kinesin antibodies (Fig. 4M–O). Quantitative analysis of spheroids in cervical spinal cross sections showed that an average of 4 spheroids per section was counted in the 7 pathologic cases that were analyzed (Table). All these spheroids were stained with antibodies recognizing neurofilaments and STOP proteins. However, only 82% and 58% of the spheroids were labeled with tubulin and kinesin antibodies, respectively. Therefore, compared to tubulin and kinesin, STOP was found to be systematically present with neurofilaments in the axonal spheroids.

DISCUSSION

STOP proteins were previously shown to be responsible for cold and drug microtubule stabilization using cell culture, as well as rat and mouse tissue homogenates (18). However, little is known about this protein in human tissues. Based on sequence homologies, Bosc et al (26) suggested that a neuronal N-STOP isoform might exist in humans with 79% homology to the rat N-STOP. Variations between these species are mainly found in the central repeats. Five repeats are present in rodents while only 1 is present in human N-STOP. In this study we perform Western blot analysis of human nervous tissues using 3 available antibodies. The 2 polyclonal antibodies (23N and 23C) recognize the N- and C-terminal regions, respectively, of the repeated sequences present in the central domain of each STOP isoform. The monoclonal anti-STOP antibody recognizes a repeated KDQG motif present only in the C-terminal end of the neuronal isoform (21). This motif is repeated 8 times in the human N-STOP protein and 5 times in the mouse sequence. Western blot analysis confirms the presence of STOP proteins in human tissue and reveals a pattern similar to mouse brain and spinal cord extracts, with a major band migrating at 145 kDa (Fig. 1). This apparent molecular weight is higher than the mass predicted from the amino-acid sequence (86 kDa), and also slightly higher than the mouse neuronal isoform. Such a peculiar migration profile suggests that the protein undergoes post-translational modifications. Among the possibilities, phosphorylation and glycosylation have been shown to modify the electrophoretic mobility of neurofilaments (27, 28), and could also act on STOP proteins. However, the analysis of the migration pattern of STOP proteins shows no difference between normal and pathological samples (Fig. 1B). This indicates that the post-translational modification of STOP proteins (detected by their migration profile on SDS-PAGE) is not altered in the ALS cases.

Neurons contain numerous microtubules that are cold- and drug-stable, properties conferred by STOP proteins (15). Here, we show that STOP proteins are present predominantly in the cold-stable microtubule fraction together with neurofilaments during a classical microtubule

Fig. 2. Human N-STOP distribution in the normal central nervous system as revealed by immunohistochemistry. Using both polyclonal (23N) and monoclonal antibodies directed against STOP proteins, staining is seen in the cytoplasm of neurons in the hippocampus (A), of motoneurons present in the gray matter of the spinal cord, and in axons present in the white matter (C). This staining is similar to that observed for neurofilaments using an anti-NFM antibody (B, D). Corpora amylacea recognizable using standard staining (F) are also well labeled with anti-STOP antibodies (E). Scale bars: A, B = 50 \mu m; C, D = 25 \mu m.

Fig. 3. Spheroids of ALS patients are labeled with anti-STOP antibodies. On a fixed spinal cord sample from an ALS patient, a typical spheroid is observed using standard hematein-phloxine-Luxol staining (A). On the same tissue, such spheroids are well labeled with antibodies against the neurofilament NFM subunit (B) or STOP proteins (C). Scale bar: 10 \mu m.
STOP proteins co-aggregate with intermediate filaments in spheroids of ALS patients, but not tubulin or kinesin. A frozen sample of the spinal cord of an ALS patient (ALS case 1) was double labeled with specific antibodies recognizing neuronal intermediate filaments (peripherin and NFM) and STOP proteins. Confocal microscope analysis showed the co-localization between STOP and neuronal intermediate filaments in spheroids. However, peripherin present in spheroids did not co-localize with either tubulin or kinesin proteins. A: anti-NFM; B: anti-peripherin; C: anti-NFM and anti-peripherin; D: anti-STOP (monoclonal); E: anti-peripherin; F: anti-STOP and anti-peripherin; G: anti-NFM, H: anti-STOP (polyclonal); I: anti-NFM and anti-STOP; J: anti-βIII-tubulin; K: anti-peripherin; L: anti-βIII-tubulin and anti-peripherin; M: anti-kinesin; N: anti-peripherin; O: anti-kinesin and anti-peripherin. Scale bar: 10 μm.

