

Evaluation of loss of function as an explanation for *SPG4*-based hereditary spastic paraplegia

Joanna M. Solowska¹, James Y. Garbern² and Peter W. Baas^{1,*}

¹Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, USA and ²Department of Neurology and Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201, USA

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The spectrum of mutations (missense, non-sense and splice-site) associated with hereditary spastic paraplegia 4 (HSP-*SPG4*) (*SPG4*:OMIM#182601) has suggested that this autosomal dominant disease results from loss of function. Because the protein encoded by *SPG4*, termed spastin, is a microtubule-severing enzyme, a loss-of-function scenario for the disease suggests that corticospinal axons degenerate due to inadequate microtubule severing resulting from inactivation of one spastin allele. Lending more complexity to the situation, there are two major isoforms of spastin (M1 and M87) translated from two start codons. M87 is widely expressed, while M1 is appreciably detected only in adult spinal cord. Here, we focused on four HSP-associated mutations of the *SPG4* gene located outside of the AAA region essential for microtubule severing. We found that none of these mutations affected the enzymatic activity or expression levels of either M1 or M87. Three of the mutations resulted in dominant-negative activity of M1. Surprisingly, the S44L mutation, which is asymptomatic when present heterozygously, conferred dominant-negative activity, while the E112K mutation, which is symptomatic when present heterozygously, did not. Clinical symptoms reported for patients carrying the dominant-negative mutations L195V or 46Stop are not more severe than those reported for patients carrying the non-dominant-negative E112K mutation. These results indicate that there are cases of HSP-*SPG4* that cannot be explained by insufficient spastin microtubule-severing activity.

INTRODUCTION

The severing of microtubules is critically important for the development of the nervous system, and also for its maintenance throughout adult life (1). The severing of long microtubules into short ones is important, for example, for the ongoing transport of microtubules, given that only short microtubules are able to move in a rapid and concerted fashion within the axon (2,3). In addition, the severing event transforms single microtubules into many, thus creating greater numbers of free ends of microtubules that are able to interact with plus-end associated proteins and cellular cortical structures (4). Greater mobility of microtubules and higher numbers of microtubules are especially important for the formation of new axonal branches (3,5,6) and the same is presumably true of dendritic sprouting, which can occur throughout the life of the neuron. Indeed, studies have

shown that experimental manipulation of microtubule-severing activity can have profoundly detrimental effects on neurons (6–8).

Neurons express more than one type of microtubule-severing protein, but the degree to which the different severing proteins are functionally redundant still remains unknown. Mutations in the *SPG4* gene, which encodes the microtubule-severing protein termed spastin, are associated with roughly 40% of the known cases of hereditary spastic paraplegia (HSP) (9). The vast majority of more than 200 different mutations identified in patients suffering from HSP-*SPG4* are located in sites within the AAA region of the protein essential for microtubule-severing or in sites that, when mutated, delete the AAA region entirely (10). For this reason, it is generally believed that HSP-*SPG4* results from insufficient microtubule-severing activity (11,12). Knockdown of spastin results in pupal lethality in *Drosophila* (13) and

*To whom correspondence should be addressed. Tel: +1 2159918298; Fax: +1 2158439082; Email: pbaas@drexelmed.edu or peter.baas@drexelmed.edu

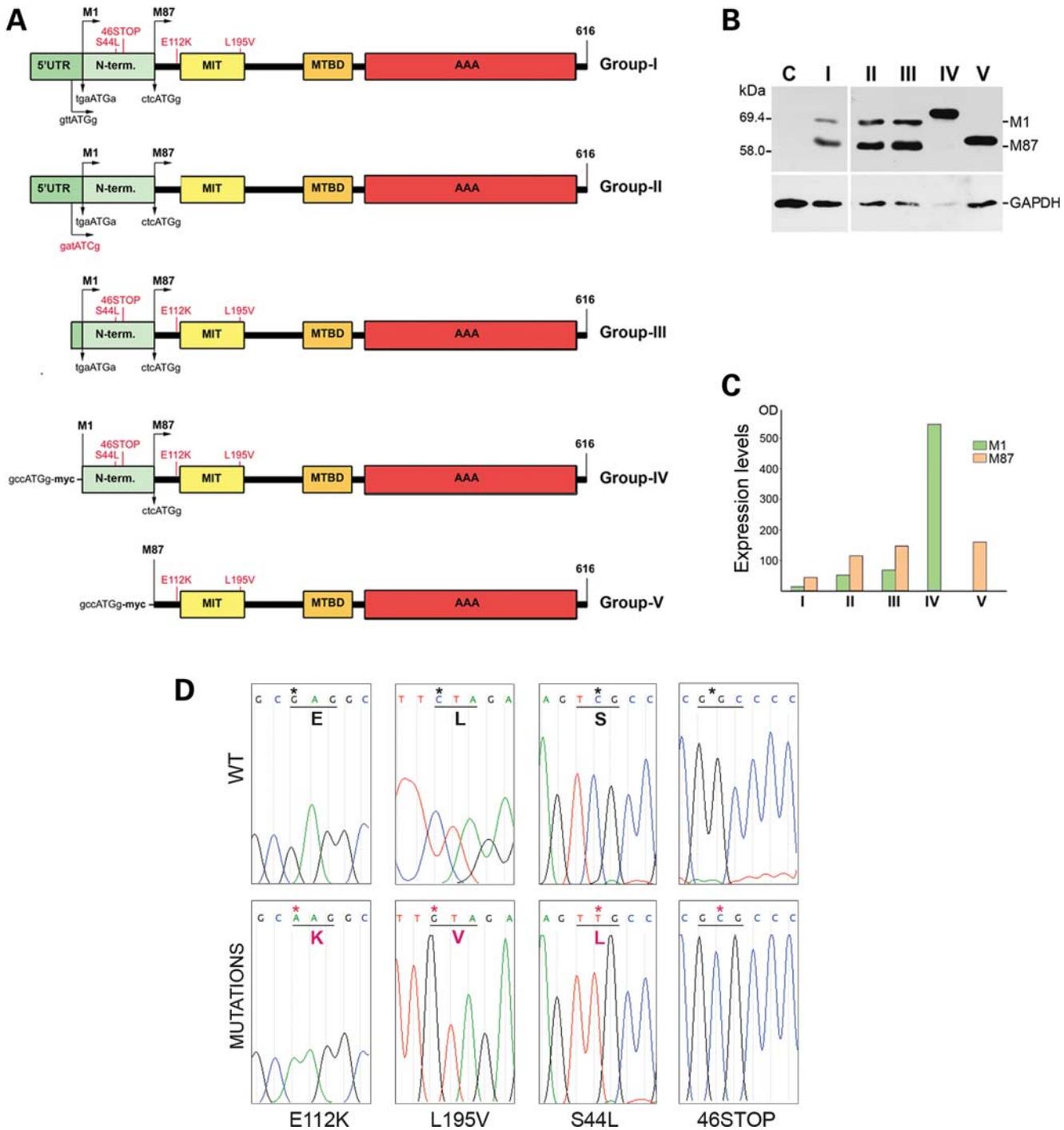


Figure 1. Human WT and mutated spastin constructs. (A) Schematic representation of spastin structural domains. 5'-UTR, 5' untranslated region, nucleotides -1 to -221, with an upstream ORF (uORF) with ATG within a good Kozak's consensus sequence (gttATGg); M1 initiation codon tgaATGa within a weak Kozak's consensus sequence with pyrimidine not purine in position -3 and A not G in position +4; M87 initiation codon ctcATGg in a better Kozak's consensus sequence with pyrimidine not purine in position -3 but G in position +4; N-term, N-terminal sequence present only in M1 spastin isoform; MIT, microtubule interacting and trafficking domain, amino acids 116-197; MTBD, microtubule binding domain, amino acids 270-328; AAA, ATPase associated with various cellular activities domain, amino acids 342-599. The ATG start codon in uORF was mutated to ATC in Group-II spastin construct or deleted from Group-III spastin constructs. To generate Group-IV and Group-V constructs, cDNAs encoding M1 or M87 were cloned into pCMV-Tag vector. In these constructs, endogenous M1 or M87 Kozak's sequences were replaced by perfect Kozak's sequences provided by c-myc tag. Locations of mutations S44L, 46Stop, E112K and L195V are indicated in red. (B) Detection of WT spastin isoforms with anti-spastin Sp/AAA antibody in extracts from control RFL-6 cells (lane C) and RFL-6 cells transfected with Group I-V constructs (lanes I-V). To detect low levels of spastin in cells transfected with Group-I construct and to avoid overexposure in case of Group-IV construct, varying volumes of extracts (as indicated by GAPDH levels) were used for immunoblotting. Transfection efficiency as assessed by the number of spastin positive cells per 200 cells was about 15-17% for all constructs. Therefore, differences observed in expression levels of spastin isoforms after transfection with constructs from different groups resulted from changes in translation levels and not from differences in levels of

causes dramatic defects in axonal outgrowth from motor neurons in the zebrafish embryo (8). However, in mouse models lacking active spastin (14,15), the developing CNS is not notably affected and even the homozygous *SpΔ/Δ* animals have only mild motor defects compared with most human patients suffering from HSP-*SPG4*.

