

Recognition of C-terminal amino acids in tubulin by pore loops in Spastin is important for microtubule severing

Susan Roehl White,^{1,2} Katia J. Evans,^{1,2} Jeffrey Lary,³ James L. Cole,^{3,4} and Brett Lauring^{1,2}

¹Department of Pathology and ²Taub Institute, College of Physicians and Surgeons, Columbia University, New York, NY 10032

³National Analytical Ultracentrifugation Facility and ⁴Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269

Spastin, an AAA ATPase mutated in the neurodegenerative disease hereditary spastic paraplegia, severs microtubules. Many other AAA proteins form ring-shaped hexamers and contain pore loops, which project into the ring's central cavity and act as ratchets that pull on target proteins, leading, in some cases, to conformational changes. We show that Spastin assembles into a hexamer and that loops within the central pore recognize C-terminal amino acids of tubulin. Key pore loop amino acids are required for severing, including one

altered by a disease-associated mutation. We also show that Spastin contains a second microtubule binding domain that makes a distinct interaction with microtubules and is required for severing. Given that Spastin engages the MT in two places and that both interactions are required for severing, we propose that severing occurs by forces exerted on the C-terminal tail of tubulin, which results in a conformational change in tubulin, which releases it from the polymer.

Introduction

Hereditary spastic paraplegia (HSP) is a genetic disease characterized by neurodegeneration of the long axons of spinal neurons of the dorsal columns and cortical spinal tract, with clinical symptoms resulting from this pathology. HSP is a heterogeneous disorder both clinically and genetically with dominant, recessive, and X-linked forms linked to >20 loci (Fink, 2002). One of the most common forms of autosomal dominant HSP is caused by mutations in Spastin (*SPG4*; Hazan et al., 1999). We and others have shown that Spastin is a microtubule (MT)-severing enzyme (Evans et al., 2005; Roll-Mecak and Vale, 2005), meaning that Spastin makes internal breaks in MTs. The mechanisms of enzyme assembly, tubulin recognition or engagement, and severing are unknown. Many disease-associated missense mutations in Spastin impair both ATPase and MT-severing activities, suggesting that HSP and axon loss paradoxically result from failure of MT severing.

Spastin is a member of the AAA ATPase (ATPases associated with various cellular activities) family (for review see Hanson and Whiteheart, 2005). The defining feature of these

enzymes is the ~250-amino-acid module known as the AAA ATPase domain. In the majority of cases examined, AAA proteins assemble into ring-shaped hexamers, sometimes in an ATP-dependent manner. In part, this is because the ATP binding pockets are constituted from residues from adjacent monomers in the oligomer.

Many AAA proteins disassemble protein complexes without destroying their substrates. For instance, NSF apparently unwinds the coiled coils that define the SNARE complex, resulting in SNARE complex disassembly so that the individual SNARE components can be reused for successive rounds of membrane fusion (Sollner et al., 1993).

Particularly well understood AAA proteins are the so-called translocators. These enzymes, whose members include the Clp family, are often involved in protein quality control. The hexameric forms of these proteins engage target proteins by recognizing C-terminal peptides (Gottesman et al., 1998) and pulling or translocating these polypeptides through the central pore of the hexamer (Hoskins et al., 1998; Ishikawa et al., 2001). Successive cycles of ATP hydrolysis lead to unfolding of target proteins and complete and directional translocation of the substrate through the pore often into an associated proteolytic machine (Weber-Ban et al., 1999; Reid et al., 2001). Therefore, action of the translocating type AAAs results in denaturation or destruction of the native target protein.

Correspondence to Brett Lauring: bl320@columbia.edu

Abbreviations used in this paper: EDC, ethyl-3-(dimethylaminopropyl)-carbodiimide; HSP, hereditary spastic paraplegia; MT, microtubule; MTBD, MT binding domain; WT, wild-type.

The online version of this article contains supplemental material.

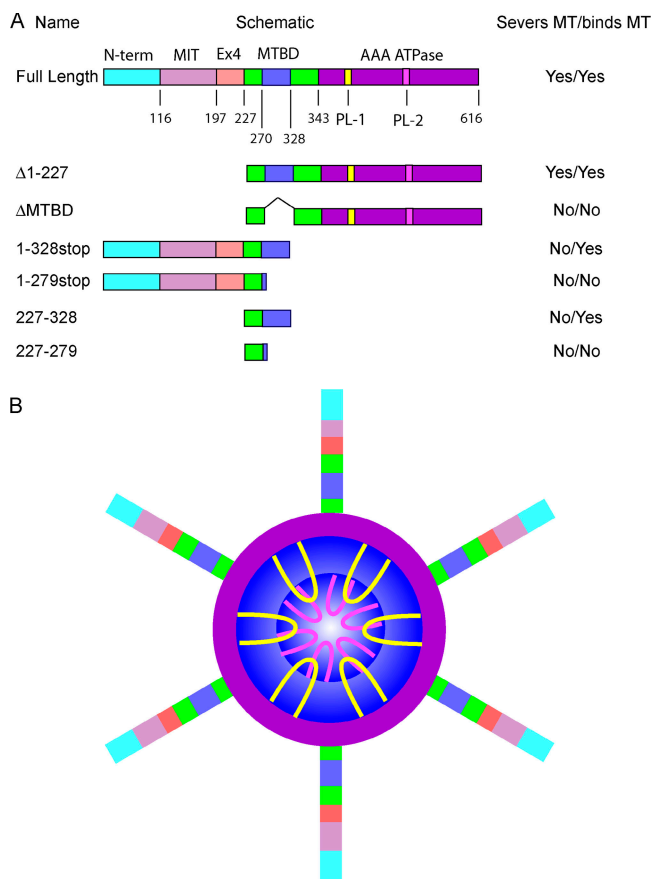


Figure 1. **Schematic depiction of domain architecture of Spastin and constructs used in this study.** (A) The numbers below each box represent the amino acid boundaries of individual domains/deletions. Numbering based on human Spastin. PL, pore loop. The column on the right indicates whether the mutant severs and/or binds to MTs. (B) Model of hexameric Spastin showing central pore and location of pore loops and N-terminal MTBD. The majority of the ring is formed by the AAA ATPase domain (purple). Image is looking down into the pore cavity. Each monomer of Spastin contributes two pore loops. Pore loop 1 (yellow) lies near the mouth of the pore, whereas pore loop 2 (pink) resides deeper in the pore. The N-terminal region projects from the ring. The cartoon is loosely based on structures of many AAA ATPases.

Structural studies of several AAA proteins, particularly translocators, revealed the existence of loops that project into the pore cavity in the hexamer. In a hexamer, each monomer contributes two loops. Mutational analysis of many AAA proteins has shown that pore loop integrity is critical for function. Pore loop 1 lies near the surface of the pore and contains a conserved YVG motif also found in Spastin (Wang et al., 2001; Yamada-Inagawa et al., 2003; Siddiqui et al., 2004; Graef and Langer, 2006). Mutation of these residues often results in retained ATPase activity and enzyme assembly but loss of function. In the P4 phage packaging motor, the equivalent of pore loop 1 has been shown to alternate between “up” and “down” conformations throughout the ATPase cycle (Mancini et al., 2004). With ClpA and ClpB (Schlieker et al., 2004; Hinnerwisch et al., 2005) or p97/VCP (DeLaBarre et al., 2006), cross-linking studies have shown that substrate proteins are in direct contact with pore loop 1. These data suggest that pore loop 1 may engage target proteins and be responsible for translocation.