preparation from human brain (Fig. 1C). Moreover, when the first supernatant was incubated in the presence of 4 M glycerol to induce the formation of reticulated neurofilament networks (25) (but at 4°C and in the absence of GTP to avoid the polymerization of tubulin into microtubules), the majority of STOP proteins sedimented together with neurofilaments in the second pellet (Fig. 1D) and were deficient in the supernatant fraction enriched for unpolymerized tubulin. This indicates that STOP proteins interact preferentially with neurofilaments, and therefore could be considered as a new “neurofilament-associated protein.” The electrophoretic mobility of NFH proteins was similar in these different fractions (Fig. 1C, D), suggesting that the association of STOP proteins is not restricted with the phosphorylated isoform of NFH.

The immunohistochemical study of normal human nervous tissues shows that STOP proteins are primarily localized to neurons. Some blood vessels were also labeled with the polyclonal antibodies, which might be due to the presence of the F-STOP isoforms in fibroblasts. In neurons, N-STOP is distributed throughout the neuronal cytoplasm, including the cell body, dendrites, and axons. In the white matter of the spinal cord and in dorsal roots, staining was restricted to axons and was similar to that observed for neurofilaments. No staining of the myelin sheaths was observed. While a similar distribution was also observed in pathological
conditions, a striking observation was the systematic co-aggregation of STOP proteins with intermediate filaments (neurofilaments and peripherin) in spheroids of ALS patients. It has been shown previously that several components, such as kinesin (23), galectin-1 (29), SOD-1 (30), and β-APP (31), could also be found in axonal spheroids. While we did not analyze all these proteins, we found that only 82% and 58% of these spheroids contain tubulin or kinesin, respectively, whereas they were all positive for neurofilament and STOP proteins (Fig. 4J–O). Therefore, the co-immunolabeling of spheroids for STOP and neuronal intermediate filament proteins represents an excellent cytopathological marker for spheroids.

In addition to its role in the stability of microtubules, STOP has several calmodulin-binding domains, suggesting possible regulation by calcium (26). Despite a limited proteolysis occurring during their axonal transport (32), neurofilaments are normally degraded at the synaptic level (33). However, the proteolytic mechanism responsible for their elimination when they abnormally accumulate is still unknown. One hypothesis could be that STOP proteins may be involved in the regulation of neurofilament degeneration through their Ca/calmodulin-binding domains. As a consequence, when neurofilaments abnormally accumulate in axons, STOP proteins could be retained in spheroids to remove neurofilaments by activating the local degradation of these filaments. Alternatively, the accumulation of STOP proteins could be a primary event in this disease, which induces a dysregulation of the local limited proteolysis of neurofilaments, and therefore their aggregation. Another possibility could be that the microtubule network is altered in spheroids, as shown by the deficiency of tubulin and/or kinesin observed in several spheroids. As a consequence, STOP proteins are accumulated in these lesions in order to stabilize the last microtubules that are still present. Further investigations are necessary to elucidate the molecular mechanism by which such perturbations contribute directly or indirectly to the formation of axonal spheroids containing numerous neurofilaments and rare microtubules (34). In such a perspective, transgenic mice in which the STOP gene has been inactivated by homologous recombination display abnormal synaptogenesis probably as a result of an abnormal axonal transport (35). Therefore, the aggregation of STOP in axonal spheroids present in ALS patients could similarly alter the synaptic functions.

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