The key to understanding how spastin mutations cause HSP may lie in the fact that *SPG4* has two start codons, one which produces a full-length isoform called M1 and the other which produces a slightly shorter isoform called M85 in rodents or M87 in humans (16–18). We recently reported that a truncated form of mouse M1 is deleterious to the axon, while the corresponding truncated form of M85 is harmless (18). This finding is particularly intriguing in light of our findings on rodents that M1 and M85 are distributed differently in the nervous system, with M1 only appreciably expressed in the adult spinal cord. In HSP-*SPG4* patients, degeneration of axons occurs almost exclusively in the corticospinal tracts, which are located within the spinal cord. Thus it may be that toxicity of the longer isoform of spastin is the main culprit in the nerve degeneration observed in people carrying the pathogenic mutations. This would be a gain-of-function scenario for the disease.

Further studies on the potential toxic properties of mutant spastin isoforms will be needed to directly test the merits of a gain-of-function model for HSP-*SPG4*. In the meantime, we have pursued a completely different approach by focusing on four spastin mutations that are associated with the disease but map to regions of the protein in which mutations would not be expected to diminish its microtubule-severing activity. We assessed whether these mutants are viable in terms of severing microtubules, and we investigated other properties of these mutants potentially relevant to their participation in a gain-of-function scenario for the neuropathy.

RESULTS

Human spastin constructs

In its simplest form, a loss-of-function scenario for HSP-*SPG4* posits that a reduction in the levels of functional spastin is responsible for an insufficient degree of microtubule-severing, and this in turn results in axonal degeneration (11,12). In our experiments, we tested whether mutations of spastin found in HSP-*SPG4* patients do indeed always lead to the loss of microtubule-severing activity. As a reduction of spastin function might result not only directly from the decreased enzymatic activity but also from decreased expression levels of mutated protein or from dominant-negative activity, we designed our experiments to test all of these possibilities.

To perform our studies, we developed a panel of new human spastin constructs encoding wild-type (WT) spastin or spastins with mutations located outside of the AAA

domain, identical to the mutations found in HSP-*SPG4* patients (Fig. 1A). The human *SPG4* gene has two start codons that produce a long isoform termed M1 and a shorter protein lacking the first 86 amino acids, termed M87 (16,17). To express M1 and M87 spastin isoforms simultaneously, we prepared Group-I WT or mutated spastin constructs that include 221 bp of 5'-UTR with an upstream open reading frame (uORF) containing ATG start codon in a good Kozak's consensus sequence gttATGg and endogenous Kozak's sequences at M1 and M87 translation initiation sites. The ATG start codon in uORF was mutated to ATC in the Group-II spastin construct or deleted from Group-III spastin constructs (Fig. 1A). In order to be able to express WT or mutated M1 and M87 isoforms separately, we cloned cDNA encoding M1 or M87 into pCMV-Tag vector, generating Group-IV and Group-V constructs. In these constructs, endogenous M1 or M87 Kozak's sequences were replaced by perfect Kozak's sequences provided by c-myc tag (Fig. 1A). Transcription of spastin mRNA in all constructs is driven by a strong CMV promoter.

To study the effects of mutations on microtubule-severing activity of human spastin isoforms, we chose to use RFL-6 rat fibroblasts. While these cells do not perfectly reflect the conditions within neurons, they have several advantages for the present studies. Unlike cultured neurons, this cell line expresses very little endogenous spastin, undetectable when compared with over-expressed human spastins (Fig. 1B, lane C). Thus, results from our functional experiments in these cells could be interpreted in terms of the ectopically expressed spastin isoforms and mutants, without the complication of endogenous spastin. In addition, these cells allowed us to use the Sp/AAA antibody directed against amino acids 337–465 in AAA domain, which are identical in rat and human spastins, to study the expression levels and stability of human spastin isoforms. The flat morphology of RFL-6 cells also affords greater resolution for observations and measurement of microtubule levels.

Transfection of RFL-6 cells with the WT Group-I construct resulted in simultaneous expression of low levels of M1 and M87 spastin isoforms (Fig. 1B, lane I). In the Group-I constructs, the M1 ORF starts with an AUG with Kozak's sequence deviating significantly from the consensus motif, with neither the purine in position –3 nor the guanine in position +4. This leads to a leaky scanning of the first AUG and preferred initiation of translation at the second AUG with a guanine in position +4. Moreover, uORF can further reduce translation from the M1 AUG resulting in a low level of M1 expression (17,19). Consistent with this idea mutation of AUG in the uORF to AUC (Group II) as well as deletion of uORF (Group III) resulted in considerably higher expression of both WT M1 and M87 spastin isoforms (Fig. 1B, lanes II and III). Replacement of the endogenous imperfect Kozak's sequences by a good Kozak's sequence, as in Group IV

transfection. (C) Quantification of the expression levels of WT spastin isoforms encoded by Group I–V constructs, normalized against GAPDH expression to adjust for number of transfected cells used for western blotting presented in (B). (D) Human spastin mutations associated with HSP-*SPG4* and generated for present studies. Mutation Glu112Lys (E112K) in exon 1 resulted from c.334G>A change, mutation Leu195Val (L195V) in exon 3 resulted from c.583C>G change, mutation Ser44Leu (S44L) located in the N-terminal region of M1 isoform in exon 1 resulted from c.131C>T change and mutation 46Stop in exon 1 resulted from insertion 114insC which created a frame shift and premature termination codon TAA in positions 138–140. Nomenclature of mutations refers to the cDNA sequence (GenBank NM_014946) with the A of the M1 translation initiation codon as +1.

constructs, further increased levels of WT M1 spastin expression. In contrast, such replacement did not increase expression levels of WT M87 spastin (Group V) (Fig. 1B, lanes IV and V). Particularly high levels of WT M1 protein detected in cells transfected with Group-IV construct might be attributed to increased translation of M1 from AUG codon in the context of a good Kozak's sequence, accumulation of insoluble M1 aggregates resisting degradation, or both. The expression levels of WT spastin isoforms encoded by Group I–V constructs, normalized against GAPDH expression to adjust for number of cells used for western blotting, are presented in Figure 1C. Transfection efficiency as assessed by number of spastin positive cells per 200 cells was about 15–17% for all constructs. Group IV and V constructs allow study of the severing activity of spastin isoforms expressed individually, while Group I–III constructs provide the unique opportunity to study interactions between untagged spastin isoforms expressed simultaneously at lower levels.

Human spastin mutations associated with HSP-SPG4

To assess microtubule-severing activity of spastins with mutations that lie outside of the AAA domain, we performed site-directed mutagenesis to create E112K, L195V, S44L and 46Stop mutations, identical to the mutations in HSP-SPG4 patients (Fig. 1D). Mutation Glu112Lys (E112K) in exon 1 affects a moderately conserved residue just upstream from the MIT domain (Fig. 1A). This mutation is associated with a case of pure HSP that started at the age of 41 and had a severity score 3 on a 4-point scale (20).

Mutation Leu195Val (L195V) in exon 3 affects a conserved residue in the MIT domain (Fig. 1A). This mutation was found in two cases of pure HSP with the onset of symptoms at the ages of 37 and 55 years. The severity scores were 2 and 3 on a 5-point scale where 1 indicates normal or very slight stiffness in the leg and 5, wheel chair bound (21).

Mutation Ser44Leu (S44L) is located in the N-terminal region of M1 isoform in exon 1 (Fig. 1A). This mutation is asymptomatic when heterozygous and is associated with a case of pure HSP with mild symptoms first noticed at the age of 60 years when homozygous (22). When present in addition to another mutation in the second spastin allele, the S44L mutation is associated with early childhood onset of HSP (21).

A frame-shift 46Stop mutation in exon 1 (Fig. 1A) is associated with a case of pure HSP (23). The premature stop codon created as a result of the mutation is located outside of M87 coding region and should only truncate M1.