Pore loop 2 lies deeper inside the pore. In translocators, pore loop 2 has not been cross-linked to target proteins, but mutations in this loop block function and sometimes lead to an inability to bind target proteins (Hinnerwisch et al., 2005; DeLaBarre et al., 2006). Although pore loops are present in AAAs that are not known to translocate target proteins, the contribution of these loops to the function of these enzymes is not as well investigated.

Here, we show that Spastin assembles into a hexamer in the presence of ATP, that hexameric but not unassembled Spastin recognizes the extreme C-terminal amino acids of its target protein, tubulin, and that this interaction is required for MT severing. Mutational analysis of the pore loops, including analysis of a disease-associated mutation in pore loop 2, shows that both pore loops are critical for MT severing and probably are directly involved in recognizing the extreme C terminus of tubulin. Despite the fact that the tubulin C-terminal tail is present in tubulin dimer, Spastin does not bind well to tubulin dimer compared with MT polymer. In addition to the pore interaction with tubulin, we define an N-terminal MT binding domain (MTBD) in Spastin that mediates the attachment of Spastin to the MT. This interaction is also required for severing.

Results

N-terminal domains in Spastin are not required for MT severing

We have previously shown that recombinant Spastin severs MTs (Evans et al., 2005). To simplify the task of addressing the mechanism by which Spastin severs MTs and to produce a more soluble recombinant protein that expresses better in *Escherichia coli*, we first sought to define which domains of Spastin are dispensable for MT severing. Like many other AAA proteins, Spastin contains a C-terminal AAA cassette (roughly aa 343–616) and an N-terminal region (aa 1–342). Homology to other AAA proteins is only within the AAA domain. The N-terminal region consists of four subdomains (see Fig. 1 A for schematic): the Atlastin binding domain (Evans et al., 2006), the MIT domain, alternatively spliced exon 4 (Charvin et al., 2003), and as we show in Fig. 2, an MTBD that is both necessary for MT severing and sufficient for MT association.

To qualitatively assay MT severing, we exploited the fact that Spastin overexpression results in nearly complete loss of MTs (Errico et al., 2002; Evans et al., 2005). Expression of the Δ1–227 deletion mutant resulted in loss of MTs, suggesting that the first 227 amino acids of Spastin are not required for severing activity (Fig. 2 A). Deletion of the first 279 amino acids of Spastin did not abolish severing (Fig. 2 A, Δ1–279 Spastin). However, Spastin lacking the first 328 amino acids did not sever MTs in transfected cells (Fig. 2 A, Δ1–328). In aggregate, the deletion experiments suggest that a region between residues 279 and 328 is important for MT severing.

Unexpectedly, we found that recombinant Spastin lacking this region (aa 279–328) could still sediment with purified MTs in vitro (unpublished data). Therefore, we deleted a few more amino acids and produced recombinant Spastin proteins lacking either aa 1–227 (Δ1–227 Spastin) or both aa 1–227

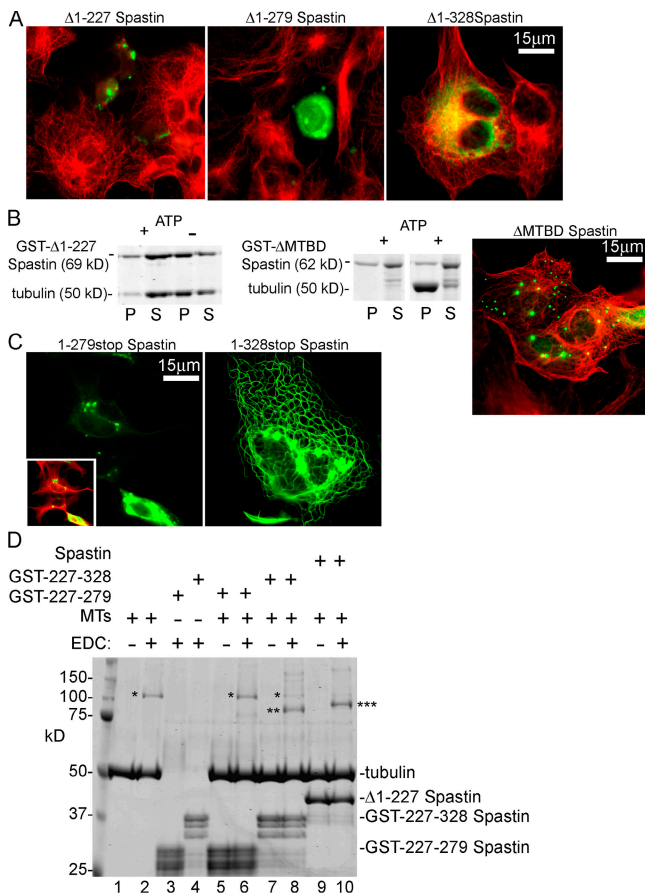


Figure 2. Identification of an N-terminal MTBD in Spastin. (A) Cos-7 cells transfected with plasmids encoding YFP-Spastin (green) with the indicated amino acids deleted were fixed and stained for tubulin (red). Deletion of the first 227 or even the first 279 amino acids did not abolish severing, as the transfected cell lacked MTs (compared with surrounding nontransfected cells). In contrast, deletion of the first 328 amino acids did abolish severing activity, as the transfected cell still has an MT array. This suggested that the approximate boundaries for a MTBD are between aa 280 and 328. (B) Recombinant $\Delta 1-227$ binds to taxol-stabilized MTs in the absence of ATP and severs them in the presence of ATP. $\Delta 1-227$ Spastin was incubated with taxol-stabilized MTs either in the absence or presence of ATP as indicated. MTs were recovered in the pellet (P) fractions after centrifugation. In the absence of ATP, some of the $\Delta 1-227$ Spastin bound to MTs. In the presence of ATP, most of the tubulin was recovered in the supernatant fraction, indicative of MT disassembly. Most of the Spastin was recovered in the supernatant as well, except for a small fraction that sedimented nonspecifically. In contrast, Δ MTBD Spastin neither binds nor severs MTs, even in the presence of ATP. Δ MTBD Spastin was incubated with ATP, as indicated, in either the absence (left two lanes) or presence of taxol-stabilized MTs (right two lanes). Samples were centrifuged, and pellet and supernatant fractions were examined by SDS-PAGE. A small percentage of the mutant Spastin sedimented nonspecifically in the absence of MTs. Note that there was no specific binding of the mutant Spastin to MTs (no additional Spastin in the pellet fraction with the MTs) and that the vast majority of the tubulin was recovered in the pellet, indicating that severing did not occur. In the gel on the right, samples were all from the same experiment but were run on opposite ends of a single gel and so are shown separately here. The micrograph shows that cells transfected with a plasmid encoding YFP- Δ MTBD Spastin still have MT arrays showing that the enzyme does not sever in cells (compare to $\Delta 1-227$ Spastin in Fig. 2 A). (C) To determine whether the MTBD is sufficient to confer MT association, stop codons were introduced at the indicated positions in the full-length YFP-Spastin cDNA, and the indicated plasmids were used to transfect cells. 1-279 stop Spastin did not decorate MTs (inset shows MTs). In contrast, 1-328 stop Spastin decorated MTs, suggesting that a region between 280 and 328 may be sufficient for MT binding. (D) Chemical cross-linking demonstrates an interaction between the MTBD and MTs. Taxol-stabilized MTs were incubated with