Microtubule-severing activity of WT and mutated spastins

To test microtubule-severing activity of M87 spastin, RFL-6 cells were transfected with Group-V constructs encoding WT or mutated M87 isoforms. Twenty-four hours after transfection, cells were fixed and stained with anti-tubulin and anti-spastin antibodies (Fig. 2A–F). Fluorescence intensity in spastin positive cells was used to assess the levels of spastin expression. Microtubule levels were quantified by measuring fluorescence intensity of tubulin staining in cells expressing approximately equivalent levels of spastin. This experiment

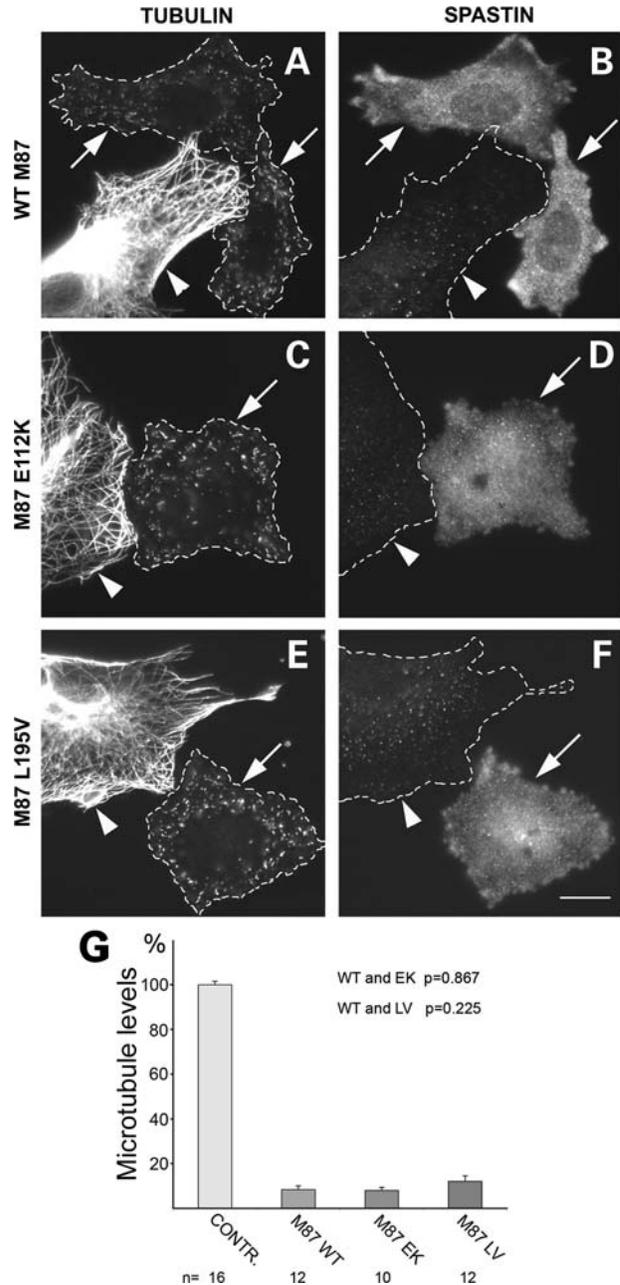


Figure 2. Microtubule severing by WT and mutated M87 spastin isoform. Levels of microtubule severing were assessed in RFL-6 cells expressing WT or mutated M87 spastin. Microtubules were visualized by immunostaining with anti-tubulin antibody (A, C and E). Spastin was detected by immunostaining with Sp/AAA anti-spastin antibody (B, D and F). Control RFL-6 cell had numerous, long microtubules (A, C and E, arrowheads) and did not express any detectable levels of spastin (B, D and F, arrowheads). In cells expressing WT M87 (A and B), M87 E112K mutant (C and D) and M87 L195V mutant (E and F) (arrows) microtubules were cut to small almost invisible fragments. WT or mutated M87 spastins were diffused evenly in the cytoplasm of transfected cells. To quantify levels of microtubule severing (G), fluorescence intensity of tubulin staining was measured in control or transfected cells expressing approximately equivalent levels of spastins. Microtubule levels in non-transfected, control cells. The differences in microtubule levels between non-transfected cells and cells transfected with WT and mutant M87 were statistically significant ($P > 0.0001$), but the differences in microtubule levels between cells expressing approximately equivalent levels of WT or mutated M87 spastins were not statistically significant. The P -values were calculated using unpaired t -test. Error bars: SEM. Scale bar: 20 μ m.

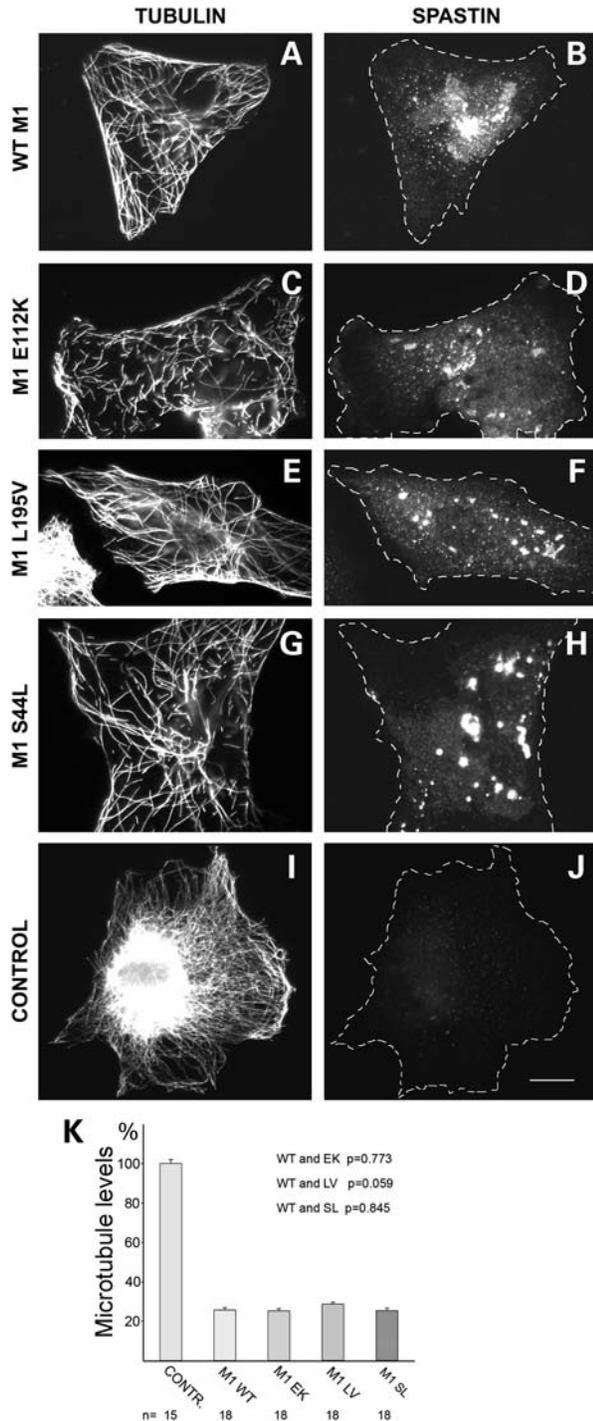


Figure 3. Microtubule severing by WT and mutated M1 spastin isoform. Levels of microtubule severing were assessed in RFL-6 cells expressing WT M1 (A and B), M1 E112K (C and D), M1 L195V (E and F) or M1 S44L (G and H) mutant. Spastin was detected by immunostaining with Sp/AAA anti-spastin antibody. Microtubules were visualized by immunostaining with anti-tubulin antibody. Control RFL-6 cell had numerous, long microtubules (I) and did not express any detectable levels of spastin (J). Microtubules in cells expressing WT (A) or mutated M1 spastin (C, E and G) were severed but were clearly longer than microtubules in cells transfected with WT or mutated M87 isoform (Fig. 2). Also, WT and mutated M1, unlike M87 (Fig. 2), formed clearly visible aggregates in cytoplasm of transfected cells (B, D, F and H). No significant differences in microtubule severing were detected in cells expressing WT or mutated M1 spastin. Quantification of

revealed that there was a statistically significant difference ($P < 0.0001$) in microtubule levels between control, spastin negative cells (Fig. 2A–F, arrowheads) and cells transfected with either WT or mutated M87 spastin (Fig. 2A–F, arrows and G). There was, however, no statistically significant difference in microtubule levels between cells transfected with WT or mutated M87 spastin (Fig. 2G). These results show clearly that M87 spastins with mutations outside of the AAA domain, which are found in HSP patients, have severing activity statistically indistinguishable from that of WT M87 spastin. The level of microtubule severing was only slightly (not statistically significantly) lower in cells transfected with M87 L195V mutant than in cells transfected with WT or E112K mutant.

The same experiment was repeated using M1-expressing Group-IV constructs. Besides WT M1, M1 E112K and M1 L195V mutants, the M1 S44L mutant was also included in these studies. Again, there was a statistically significant difference in microtubule levels between control, spastin negative cells and cells transfected with either WT or mutated M1 spastins ($P < 0.0001$), but no statistically significant difference in microtubule levels among cells transfected with WT or mutated M1 spastins (Fig. 3K). Again, the level of microtubule severing was slightly lower, but not statistically significantly lower in cells transfected with the L195V mutant. Overall however, severing activity of the M1 isoform was significantly lower than that of the M87 isoform. In cells transfected with WT or mutated M87 isoforms, only 8–12% of microtubule staining observed in control cells remained, compared with 25–29% for cells transfected with WT or mutated M1 isoforms (Figs 2 and 3). Also in M87 transfected cells, the levels of microtubule-severing correlated well with the concentration of this spastin isoform, and above some threshold level almost all spastin positive cells lost >95% of their microtubules and the remaining microtubules were very short. In contrast, regardless of expression levels, <4% of cells transfected with M1 had very short microtubules. Interestingly, higher expression levels of M1 resulted in the formation of spastin aggregates not observed in M87 expressing cells (compare Fig. 2B, D and F and Fig. 3B, D, F and H). These results indicate that, as observed for mouse spastin isoforms, the human M87 isoform has higher microtubule-severing activity, compared with the M1 isoform.

Expression levels of WT and mutated spastin isoforms

The mutations outside of the AAA domain tested in this work did not affect M1 or M87 microtubule-severing activity. Still, it was possible that while the enzymatic activity was not changed, the mutated spastins were more prone to misfolding and/or aggregation, and therefore more likely to be degraded by the ubiquitin-proteasome system (UPS) or by autophagy. In such a scenario, it is not the loss of enzymatic activity

microtubule levels is presented in (K). The differences in microtubule levels between non-transfected cells and cells transfected with WT and mutant M1 were statistically significant ($P > 0.0001$), but the differences in microtubule levels between cells expressing approximately equivalent levels of WT or mutated M1 spastins were not statistically significant. The P -values were calculated using unpaired t -test. Error bars: SEM. Scale bar: 15 μ m.

but the lower levels of active spastins that might affect the level of microtubule severing. To investigate this possibility, we first performed western blot analyses using extracts from RFL-6 cells transfected with Group-IV or Group-V constructs. The results indicated that there was no decrease in levels of M1 or M87 mutated proteins compared with WT M1 or M87 (Fig. 4A). Group-IV and -V constructs, however, expressed very high levels of spastins, particularly of the M1 isoform, and these high levels of exogenous proteins might overwhelm the transfected cells ability to degrade them.

To assess the expression of mutated spastins at more physiological levels, we used Group-I constructs that direct simultaneous synthesis of significantly lower levels of M87 and M1 (Fig. 4B and C, no MG132). The results presented in Figure 4B and C were obtained using greater volumes of cell extracts and longer exposure times, so the intensity of the protein bands in Figure 4B and C cannot be directly compared with that in Figure 4A. Figure 4B compares the expression levels of WT M1 and M87 with that of mutated M1 and M87 where both M1 and M87 carry the E112K or L195V mutation. Spastin mutants S44L and 46Stop, shown in Figure 4C, carry mutations located outside the M87 coding sequence and are therefore present only in the M1 isoform (Fig. 1A, Group-I constructs). Using Group-I constructs, we were again not able to detect any significant differences in expression levels between WT and mutated spastin isoforms (Fig. 4B and C, no MG132). Interestingly, the 46Stop mutation did not affect the levels of M87 synthesis (Fig. 4C, stop lane), and therefore we might expect HSP-*SPG4* patients carrying this mutation to have normal levels of WT M87 and possibly a very short N-terminal fragment of M1. The S44L mutation also did not affect expression levels of WT M87 (Fig. 4C, SL lane). These results indicate that there are *SPG4* mutations associated with HSP that do not affect spastin microtubule-severing activity or expression levels, and therefore clinical symptoms observed in patients carrying these mutations cannot be explained by loss of function. When proteolysis was inhibited for 6 h with the proteasome inhibitor MG132, accumulation of both M1 and M87 isoforms was observed in cells transfected with Group-I constructs. However, we did not detect any significant differences in accumulation of WT versus mutated spastins (Fig. 4B and C, MG132). There was, however, a significant difference in accumulation of M1 and M87 isoforms. Inhibition of UPS by MG132 resulted in roughly a 50% increase of the levels of WT or mutated M87 and about 3-fold increase of the levels of WT or mutated M1. This result indicates that M87 is the more metabolically stable isoform, while M1 is more readily degraded when expressed at low levels in cells with an active UPS. However, when the UPS system is compromised, the accumulation of M1 is significantly greater than the accumulation of M87 isoforms.

Our earlier experiments indicated that mutated M1 might be neurotoxic (18). The accumulation of neurotoxic proteins is a hallmark of many neurodegenerative diseases (24,25). Our short-term experiments using fibroblasts did not reveal any differences in accumulation of WT and mutated M1 isoforms, but this does not mean that these differences do not exist in neuronal tissues affected in HSP-*SPG4*. As HSP-*SPG4* in many cases develops over a long period of time with an

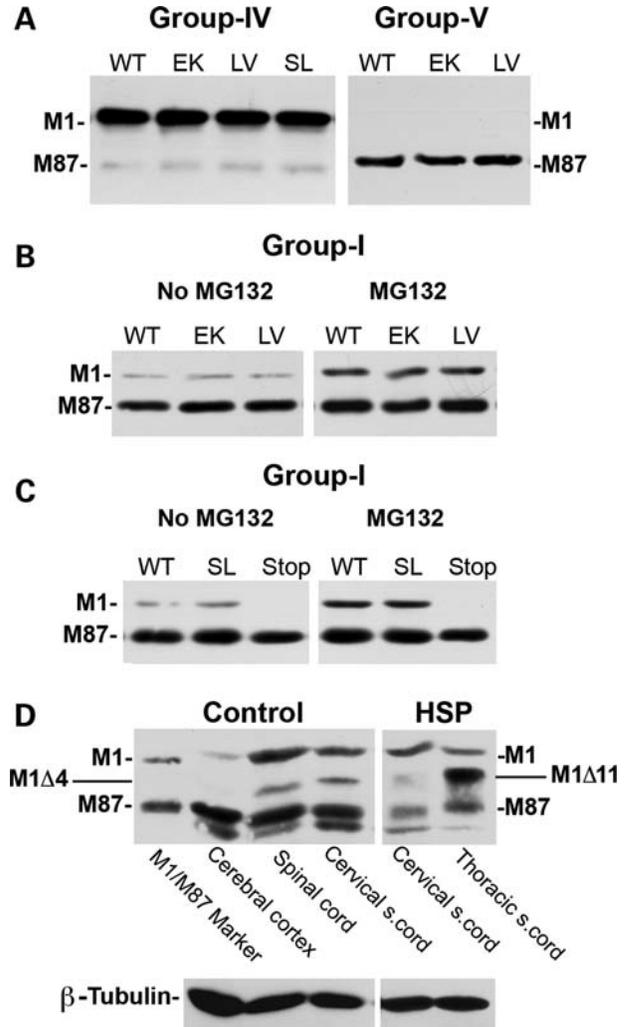


Figure 4. Effect of mutations on spastin expression levels. Expression levels of WT and mutated spastin isoforms were assessed in RFL-6 cells transfected with Group-I, IV or V constructs. The transfection efficiency was about 15–17% for all constructs. Extracts from transfected cells were subjected to SDS-PAGE electrophoresis and western blotting with Sp/AAA anti-spastin antibody. When indicated, 10 μ M proteasome inhibitor MG132 was added to the medium 23 h post-transfection, for 6 h. (A) No differences in the expression levels of the WT and mutated M1 or the WT and mutated M87 were detected in cells transfected with Group-IV or Group-V constructs, respectively. (B, no MG132) Owing to leaky scanning and re-initiation of translation, cells transfected with Group-I constructs express simultaneously M1 and M87 spastin isoforms. The levels of co-expressed M1 and M87 carrying E112K or L195V mutation were not lower than the levels of co-expressed WT M1 and M87. (C, no MG132) Mutations, S44L or 46Stop, outside M87 coding sequence did not affect translation from the downstream M87 start site, resulting in synthesis of normal levels of WT M87. Inhibition of UPS by MG132 (B and C, MG132) resulted in significantly greater accumulation of M1 than M87 isoform. No differences, however, were detected in accumulation of WT M1 and mutated M1 isoforms during this short-term proteasome inhibition. (D) Comparison of spastin expression in control human brain and spinal cord and HSP-*SPG4* spinal cord. The M1 and M87 spastin were detected in control and HSP-*SPG4* spinal cord. In control cerebral cortex, a prominent M87 and only a very weak M1 band was detected. Besides the 60 kDa M87 and 68 kDa M1 spastin, a 64 kDa band (M1 Δ 4), likely to correspond to spastin translated from a common splice variant lacking exon 4 encoding 32 amino acids, was also found in the control spinal cord. In the thoracic spinal cord sample from a patient carrying a splice mutation that skips exon 11, a strong band of 64 kDa (M1 Δ 11) that might correspond to M1 isoform missing 31 amino acids encoded by exon 11 was detected.