and aa 270-328 (called Δ MTBD Spastin; Fig. 1 A). Using a previously published sedimentation assay for MT destruction (Evans et al., 2005) where MTs are recovered in the pellet and released tubulin in the supernatant after centrifugation, we found that $\Delta 1-227$ Spastin could associate with MTs in the absence of ATP and caused MT disassembly in the presence of ATP (Fig. 2 B). In contrast, Δ MTBD Spastin neither bound nor severed MTs, demonstrating that aa 270-328 are necessary for MT severing in vitro. Consistent with this in vitro data, overexpression of Δ MTBD Spastin in cells did not result in MT loss (Fig. 2 B). We next examined whether the MTBD was sufficient to confer MT association both in cells and in vitro. In transfection experiments, 1-328 stop Spastin but not 1-279 stop Spastin decorated MTs, suggesting that amino acids 280-328 are important for MT binding (Fig. 2 C).

We next wanted to examine whether this domain was sufficient for direct MT interaction. We found that we could use the heterobifunctional chemical cross-linker ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC) to detect a direct interaction between Spastin and tubulin/MTs (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>). This cross-link did not require ATP. A GST fusion protein containing only aa 227-279 of Spastin was not able to cross-link to MTs (Fig. 2 D). However, a fusion protein containing aa 227-328 of Spastin was able to cross-link to MTs, suggesting that this MTBD is sufficient for direct interaction with MTs. Despite the fact that GST-227-328 Spastin could be cross-linked to MTs, it did not sediment with MTs (unpublished data). This may suggest a low-affinity interaction.

Together, these data suggest that an N-terminal MTBD encompassing aa 270-328 in Spastin is also important for MT severing. This domain also appears to be sufficient for MT association in a nucleotide-independent manner. We made internal deletions within this region to refine it further (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>) and found that both halves of this domain seem to mediate MT association in cells but one half appears more critical for MT severing than the other. Consistent with our results, others have shown that deletion of the entire N-terminal region preceding the AAA domain in *Drosophila melanogaster* Spastin resulted in an enzyme incapable of MT severing (Roll-Mecak and Vale, 2005). They did not address whether this N-terminal domain bound to MTs directly.

Our data demonstrating that Spastin lacking the first 270 amino acids can still associate with MTs and sever in vivo seem inconsistent with the claim by others of a MTBD in the first 269 amino acids of Spastin (Errico et al., 2002). Those experiments did not use purified proteins, and it is possible that

Spastin, GST-227-279 Spastin, or GST-227-328 Spastin, as indicated in the figure, in the presence of EDC as indicated. The Coomassie blue-stained gel shows that neither GST fusion protein cross-linked to itself in the absence of MTs (lanes 3 and 4) but that GST-227-328 Spastin cross-linked nearly as efficiently to MTs (lane 8; cross-link marked by two asterisks), as did Spastin (lane 10; cross-link marked by three asterisks). The cross-link marked by a single asterisk is a tubulin-tubulin cross-link (see Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>).

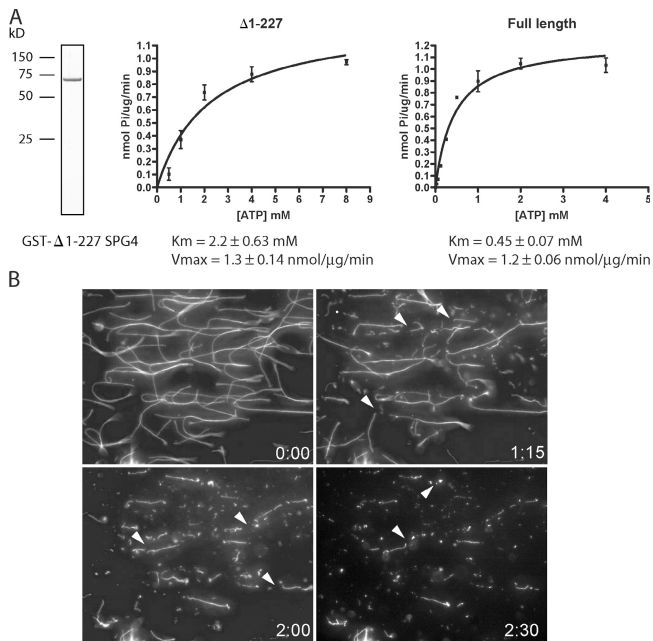


Figure 3. Biochemical characterization of $\Delta 1-227$ Spastin. (A) An aliquot of purified $\Delta 1-227$ Spastin was analyzed by SDS-PAGE. ATPase activity as a function of ATP concentration is shown. The data was fit to a curve using Prism 4.0 software, and nonlinear regression analysis was used to calculate K_m and V_{max} . K_m and V_{max} were also recalculated from our original data (Evans et al., 2005) for full-length Spastin using nonlinear regression method rather than the Lineweaver-Burke plot originally used. Error bars indicate \pm SD. (B) Spastin $\Delta 1-227$ severs MTs. Rhodamine-labeled taxol-stabilized MTs ($1 \mu\text{M}$ tubulin) were immobilized in a perfusion chamber, and 400 nM Spastin with 1 mM ATP was added. Four frames from the time lapse are shown with the time stamped (min:s) in each frame. Note the appearance of internal breaks indicative of severing. Representative breaks are indicated by arrowheads. Conditions are as published previously (Evans et al., 2005).

the observed MT association in crude cell lysates was indirect, perhaps mediated by endogenous Spastin in the cells. Our deletion results suggested that $\Delta 1-227$ Spastin severs MTs. Compared with recombinant full-length Spastin, this deletion mutant expressed much better, was more soluble, and was less prone to aggregate. Therefore, we characterized this version of recombinant Spastin and, unless otherwise indicated, all further experiments and effects of point mutations were examined in this “backbone.”

We performed ATPase assays for $\Delta 1-227$ Spastin and calculated the K_m and V_{max} for the enzyme (Fig. 3 A). Because these values were previously determined for full-length Spastin using a Lineweaver-Burke plot (Evans et al., 2005), we recalculated K_m and V_{max} using the nonlinear regression method; these numbers are also shown in Fig. 3 A. $\Delta 1-227$ Spastin severed MTs in a videomicroscopy-based assay that we had previously used to study Spastin (Fig. 3 B). We conclude that the first 227 amino acids of Spastin are not required for MT severing. Although $\Delta 1-227$ Spastin severs MTs and has similar ATPase kinetics compared with full-length protein, severing as assessed by the videomicroscopic assay is not amenable to kinetic analysis in our experience. Therefore, we cannot ascertain whether the truncated enzyme severs as well as full-length protein.

The extreme C-terminal residues of α -tubulin are recognized by Spastin

Limited proteolysis of MTs with subtilisin, which cleaves off small peptides from the extreme C terminus of α - and β -tubulin (Paschal et al., 1989), renders them resistant to severing by Spastin (Roll-Mecak and Vale, 2005). Therefore, we hypothesized that Spastin might engage the free C terminus of tubulin during the severing reaction. Examination of MT structure shows that the last ~ 100 amino acids of α - and β -tubulin project from the surface of the MT primarily as two helices (Nogales et al., 1998, 1999). The last several amino acids of α - and β -tubulin are unstructured and are not resolved in crystal structures. We prepared recombinant GST fusion proteins representing the C-terminal 104 amino acids of α -tubulin. Because of posttranslation detyrosination of α -tubulin, we prepared tails ending either in Tyr or Glu and predicted that Spastin should bind directly to these tails. Accordingly, we immobilized the GST-tubulin tails on glutathione-Sephacryl and found that wild-type (WT) Spastin did not bind to the bead-immobilized tubulin tails (Fig. 4 A). Given that Spastin can associate with MTs in vitro in the absence of ATP (Fig. 2 B), this result was surprising.

Because WT Spastin could not bind to the tubulin tail and because AAA proteins harboring a canonical Walker B motif point mutation (E442Q in Spastin) often remain kinetically trapped on their cellular target proteins (Dalal et al., 2004; Evans et al., 2005), we tested whether E442Q Spastin could bind to the tubulin tail. Indeed, E442Q Spastin bound to the tail only in the presence of ATP. E442Q Spastin bound equally well to both Glu and Tyr tails. E442Q Spastin did not bind well to truncated GST-tubulin tails lacking the last 22 amino acids (Fig. 4 B), suggesting that the most C-terminal region of these peptides are important for Spastin binding.

This ATP-dependent binding appears to represent a distinct interaction with tubulin as compared with the ATP-independent interaction between tubulin and the N-terminal MTBD discussed earlier (Fig. 2). In other AAA proteins, the E to Q Walker B mutation allows for ATP binding but not hydrolysis, trapping the oligomeric form of the enzyme. This suggests that oligomeric Spastin is binding to the tubulin tail.

Spastin assembles into a hexamer

To specifically test whether Spastin can assemble into a homooligomer and whether the E442Q mutation traps the oligomer as a stable complex, we analyzed Spastin by gel filtration. E442Q Spastin, which is predicted to bind but not hydrolyze ATP, assembled into a homooligomer in an ATP-dependent manner (Fig. 4 C, compare top two panels). In contrast to the relatively symmetrical elution profile of oligomeric Spastin shown in Fig. 4 C, in some experiments, the peak was less symmetrical and left shifted. Neither ATP nor ATP γ S trapped WT Spastin as a stable oligomer. We presume that WT Spastin can form a hexamer but that even with ATP γ S, the hexamer is not as stable as that formed by the E442Q mutant.

To more accurately characterize the Spastin homooligomer, we collected the high molecular mass oligomer fraction of E442Q Spastin obtained by gel filtration and analyzed the

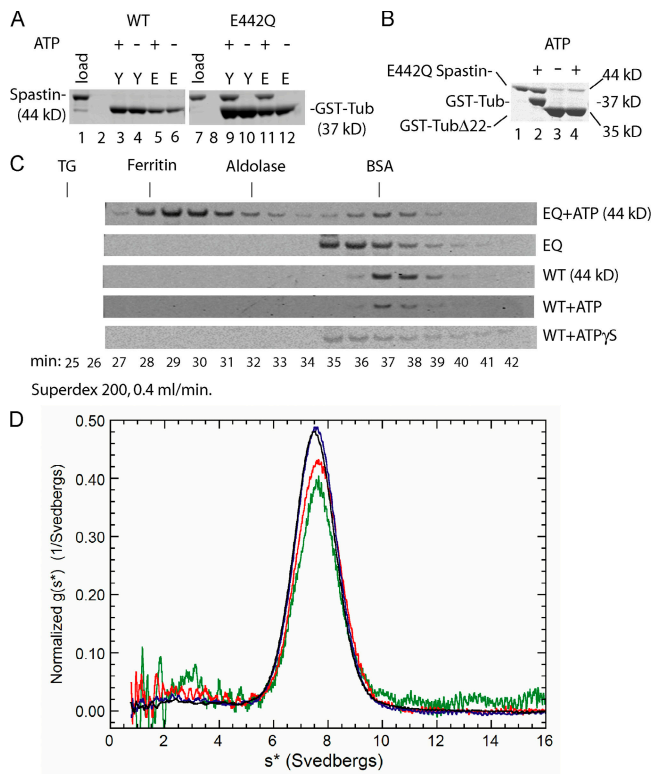


Figure 4. Spastin binds the C-terminal tail of tubulin and hexamerizes in an ATP-dependent manner. GST-tubulin tails (ending in either Tyr or Glu) were immobilized on glutathione-Sephadex. WT or E442Q Spastin was applied in either the absence or presence of ATP. After binding and washing of beads, samples were analyzed by SDS-PAGE. (A) E442Q but not WT Spastin binds in the presence of ATP. The first lane of each series shows an aliquot (20% of total) of Spastin starting material. About 25–33% of the Spastin bound to the tails. (B) Compared with full-length tail, deletion of the last 22 amino acids of the tail nearly completely abrogates the binding of E442Q Spastin. (C) Purified $\Delta 1-227$ Spastin was subjected to gel filtration chromatography on a Superdex 200 column. 0.4-ml fractions were analyzed by SDS-PAGE and Coomassie blue staining. Elution positions of size standards are shown. Elution times in minutes are shown below each fraction. (D) Sedimentation velocity analysis of E442Q Spastin in the presence of ATP. Normalized $g(s^*)$ versus s^* plots were obtained using the program DcDt+ (Philo, 2000, 2006). Sample concentrations: 0.94 mg/ml (black), 0.51 mg/ml (blue), 0.22 mg/ml (red), and 0.08 mg/ml (green).

protein complex by sedimentation velocity in the presence of ATP. Fig. 4 D shows that the normalized sedimentation coefficient distributions obtained over a broad concentration range are nearly superimposable, with a major peak at 7.6 ± 0.05 Svedbergs (uncorrected). Thus, Spastin forms a stable, non-equilibrating oligomer over the concentration range examined. As described in Materials and methods, we also calculated the translational diffusion constant from the sedimentation velocity data. We then used the Svedberg equation to calculate the molecular mass of Spastin. The sedimentation data fit to a mean mass of 250 ± 10 kD, which agrees well with a predicted hexamer mass of 258 kD ($\Delta 1-227$ Spastin has a mass of 43 kD). We conclude that Spastin assembles into a hexamer in the presence of ATP.