average age of onset at 30 ± 16 years (26), it is conceivable that even very small differences in metabolic stability lead to accumulation of mutated but not WT M1. To test this possibility, we compared spastin expression in control human cerebral cortex and spinal cord with that in the spinal cord of an HSP-*SPG4* patient (Fig. 4D). Human M87 spastin was expressed both in control cerebral cortex and spinal cord. A significant level of M1 expression, however, was detected only in control spinal cord and not brain. This expression pattern of human spastin isoforms resembles the distribution of M1 and M85 spastin isoforms in mouse tissues (18). Besides 60 kDa M87 and 68 kDa M1 spastin, a 64 kDa band (M1 Δ 4), likely corresponding to spastin translated from a common splice variant lacking exon 4 (27), was also found in the control spinal cord. The HSP spinal cord samples were obtained postmortem from a patient carrying a splice mutation that causes in-frame skipping of exon 11 (28). Exon 11 encodes 31 amino acids and the M1 isoform missing 31 amino acids should migrate as a band of approximately the same molecular weight of 64 kDa as M1 Δ 4, which is missing 32 amino acids. Interestingly, a prominent band of this size was present in the thoracic but not cervical spinal cord from the HSP-*SPG4* patient (Fig. 4D). Because the intensity of this band not only greatly exceeded the intensity of M1 Δ 4 but also M87 band, we concluded that this band might represent accumulated M1 Δ 11. The protein band corresponding to the M87 isoform lacking 31 amino acids encoded by exon 11 was not found. This might indicate that M87 Δ 11 but not M1 Δ 11 is prone to degradation. Interestingly, no prominent 64 kDa band was found in the cervical spinal cord from the same patient. This suggests that mutated M1 Δ 11 accumulates preferentially in the more distal axons. If the mutated M1 spastin is cytotoxic (as we have proposed), this observation may explain why axonal degeneration in HSP classically occurs as a 'dying back' from the synapse.

Co-aggregation of M1 and M87 spastin

The possibility that mutated M1 spastin might accumulate begs the question as to whether such accumulated M1 affects M87 microtubule-severing activity. When compared with M87, M1 has the ability to form presumably insoluble aggregates (compare Fig. 2B, D and F and Fig. 3B, D, F and H). To compare solubility of individually expressed WT and mutated M1 and M87 isoforms, we transfected cells with Group-V construct expressing WT or mutated M87 spastin or with Group-IV construct expressing WT or mutated M1 spastin. Transfected cells were then extracted with cell lysis reagent M-PER. After centrifugation, supernatants were collected and pellets were re-suspended in SDS-PAGE sample buffer. As expected, western blot analysis revealed that the majority of WT and mutated M87 is soluble and remains in supernatants (Fig. 5A), while WT and mutated M1 are evenly divided between soluble fraction in supernatants and insoluble fraction in pellets (Fig. 5B).

To test whether WT or mutated M1 affects the solubility of co-expressed M87, we transfected cells with Group-III constructs that simultaneously express elevated levels of M1 and M87. Western blot analyses revealed that the M87 isoform, which is present predominantly in supernatant when

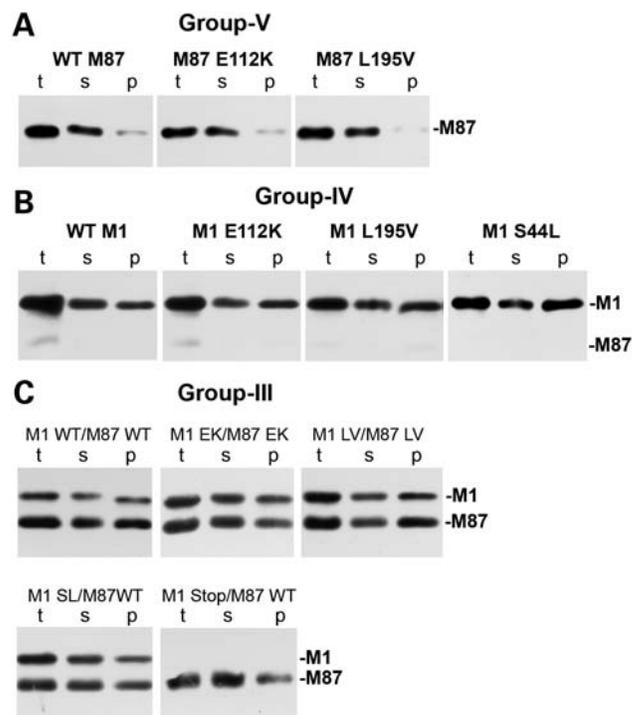


Figure 5. Solubility of M1 and M87 spastin. Solubility of individually expressed and co-expressed WT and mutated M1 and M87 isoforms was compared in extracts (t) from cells transfected with Group-V, IV or III constructs. After centrifugation, supernatants (s) were collected and pellets (p) were re-suspended in SDS-PAGE sample buffer. (A) Western blot analysis revealed that WT and mutated M87 isoform were mostly soluble and remained in supernatants (s). (B) WT and mutated M1 isoform were evenly divided between the soluble fraction in supernatants (s) and the insoluble fraction in pellets (p). (C) The M87 isoform when co-expressed with the WT or mutated M1 isoform was found both in supernatants (s) and pellets (p). Even a short 46-amino acid fragment of M1 undetectable with Sp/AAA anti-spastin antibody, resulting from the 46Stop mutation, seemed to be able to trap WT M87 into insoluble aggregates found in a pellet. The WT and mutated spastin levels in the insoluble fractions were not significantly different.

expressed alone, becomes evenly divided between supernatant and pellet when expressed in the presence of M1 (compare Fig. 5A with C). These results indicate that the solubility of M87 can be modulated by M1. The levels of insoluble M87 did not seem to be affected by HSP-related mutations, and no significant differences in solubility were found between WT and mutated spastins (Fig. 5C). Interestingly, in cells transfected with the construct co-expressing WT M87 and a short 46-amino acid fragment of M1 (M1Stop mutant), some M87 isoform was found in the pellet, suggesting that even a very short fragment of M1 (not recognized by our anti-spastin antibody directed against AAA domain) can co-aggregate with M87. We do not know whether M1/M87 co-aggregation is in any way specific, but it is reasonable to assume that M87 in the insoluble fraction does not sever microtubules. Therefore, accumulated M1 might decrease the severing activity of M87 by trapping this isoform in insoluble aggregates.

Dominant-negative activity of mutated M1 spastin

As the expression of M1 seems to be tightly regulated, the levels of M1 in spinal cord are detectable but low compared

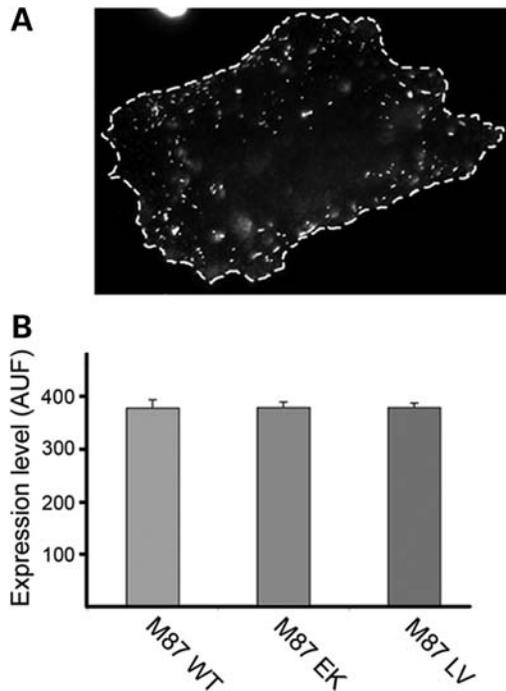


Figure 6. Microtubule-severing activity of individually expressed WT and mutated M87 isoforms. To compare the severing activity of WT and mutated M87, the fluorescence intensity of spastin staining was measured in 25–30 randomly chosen cells with very short microtubules as in (A). The 8–10 lowest values were then used to calculate the lowest levels of spastin required for severing. Quantitative analysis (B) revealed that the lowest expression levels of WT and mutated M87 needed for microtubule severing were statistically the same ($P > 0.5$).

with M87 (the expression level of putative M1 Δ 11 in the thoracic spinal cord of the HSP patient was an exception). M1 at this low concentration is probably soluble and would not affect the M87 isoform by co-precipitation, but it is still possible that mutated soluble M1 affects M87 activity in a dominant-negative manner. Our next set of experiments was designed to test this possibility. First, we expressed WT and mutated M87 isoforms individually and compared spastin levels required for severing of all microtubules in a given cell to very short fragments, as shown in Figure 6A. The fluorescence intensity of spastin staining in 25–30 randomly chosen cells with short microtubules was measured and 8–10 lowest values were used to calculate the lowest levels of spastin required for severing. As expected from results of our earlier experiments, the expression levels of WT and mutated M87 isoforms sufficient to cut microtubules to short fragments were statistically the same (Fig. 6B), indicating again that the mutations we tested did not affect severing activity of individually expressed M87.