The binding data in Fig. 4 A suggest that the hexameric form of Spastin binds to the tubulin tail. In light of the assembly data, it is likely that the WT Spastin hexamer dissociates upon

ATP hydrolysis, whereas the E442Q hydrolysis-deficient mutant remains trapped as a stable hexamer. We imagine that WT Spastin does form a hexamer and binds transiently but that we cannot detect binding on account of hexamer instability.

Spastin pore loops are required for MT severing and recognition of the C-terminal amino acids in tubulin

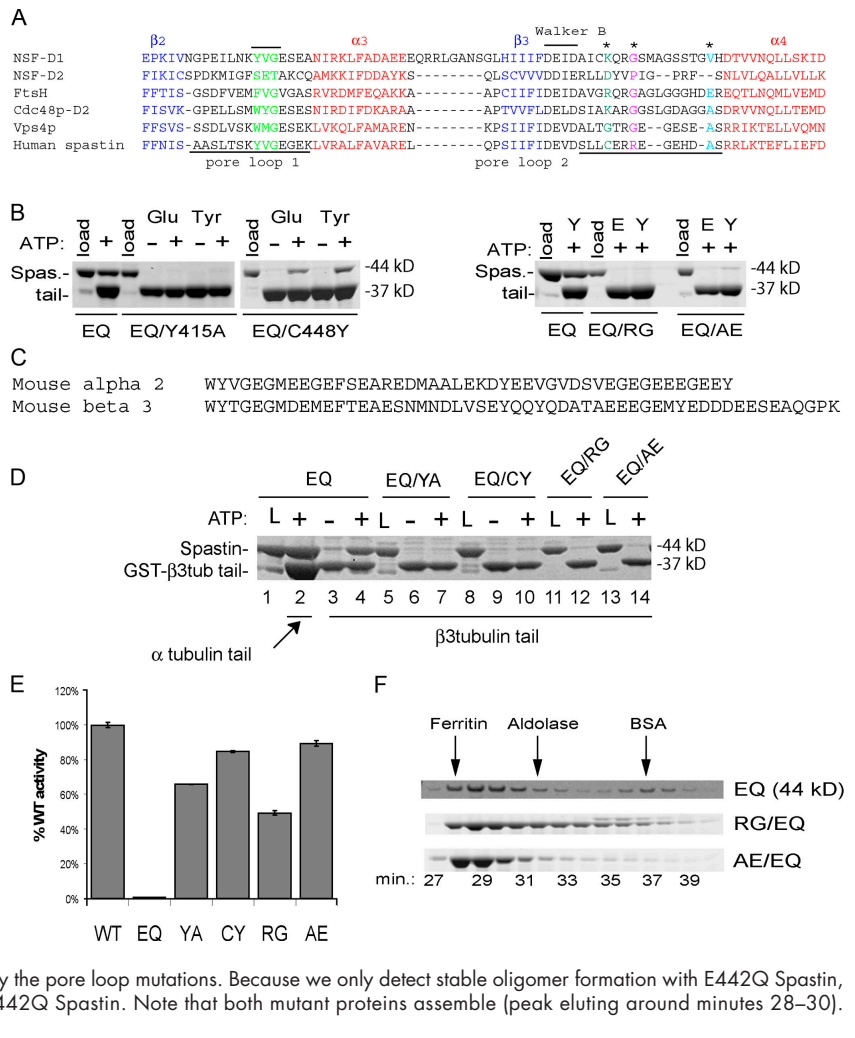
Why might hexameric but not monomeric Spastin bind to the tubulin tails? Assembly of AAA proteins results in the formation of a central pore (Fig. 1 B). Because pore loops in some AAA proteins have been shown to directly contact substrate proteins, we hypothesize that the observed binding to the tubulin tails is mediated by pore residues in Spastin and that mutations in these pore loops should abrogate the tubulin tail–Spastin interaction and MT-severing activity (Fig. 5 A). We introduced several pore loop mutations into E442Q Spastin and asked whether these pore mutations could suppress the ATP-dependent binding to the tubulin tails (Fig. 5 B). Residues 415–417 in Spastin constitute the YVG motif in pore loop 1 (Fig. 5 A). Many groups have found that the aromatic residue in pore loop 1 is crucial for function (Yamada-Inagawa et al., 2003; Siddiqui et al., 2004; Park et al., 2005). E442Q/Y415A Spastin did not bind to the GST- α -tubulin tails (Fig. 5 B).

Because the tubulin tail is extremely acidic, we predicted that basic amino acids in pore loop 2 might be important for tail binding. Compared with some other AAA proteins, pore loop 2 in Spastin lacks a conserved acidic residue and harbors an extra basic residue (Fig. 5 A). Therefore, we engineered missense mutations in pore loop 2: two that alter charged amino acids (R451G and A457E) and one representing a disease-associated mutation (C448Y; Hazan et al., 1999). Fig. 5 B shows that C448Y/E442Q Spastin but not R451G/E442Q or A457E/E442Q Spastin bound to the GST- α -tubulin tail. The Arg residue that we mutated is not conserved in all Spastins (e.g., *D. melanogaster* Spastin). However, in dSpastin, there is a Lys residue in the loop at a more C-terminal position, and the “charge balance” in the loop is therefore the same as human Spastin.

Tubulin is a heterodimer, and we therefore examined the binding of Spastin to $\beta 3$ -tubulin tails. We chose to examine this neuron-specific β -tubulin isoform both because HSP is a neuronal disorder and because, although, like α -tubulin, it has an acidic tail, the sequence, particularly the last four amino acids, is quite distinct from α -tubulin (Fig. 5 C). Similar to the results for the α -tubulin tail, we found that E442Q Spastin bound to GST- $\beta 3$ -tubulin tail in an ATP-dependent manner (Fig. 5 D). As with the α -tubulin tail, introduction of the Y415A, R451G, or A457E pore loop mutations into E442Q Spastin abolished binding. In contrast to the result obtained with α -tubulin tail, the C448Y HSP-associated mutation abrogated binding to the $\beta 3$ -tubulin tail.