To test whether M1 affects the microtubule-severing activity of M87, we transfected cells with Group-III constructs co-expressing elevated levels of WT or mutated M1 and M87 and evaluated the lowest levels of spastin required for severing of microtubules to short fragments as described above. We found that co-expression of WT M1 did not significantly affect the microtubule-severing activity of WT M87 (compare the expression levels of spastin in Figure 7A, WT M87 alone and Fig. 7C WT M1/WT M87). However, even

at this relatively low level of expression, some cells co-expressing WT M1 and M87 showed some aggregates (Fig. 7C). In contrast to WT M1, M1 isoforms carrying S44L or 46Stop mutations localized outside of the M87 coding sequence affected severing activity of co-expressed WT M87. The levels of spastin expression required for severing were significantly higher in both cases (Fig. 7I), suggesting dominant-negative activity of mutated M1 (compare spastin levels in cells in Fig. 7C, E and G). The decrease in severing activity cannot be attributed to the presence of spastin aggregates because cells transfected with mutant spastin showed similar levels of aggregates as cells transfected with WT spastin. Quantitative analyses of these experiments are shown in Figure 7I. Interestingly, these results indicate the same level of dominant-negative activity for HSP-associated 46Stop mutation and for the S44L mutation, which is non-pathogenic when heterozygous.

Our next experiment tested the dominant-negative activity of mutated M1 E112K or M1 L195V co-expressed with mutated M87 E112K or M87 L195V. Expressed individually, none of these mutated isoforms showed lower severing activity than WT spastin (Fig. 6B). Surprisingly, the severing activity of co-expressed M1 E112K/M87 E112K mutant was the same as WT M1/M87 (compare Fig. 8A and B with C and D). This result indicates that not all mutations confer dominant-negative activity and that HSP-*SPG4* can be associated with a mutation (E112K) that has virtually no effect on spastin severing activity, while on the other hand a mutation S44L, which is non-pathogenic when present heterozygously, might have a dominant-negative effect. In contrast to the E112K mutant, the severing activity of M1 L195/M87 L195V was significantly diminished (compare Fig. 8A and B with E and F). To achieve the same level of microtubule severing, the expression level of M1 L195/M87 L195V had to be significantly higher than that of WT M1/M87 (compare Fig. 8A and G). Again, the decrease in severing activity could not be attributed to spastin aggregation because there were fewer aggregates in low expressing cells with less-severed microtubules than in higher expressing cells with very short microtubules (compare Fig. 8E and F with G and H). We cannot rule out the possibility that aggregation of mutated M1 might protect M87 activity by decreasing the pool of soluble, dominant-negative M1. The quantitative analyses of expression levels of E112K and L195V mutants required for severing of microtubules to short fragments are shown in Figure 8I.

DISCUSSION

Genetic analyses on the types of mutations found in the *SPG4* gene of HSP patients have suggested a loss-of-function model for the disease (29,30). However, the idea that there is not enough active spastin available to perform its necessary functions is hard to reconcile with the fact that there are no developmental abnormalities in the patients, and that the degeneration occurs almost exclusively in the corticospinal tracts. Also, patients carrying different mutations in the *SPG4* gene expected to produce varying levels of spastin inactivation show no significant difference in age of onset, clinical

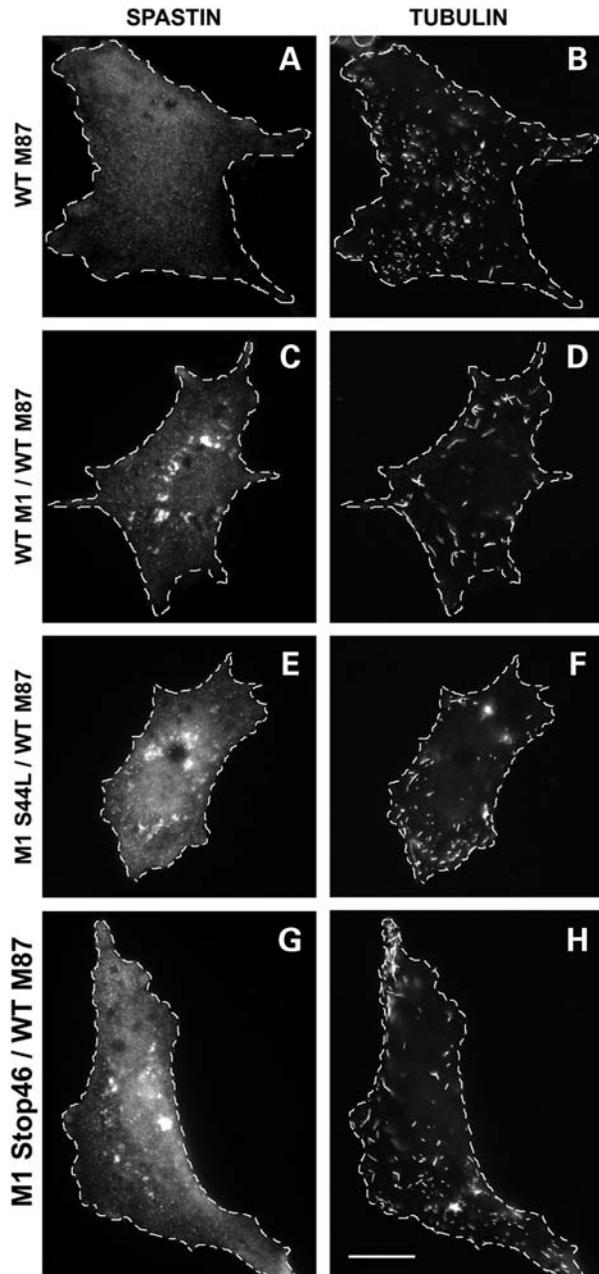


Figure 7. Dominant-negative effect of mutated M1 isoform on microtubule-severing activity of WT M87 isoform. To test whether mutated M1 isoform had a dominant-negative activity, the lowest levels of spastin required for

progression or severity of symptoms. On the other hand, the age of onset and the severity of symptoms can differ greatly in patients from the same family carrying identical mutations and presumably having the same levels of active spastin (30,31). This apparent lack of correlation between the levels of spastin microtubule-severing activity and severity of HSP-*SPG4* seems to be universal and difficult to reconcile with models claiming that the loss of spastin function alone is responsible for the disease.

Our strategy for the present work was to show that HSP-*SPG4* has actually been diagnosed in patients carrying spastin mutations that do not affect microtubule-severing activity. To test this possibility, we selected four mutations outside of the AAA domain (S44L, 46Stop, E112K and L195V) that are associated with HSP and showed that these mutations did not detectably affect the microtubule-severing activity of either the M87 or M1 isoform of spastin. Also the levels of expression of the mutated spastins were indistinguishable from those of the WT proteins. Interestingly, however, we found that the enzymatic activity of M87 spastin could be diminished by dominant-negative effects produced by mutated M1 in the case of some but not all of the mutations we studied or by increased aggregation of M87 induced by insoluble mutated M1.

While the loss of spastin microtubule-severing activity in an individual patient might be the greatest in the spinal cord owing to dominant-negative activity of mutant M1, the levels of spastin activity might still differ significantly among patients carrying different mutations. However, clinical symptoms reported for patients carrying L195V or 46Stop dominant-negative mutations were not more severe than those of the non-dominant-negative E112K mutant carrier with near normal levels of active M87 spastin (20,21,23). Interestingly, the loss of spastin microtubule-severing activity might be greater in the case of an asymptomatic S44L mutation than in the case of HSP associated with the E112K mutation. The S44L mutation, located outside of the M87 coding sequence, does not affect synthesis of WT M87 but M1 S44L mutant might lower M87 activity in a dominant-negative manner, while M1 E112K is not a dominant-negative mutation. We recognize that the group of patients carrying the missense mutations outside the AAA domain is very small,

severing of all microtubules in a given cell to very short fragments were compared in cells expressing the WT M87 alone (A and B), or in cells co-expressing WT M1 and WT M87 (C and D), M1 S44L and WT M87 (E and F) or M1Stop and WT M87 (G and H). All cell images were obtained using identical imaging criteria such as gain and exposure time. The brightness and contrast of all images were adjusted in identical way using Adobe Photoshop. Quantitative analysis (I) revealed that the lowest expression levels of spastin needed for microtubule severing were the same for WT M87 expressed alone or co-expressed with WT M1 ($P = 0.41$). However even at this low level of expression, some aggregates formed in cells transfected with WT M1/WT M87 construct (C) but not in cells transfected with M87 alone (A). When compared with WT M1/M87, co-expression of M1 isoforms carrying S44L or 46Stop mutations localized outside of M87 coding sequence significantly increased the levels of spastin needed for microtubule severing ($P = 0.001$ for M1 S44L/M87 and $P = 0.0043$ for M1 Stop46/M87). The P -values were calculated using unpaired t -test. Error bars: SEM. AUF (arbitrary unit of fluorescence) represents the sum of grey values of all pixels in a selected cell divided by the number of pixels. Scale bar: 20 μ m.