In summary, mutations in both pore loops 1 and 2 can affect tail binding. Importantly, compared with many disease and engineered mutations in Spastin that we previously examined (Evans et al., 2005), the pore loop mutants all retained some ATPase activity (Fig. 5 E). Also, we found that R451G/E442Q and A457E/E442Q Spastin could still oligomerize in the presence

Figure 5. Mutations in pore loops 1 and 2 affect tubulin tail recognition. (A) Sequence alignments of part of the AAA domain from several AAA proteins. Alignments are from Karata et al. (1999). Pore loop 1 is underlined and contains the YVG motif (green). This loop, which projects into the pore near the “mouth,” lies between sheet $\beta 2$ and helix $\alpha 3$ in AAA domains. Pore loop 2 projects into the pore cavity but lies deeper in the pore and follows the Walker B motif (DEVD in Spastin) and lies between this motif and helix $\alpha 4$ in the AAA domain. Amino acids mutated in pore loop 2 are marked by asterisks. (B) Effect of pore loop mutations on α -tubulin C-terminal tail binding. (left) Although the pore loop 1 mutation (Y415A) did not bind the α -tubulin tail, the HSP-associated pore loop 2 mutation (C448Y) did not affect tail binding. (right) Either elimination of a basic residue (R451G) or introduction of an acidic residue (A457E) in pore loop 2 abolishes tail binding to both Glu (E) and Tyr (Y) tails. The tail binding assay is as described in Fig. 4. In all cases, the indicated pore loop mutations were engineered into E442Q Spastin and we were asking whether the pore loop mutations abolished binding of hexamerized Spastin. (C) Partial amino acid sequences of mouse $\alpha 2$ - and $\beta 3$ -tubulin tails are shown. Note the differences at extreme C terminus. (D) Binding of E442Q Spastin to the $\beta 3$ -tubulin tail and the effects of pore loop mutations. Samples marked L represent 20% of the input Spastin. As a positive control, lane 2 shows binding to the $\alpha 2$ -tubulin tail. Lanes 3 and 4 show that E442Q Spastin binds to the $\beta 3$ -tubulin tail in an ATP-dependent manner. As with the α tail, the YA, RG, and AE mutants do not bind. Although C448Y binds the $\alpha 2$ tail, it does not bind to the $\beta 3$ tail. (E) Relative ATPase activities of Spastin proteins harboring pore loop mutants. Error bars indicate \pm SD. (F) Selected pore loop mutants that are impaired in tubulin tail binding are not impaired in oligomer formation. Gel filtration analysis performed in the presence of ATP with the indicated mutant Spastin proteins shows that homooligomerization is not abolished by the pore loop mutations. Because we only detect stable oligomer formation with E442Q Spastin, we introduced the R451G or A457E mutations into E442Q Spastin. Note that both mutant proteins assemble (peak eluting around minutes 28–30). The elution positions of size standards are shown.



of ATP (Fig. 5 F). The ATPase measurements and gel filtration experiments suggest that pore loop mutations do not prevent pore assembly and that the point mutations in pore loops more or less specifically prevent tubulin tail binding without globally disrupting Spastin. We conclude that the tubulin tail likely binds in the pore in hexameric Spastin in a loop-dependent manner.

If the tubulin tail binds inside the central pore of the Spastin hexamer, the C-terminal Tyr residue in the tubulin tail should be relatively resistant to proteolysis by carboxypeptidase A (CpA), which specifically removes this Tyr from α -tubulin (Chapin and Bulinski, 1991). We observed partial protease protection, consistent with this notion (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>).

Pore loop mutations in Spastin inhibit MT severing

If the pore-tubulin tail interaction is important for MT severing, the pore mutations should impair Spastin's MT-severing activity, even though the enzymes can hydrolyze ATP (Fig. 5 E). Indeed, when cells were transfected with plasmids encoding YFP-Spastin harboring pore mutations, severing did not occur (Fig. 6 A). Instead, the mutant Spastin proteins decorated MTs.

Similarly, using the previously described sedimentation assay, recombinant Spastin proteins harboring pore loop mutations (Y451A, C448Y, and R451G) did not sever MTs in the presence of ATP (Fig. 6 B), as nearly all of the tubulin remained in the pellet as polymer. Similar results were obtained for the A457E mutation (not depicted). Note that a small fraction of each protein sedimented into the pellet only in the presence of MTs.

A C-terminal tubulin polypeptide acts as a competitive inhibitor of Spastin

Because we showed that pore loops engage the tubulin tail and that mutations that prevent this interaction also prevent severing, we further predicted that C-terminal peptides derived from tubulin should act as competitive inhibitors of Spastin-mediated MT severing. We incubated the GST-tubulin tail fusion protein with Spastin and taxol-stabilized MTs in either the presence or absence of ATP, as indicated in Fig. 6 C. We used the previously described MT sedimentation assay to monitor MT disassembly. In the absence of GST-tubulin tail, Spastin effectively severed MTs. In contrast, when GST-tubulin tail was added in 10-fold molar excess compared with tubulin, severing was substantially blocked. Truncated GST-tubulin tail lacking

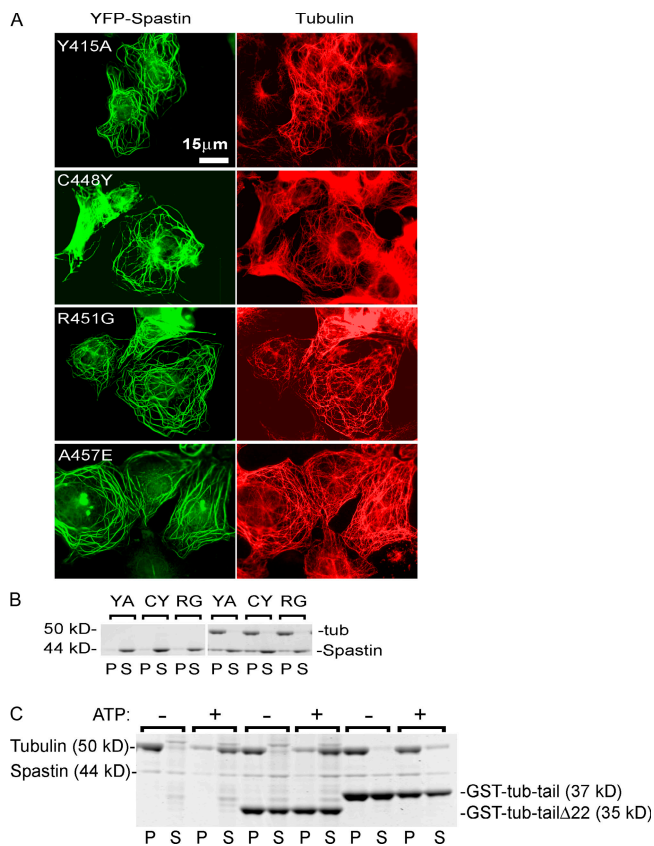


Figure 6. Pore loop mutations affect MT-severing activity. (A) Pore loop mutations abolish MT severing in vivo. Although overexpression of YFP-Spastin results in MT loss (Fig. 2 A), overexpression of Spastin harboring pore loop mutations (green) did not (tubulin shown in red). Instead, these mutant proteins decorate a subset of MTs, as do many other Spastin mutants (Evans et al., 2005). (B) Pore loop mutations abolish MT severing in vitro. The indicated mutant Spastin proteins were incubated with ATP in either the absence or presence of taxol-stabilized MTs. After incubation, samples were centrifuged. In contrast to WT Spastin, which releases severed tubulin that is recovered in the supernatant (S) fractions after centrifugation (Fig. 2 B), these mutants do not sever. MTs are in the pellet (P) fractions. Mutant proteins did not sediment in the absence of MTs. A fraction of each protein sedimented with the MTs. (C) Peptides corresponding to the C-terminal tail of tubulin are competitive inhibitors of MT severing. Taxol-stabilized MTs were incubated with Spastin and ATP as indicated. Where indicated, purified GST-tubulin tail (GST fused to the C-terminal 104 amino acids of α -tubulin) or GST-tubulin tail lacking the C-terminal 22 amino acids was added as a competitive inhibitor at 10-fold molar excess compared with tubulin (threefold molar excess compared with Spastin hexamer assuming all Spastin oligomerizes). After 15 min at 37°C, MTs were separated from released tubulin by centrifugation as described previously (Evans et al., 2005). Severing activity is evident by appearance of more tubulin in supernatant fractions. Remaining MTs are recovered in the pellet.

the C-terminal 22 amino acids did not act as a competitive inhibitor of Spastin. Although the last 22 amino acids are necessary for severing, a fusion protein consisting only of the 22 amino acids of tubulin fused to GST did not act as a competitive inhibitor (unpublished data). This may indicate that the rest of the tubulin tail is important or that when expressed alone, the last 22 amino acids of the tail are not properly ordered. The experiment in Fig. 6 C was performed with tubulin tail ending in Tyr, and similar results were obtained with peptides terminating in Glu (not depicted).