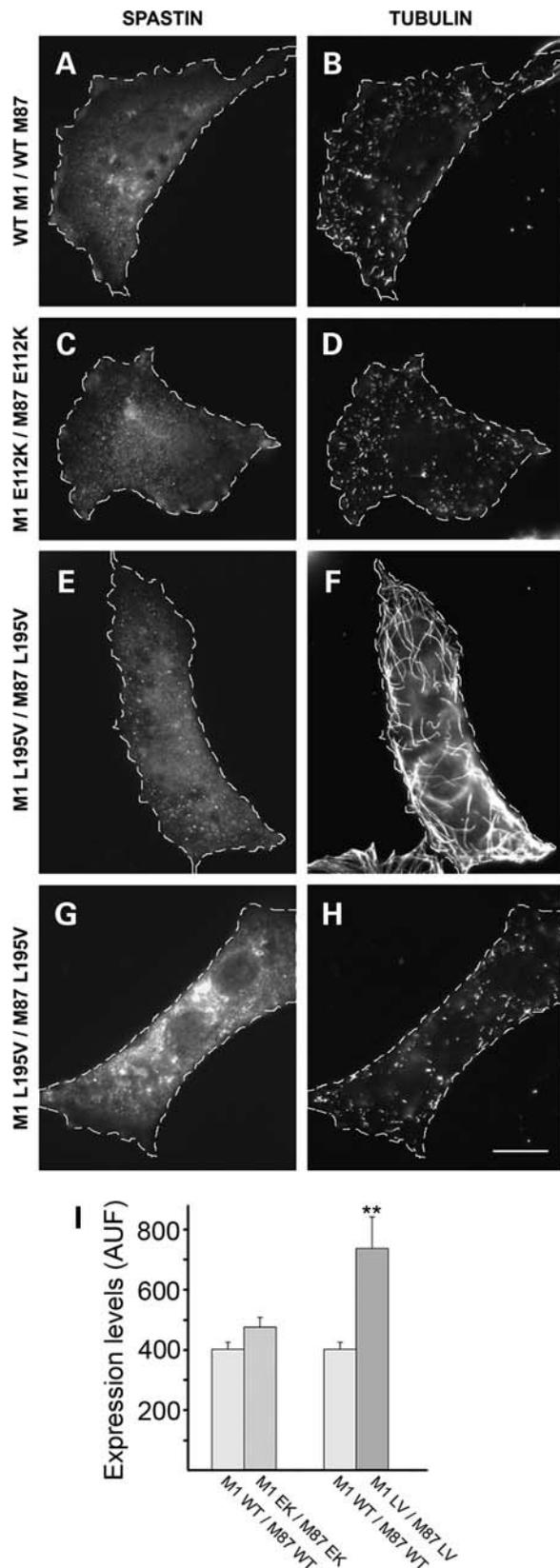


Figure 8. Not all mutated M1 have dominant-negative activity. The lowest levels of spastin required for severing of all microtubules in a given cell to

and hence these results should be interpreted with caution. Even so, it is notable that the simplest interpretation of our data is consistent with the body of existing clinical evidence, discussed above (30,31), indicating no correlation between the loss of spastin microtubule-severing activity and the severity of HSP symptoms.

In evaluating the loss-of-function scenario, we must also consider the possibility that spastin is involved in activities unrelated to the severing of microtubules. For example, spastin's MIT domain interacts with ESCRT-III complex-associated endosomal protein CHMP1B, which is important for endosomal membrane trafficking. Mutations in the MIT domain prevent spastin from localizing properly during cell division (32,33), and hence it is possible that a mutant form of spastin might sever microtubules competently but not be targeted to the right places in the neuron. The N-terminal amino acids 1–132 of spastin are also required for recruitment of atlastin, a Golgi-localized integral membrane protein GTPase. Mutations in atlastin are associated with a form of HSP, *SPG3A* (OMIM 182600), and one of the mutations, found in an *SPG3* patient, prevents the interaction of atlastin with spastin (34). Mutations in spastin might, in turn, affect its interactions with atlastin. These observations suggest the possibility of an alternative loss-of-function scenario wherein it is specifically a loss of M1 function that causes corticospinal tracts to degenerate. On one hand, this makes some sense because the levels of M1 are more limiting than the levels of M87, but the question arises as to why the corticospinal axons were healthy throughout the development, prior to the appearance of any detectable M1. Moreover, at least at present, there does not seem to be any apparent pattern in the pathological mutations that might predict a common mechanism unrelated to microtubule severing.

As an alternative explanation for HSP-*SPG4*, we propose a gain-of-function mechanism, at least in the case of certain mutations. Because the enzymatic activity of the human mutants in the present studies was intact, it would be difficult to distinguish the results of over-expression studies as being due to detrimental effects of cytotoxicity versus detrimental effects of overly severed microtubules. However, even the results of the experiments with these mutants provided some indications of their possible cytotoxicity. Recent studies from our laboratory indicate that the M1 isoform of spastin, when truncated so that it cannot sever microtubules, produces

very short fragments were compared in cells co-expressing the WT M1/M87 (A and B) with that in cells co-expressing M1 E112K/M87 E112K mutant (C and D) or M1 L195V/M87 L195V mutant (E and F). To achieve the same level of microtubule-severing, the expression level of M1 E112K/M87 E112K (C and D) was similar to that of WT M1/WT M87 (A and B), but the expression level of M1 L195V/M87 L195V had to be significantly higher (G and H). This decrease in severing activity could not be attributed to co-aggregation because there was less aggregates in low expressing cells with less severed microtubules (E and F) than in higher expressing cell with very short microtubules (G and H). As expected, quantitative analysis (I) showed that the difference in the level of expression needed for microtubule-severing was statistically not significant between WT M1/M87 and M1 E112K/M87 E112K mutant ($P = 0.0752$), but statistically significant between WT M1/M87 and M1 L195V/M87 L195V mutant ($P = 0.003$). The P -values were calculated using unpaired t -test. Error bars: SEM. AUF (arbitrary unit of fluorescence). Scale bar: 20 μm.

deleterious effects on the vitality of the axons of cultured rodent neurons, and on fast axonal transport in squid axoplasm (18). Our current results showing that M1 is considerably more prone to aggregation than M87 suggest that keeping the levels of M1 very low might be crucial for the health of the axon. *In vivo*, M1 expression seems to be tightly regulated by translation-reducing mechanisms often employed to prevent harmful overproduction of potent proteins (19). Our experiments confirmed the earlier reports (16,17) that expression of M1 and to a lesser degree expression of M87 can be significantly increased by removing the endogenous GC-rich 5'-UTR and by deleting the upstream AUG sequence. The greatest effect on expression level of M1 was accomplished when the 5'-UTR with the upstream AUG was deleted and a weak endogenous Kozak's sequence at M1 initiation codon was replaced with a good Kozak's sequence. The low levels of M1 might also be maintained by proteasome degradation. Experimental inhibition of the UPS resulted in significantly greater accumulation of M1 than M87 isoforms. Age-related decrease of UPS activity (35) might therefore contribute more to accumulation of mutated M1 than M87. It is also possible that mutated M1 impairs UPS activity, a phenomenon reported for mutated huntingtin protein (36). In addition, above a certain threshold level, the mutated M1 might form insoluble aggregates resistant to degradation.