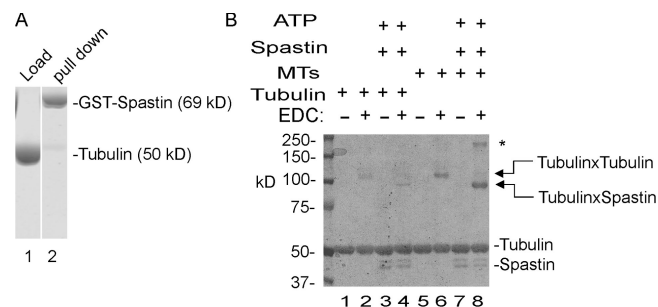


Figure 7. Spastin interacts only poorly with nonpolymerized tubulin. (A) GST-E442Q Spastin was incubated with ATP and immobilized on glutathione-Sepharose. 12 μ g tubulin was added to 6 μ l bead slurry containing 12 μ g Spastin in a volume of 100 μ l. After incubation for 30 min at room temperature, beads were washed three times. Almost none of the tubulin bound to the immobilized Spastin (lane 2). Lane 1 shows 20% of the tubulin load. These two lanes are from the same experiment but were run on opposite ends of the same gel and so are shown separately here. (B) Equivalent amounts of nonpolymerized tubulin (lanes 1–4) or taxol-stabilized MTs (lanes 5–8) were incubated with E442Q Spastin, ATP, and EDC, as indicated in the figure. The Coomassie blue-stained gel shows that Spastin only cross-links weakly to nonpolymerized tubulin (lane 4) compared with MT polymer (lane 8). The cross-link marked by the asterisk is uncharacterized (see Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>).

Spastin interacts poorly with nonpolymerized tubulin

Because the tubulin tails are present on nonpolymerized tubulin heterodimer, we might have predicted that Spastin, particularly the hexameric form, would interact with nonpolymerized tubulin heterodimer. To test this idea, GST-E442Q Spastin was immobilized on glutathione agarose, and tubulin was added in the presence of ATP (Fig. 7 A). In contrast to the tail binding experiments (Fig. 4 A), where about one third of the added Spastin could be captured on tubulin tail beads, only negligible amounts of tubulin heterodimer were captured on GST-Spastin beads. This suggested that Spastin only interacts poorly, if at all, with tubulin dimer. Consistent with this notion, soluble tubulin dimer does not cofractionate with hexameric E442Q Spastin by gel filtration analysis (unpublished data). These binding studies suggest that hexameric Spastin does not bind tubulin well despite the presence of the tail. This may indicate that the tails are perhaps more accessible to Spastin when tubulin is polymerized in a MT or that Spastin plays a role in exposing the tail to the pore loops.

Also, as mentioned earlier, we can detect interaction of Spastin with tubulin using the chemical cross-linker EDC (Fig. S1). We compared the efficiency of Spastin cross-linking obtained using MTs to that obtained using nonpolymerized tubulin and found that cross-linking to polymer is much more efficient than to nonassembled tubulin, again suggesting that Spastin interacts better with MTs (Fig. 7 B, compare cross-links in lanes 4 and 8).

Discussion

Our deletion analysis revealed the existence of an N-terminal MTBD in Spastin (aa 270–328) that is required for MT-severing activity in vivo and in vitro. Additionally, this domain may be

sufficient for MT association. Analysis of this region of Spastin did not reveal any homology to any known protein or functional motif. Although Katanin has an MTBD preceding its AAA domain, there is no homology between the N terminus of Spastin and Katanin. In fact, the only region of homology between these MT-severing proteins is in the AAA ATPase domain.

Our deletion analysis of the N-terminal region of Spastin also revealed that the first three subdomains in the N terminus of Spastin encompassing the first 227 amino acids are not required for MT severing. Previously, we (Evans et al., 2006) and others (Sanderson et al., 2006) found that Atlastin binds to a region in the first 116 amino acids of Spastin. Atlastin, an integral membrane GTPase, is encoded by another autosomal dominant HSP gene. We hypothesize that Spastin-interacting proteins, such as Atlastin, binding to the first 227 amino acids of Spastin might serve to localize Spastin to sites of activity (Evans et al., 2006). Because we mapped an MTBD between aa 270 and 328, it is unlikely that known Spastin binding proteins interacting with regions between aa 1 and 227 would interfere with MT severing. This is consistent with the observation that Atlastin binding does not appear to regulate MT severing by Spastin (Evans et al., 2006).

We demonstrated that Spastin can assemble into a hexamer during its ATPase cycle. This is significant because Vps4, another AAA protein closely related to Spastin, has been proposed to assemble into larger oligomers such as 12 mers rather than hexamers (Babst et al., 1998; Scott et al., 2005). Although slowly hydrolyzable ATP analogues allow for trapping of some WT AAA ATPases as oligomers, this is not the case for Spastin (Fig. 4 C). Similarly, Katanin (Hartman and Vale, 1999) and Vps4 cannot be trapped as stable oligomers with ATP. Instead, the demonstration of homooligomeric forms of these enzymes also necessitated the use of the Walker B E to Q mutation. Together with Fidgetin, Spastin, Katanin, and Vps4 constitute the meiotic subfamily of AAA ATPases (Frohlich, 2001), and in this respect, this subgroup appears distinct from other AAA proteins.

Subtilisin treatment of MTs, which removes small peptides from the C terminus of α - and β -tubulin, renders MTs resistant to severing by both Spastin and Katanin (McNally and Vale, 1993; Roll-Mecak and Vale, 2005), the other known AAA ATPase that severs MTs. This prompted us to investigate the role of the tubulin C terminus in MT severing. Our results show that pore loops in hexameric Spastin recognize the extreme C terminus of its target protein (tubulin) and that this interaction is critical for MT severing. Whether Katanin recognizes tubulin in a similar manner is not known. Several microtubule-associated proteins and motor proteins also bind to the tubulin tail; this raises the possibility that certain microtubule-associated proteins may protect MTs from severing as proposed (Baas et al., 2005; Qiang et al., 2006).