The accumulation of aggregated proteins is a hallmark of many neurodegenerative diseases (24,25,37). While it is disputed whether protein aggregation is beneficial or harmful, it seems certain that even when initial sequestration of toxic proteins in aggregates might be advantageous, eventually such growing aggregates will trigger apoptosis or block neuronal processes and impair axonal transport. We have shown that the over-expressed M1 can co-aggregate with M87, and therefore it is conceivable that other important proteins might be also trapped in M1 aggregates. Our analysis of spastin expression in a spinal cord of a HSP patient carrying a splice mutation that causes in-frame deletion of exon 11 does not prove but is consistent with an accumulation of mutated M1 but not mutated M87. It is important to establish whether such accumulation of mutated M1 is typical of HSP and whether it correlates with the severity of HSP symptoms. This, however, might not be easily accomplished because the spinal cord is the only tissue where this could be done in a meaningful way, if our model is correct. Data from the other laboratories confirmed that neither brain (15) nor lymphoblastoid cells from *SPG4* patients expressed mutated M1 (6), but analyses of HSP-*SPG4* spinal cords were not shown in these reports.

There is compelling experimental evidence suggesting that mutated proteins in solution might be even more problematic than the aggregates they subsequently form. It was shown, for example, that the formation of large-mutated huntingtin aggregates might improve cell survival while diffuse huntingtin may be highly reactive and cytotoxic (38). As the levels of M1 expression are tightly regulated, the concentration of this isoform is low and both WT and mutated M1 might be mostly soluble *in vivo*. In our experiments, we found that three out of four mutated M1 isoforms when expressed at low levels inhibited M87 activity in a dominant-negative manner. To sever microtubules, spastin assembles into hexamers (39,40).

The dominant-negative function might result from function-blocking insertion of soluble mutated M1 into the hexamer or from direct interaction of mutated M1 with M87 that prevents hexamer formation. The latter might explain why the M1 46Stop mutant that lacks domains required for hexamer formation can still have dominant-negative activity. As the WT soluble M1 does not have dominant-negative activity, we hypothesize that mutations affect the conformation of M1 and facilitate abnormal interactions with M87. It is not unreasonable then to consider that mutations that change interactions between M1 and M87 might also affect interactions of mutated M1 with other proteins.

In conclusion, our results demonstrate that there are spastin mutants associated with HSP that retain their full ability to sever microtubules. We have shown that the microtubule-severing activity of the M87 isoform can be diminished by dominant-negative activity of the mutated M1, but this effect occurs in some but not all cases of the pathogenic mutations. Therefore, there are some HSP-*SPG4* patients that display the full spectrum of HSP symptoms, in whom there would be no expectation of diminished microtubule-severing activity. We cannot dismiss the possibility that loss of function may contribute to the degeneration observed in patients with other types of mutations, but we believe the present results fortify the need to investigate gain of function as a principal cause of the pathology. Indeed, a gain-of-function scenario based on toxicity of the mutated M1 might help explain the notable differences in onset ages and symptom severity characteristic of HSP-*SPG4*. It seems reasonable that differences exist among patients in their ability to compensate for potential losses of spastin activity and/or their ability to tolerate or dispose of toxic proteins. Gaining insight into these mechanisms, particularly the processes involved in the disposal of misfolded proteins might prove beneficial not just for the HSP-*SPG4* patients but also to patients suffering from other neurodegenerative disorders.

MATERIALS AND METHODS

Preparation of spastin constructs

Human cDNA in Bluescript SK with 221 nucleotides of 5'-UTR and a sequence encoding the full-length spastin missing exon 4 (Clone MGC: 166832, clone sequence BC150260.1) was purchased from Invitrogen (Carlsbad, CA, USA). The *XbaI/NcoI* cDNA fragment encoding exon 4 of the human spastin was amplified by reverse-transcription PCR using total RNA isolated from HeLa cells. After sequencing, the *XbaI/NcoI* fragment with exon 4 was used to replace the *XbaI/NcoI* fragment without exon 4 and to generate cDNA with 5'-UTR encoding full-length human spastin. To generate point mutations c.334G > A, c.583C > G, c.131C > T or insertion 114insC, the QuickChange II XL site-directed mutagenesis kit (Stratagen, LaJolla, CA, USA) was used according to the manufacturer's instructions. The presence of the mutations was confirmed by DNA sequencing. The nomenclature of the mutations refers to the cDNA sequence (GenBank NM_014946) with the A of the M1 translation initiation codon as +1. To prepare Group-I constructs the WT or mutated, full-length spastin cDNA with 221 nucleotides of

5'-UTR was cloned into mammalian expression vector pCMV-Tag (Stratagen) from which the myc-tag sequence was deleted. To create Group-II construct, the gttATG sequence of the upstream ORF was mutated to the gatATCg sequence. This mutation destroyed the upstream start codon and created an *EcoRV* restriction site used to delete the 5'-UTR in Group-III constructs. To create Group-IV and Group-V constructs expressing M1 and M87 isoforms separately, the WT or mutated cDNAs encoding M1 isoforms (amino acids 2–616) or M87 isoforms (amino acids 88–616) were cloned into pCMV-Tag vector in frame with the c-myc tag that provided a start codon with a perfect Kozak's sequence.

Cell transfections and immunostaining

RFL-6 rat fibroblasts cultured in Lab-Tek chambers (Nalgene, Denmark) were transfected with spastin constructs using Lipofectamine 2000 (Invitrogen) as recommended by manufacturer. Twenty-eight hours after transfection, cells were fixed with 0.2% glutaraldehyde and 4% paraformaldehyde, and then post-extracted with 0.1% TritonX-100. The cultures were then double-labeled by exposure first to anti-spastin Sp/AAA polyclonal antibody (1:20,000) (18), overnight at 4°C and then to Alexa Fluor 488-conjugated goat anti-rabbit (1:500) (Invitrogen, Eugene, OR, USA) and Cy3-conjugated anti- β -tubulin mouse monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 37°C. Images were obtained on an Axiovert 200 microscope (Carl Zeiss) equipped with a high-resolution CCD camera (Orca ER, Hamamatsu, Japan) using identical imaging criteria such as gain and exposure time. Mean grey values (the sum of grey values of all the pixels in a selected cell divided by the number of pixels) of raw images were obtained using AxioVision software and presented as arbitrary units of fluorescence (AUF). After measuring the intensity of fluorescence, the brightness and contrast of all images were adjusted in identical manner using Adobe Photoshop.

Cell transfection and western blotting

To compare expression levels of WT and mutated spastin isoforms, 3×10^5 of RFL-6 cells were plated into one well of 24-well plate and transfected using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Transfection for each individual construct was repeated four times and transfection efficiency was evaluated in separate wells by counting the number of spastin positive cells per 200 cells. To avoid extreme over-expression of microtubule-severing spastin combined with cytotoxic effects of lipofectamine, the conditions of transfection were adjusted to keep transfections at low levels of 15–17% for all constructs tested. Twenty-three hours after transfection, fresh medium with or without 10 μ M proteasome inhibitor MG132 (Sigma-Aldrich) was added to all wells. Six hours later, cells were washed with PBS and collected in 200 μ l of SDS–PAGE sample buffer (Sigma-Aldrich) with Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Typically 5–30 μ l of lysates were used for SDS–PAGE electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose

and immunoblotted with the anti-spastin Sp/AAA antibody (diluted 1:20,000) as described previously (18). To assess the solubility of WT and mutated spastins, 6×10^5 of RFL-6 cells were plated into one well of 12-well plate and transfected using Lipofectamine 2000. Cells in three wells were transfected with each individual construct and transfection efficiency was 15–17% for all constructs tested. Twenty-nine hours after transfection, cells were washed with PBS and collected in 500 μ l of M-PER Mammalian Protein Extraction Reagent with Halt protease inhibitor cocktail (Thermo Scientific). A total of 100 μ l of lysates were saved to assess total spastin expression, the remaining 400 μ l of lysates were spun for 25 min, 16 000xg at 4°C in an Eppendorf microcentrifuge. Supernatants were collected and pellets were re-suspended in 200 μ l of SDS–PAGE sample buffer. Five to 30 μ l aliquots were used for SDS–PAGE electrophoresis. The anti-spastin Sp/AAA antibody (diluted 1:20,000) was used for immunoblotting as described above.

Human tissues

10 mg/ml solutions of SDS-solubilized proteins prepared from normal human tissues were purchased from Clontech (Mountain View, CA, USA). Normal cerebral cortex tissue was pooled from eight male/females, ages 28–60. Normal whole spinal cords were pooled from 49 male/females, ages 15–66. A normal whole cervical spinal cord was from a 49-year-old male. All normal tissue donors suffered from trauma and sudden death. The lysates of the cervical and thoracic spinal cord from a patient with HSP-*SPG4* carrying a splice mutation that resulted in skipping of spastin exon 11 (28) were prepared using 100 mg tissue per 1 ml of SDS–PAGE sample buffer. Aliquots containing 150 μ g of proteins were subjected to SDS–PAGE electrophoresis followed by western blotting with anti-spastin Sp/AAA antibody.

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Conflict of Interest statement. None declared.

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