Though the role of pore amino acids has been well studied in translocating AAA proteins involved in protein quality control, their role in other AAA proteins is not as well understood. A pore loop 1 mutation in Vps4 abolishes retroviral budding in a cell-based assay (Scott et al., 2005), just as a similar mutation in Spastin abolished severing. Our analysis of pore loop 2 in

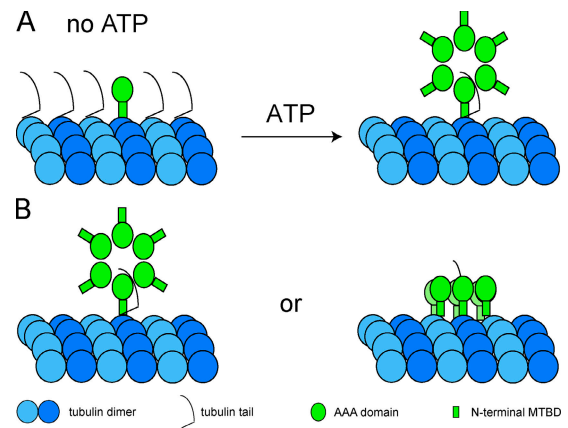


Figure 8. **Model.** (A) Unassembled Spastin can associate with MT polymer by virtue of the N-terminal MTBD. This interaction is not ATP dependent. ATP results in assembly of the hexamer and pore formation. The tubulin tails, which project from the surface of the MT polymer, bind to the pore loops (tails not drawn on all tubulins for clarity). This binding is ATP dependent. Thus, Spastin can engage the MT in two distinct places. (B) The orientation of the ring on MTs is unclear. Two possible orientations are shown. The number of N-terminal MTBDs simultaneously in contact with the MT is not known.

Spastin reveals that it plays a critical role in severing and in tubulin C-terminal tail recognition, akin to the role of pore loop 2 in *ssrA* tag recognition (Hinnerwisch et al., 2005). The C448Y disease-associated mutation uncouples ATPase from severing activities (Fig. 5 E and Fig. 6, A–C) and binds to the α -tubulin tail (Fig. 6 B). Interestingly, it cannot bind to the β 3-tubulin tail (Fig. 6 C). Thus, even in non-Clp type AAA ATPases, pore loops are critical for function and for target protein recognition. Even though pore loops are present in unassembled Spastin, hexamerization is important for stable, robust tail binding. This could mean that there is simultaneous engagement of the tail by more than one loop or that the loops adopt different structures in the hexameric Spastin.

In transfected cells, pore loop mutations in Spastin, whether they abolish tail binding or not, result in Spastin proteins that decorate MTs. Therefore, it is probable that MT association with these mutants occurs via the N-terminal MTBD and that the enzyme assembles and perhaps hydrolyzes ATP. However, we imagine that severing fails to occur either because the tubulin tail cannot engage the pore loop (YA, RG, or AE for α - and β -tubulin and these plus C448Y for the β -tubulin tail) or the normal response of the AAA ring to the engagement is blocked because of the mutated pore loops (CY). In either case, the protein apparently does not release from or sever the MTs.

Spastin lacking the N-terminal MTBD domain does not sever or decorate MTs despite having intact pore loops. This suggests that although tubulin tail-loop interaction is necessary for severing, this additional Spastin tubulin interaction must also be necessary for MT severing. Although Δ 270–328 Spastin/E442Q can hexamerize and hydrolyze ATP, it could not bind to the GST- α -tubulin tails (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>). The reason for this is not clear but may suggest that the N-terminal MTBD-MT interaction is important in positioning the hexamer such that the

tubulin tail is pore accessible or that this MTBD actually plays a role in exposing that tail to the pore. We do not know where the N-terminal MTBD contacts the MT.

We have seen that purified recombinant Spastin can bind MTs. This includes WT enzyme as well as mutants that are predicted not to bind ATP (e.g., K388A Spastin) or hydrolyze ATP (e.g., E442Q; Evans et al., 2005). Thus far, only deletion of the N-MTBD prevents MT association in vitro and in vivo. In contrast, when mutant versions of Spastin are overexpressed in cells, two distinct patterns are observed. Most mutants analyzed in this and past work (Evans et al., 2005) do not sever MTs. However, some mutant proteins decorate MTs (e.g., E442Q and pore loop mutants), whereas others do not even though they bind in vitro (e.g., K388A and R499C; Evans, et al., 2005). We imagine that decoration of MTs in cells reflects more than mere binding and may reflect kinetic trapping of hexameric Spastin on MTs. Because E442Q forms an ATP-dependent oligomer, it is possible that this mutant protein assembles into a hexamer on MTs but, because it cannot hydrolyze the bound ATP, remains kinetically trapped on the MT, resulting in decoration. Interestingly, the pore loop mutant Spastins decorate MTs even though they can hydrolyze ATP, albeit not quite as well as WT enzyme. This may imply that, although necessary for release, completion of an ATPase cycle is not sufficient to result in release from the MT.

Fig. 8 shows a schematic of the two distinct interactions Spastin makes with MTs. We propose that Spastin exerts force on the MT in part by engagement of the tubulin tail by Spastin's pore. Severing could be achieved by pulling the tail into the pore. In this model, the N-MTBD would merely anchor the hexamer and severing would be analogous to untying a shoelace by pulling on a slipknot. Alternatively, the importance of the tail binding could be that relative motions occur between the two sites where Spastin is anchored to an MT. Soluble tubulin has a curved conformation but has a straight conformation in the MT polymer (Nogales et al., 1999; Gigant et al., 2000; Nogales et al., 2003). Other proteins, such as stathmin, cause MT catastrophe by binding to and sequestering curved tubulin dimer (Cassimeris, 2002), and depolymerizing kinesins may engage tubulin tails to peel off dimers at MT ends by flipping them into the curved conformation (Wordeman, 2005). Spastin could sever MTs by flipping a tubulin heterodimer in the middle of a polymer into the curved conformation, making it incompatible with polymer packing.

Given that the tubulin tails are present on nonpolymerized dimer, one might have expected hexameric Spastin to bind to dimer. The fact that it does not (Fig. 7) may mean that the tails are not accessible in the dimer but may become exposed or properly oriented in the MT polymer either by virtue of some polymer-induced conformational change in this region of tubulin or by virtue of Spastin binding to the polymer. As mentioned earlier, Spastin could conceivably be restricted to MT polymer if it indeed interacts preferentially with tubulin in a straight conformation. As Spastin apparently does not interact well with soluble dimer, any ATP-driven change in tubulin conformation occurring during severing may result in Spastin release from the severed tubulin.

Materials and methods

Expression and purification of Spastin

Expression and purification of GST-Spastin was as previously described (Evans et al., 2005) with the following modifications. Deletions and point mutations were made using the QuikChange kit (Stratagene). $\Delta 1-227$ Spastin expressed better and was more soluble than full-length Spastin, resulting in better yields. GST-tagged protein was expressed from the pGEX-6P-3 vector (GE Healthcare). Protein was expressed using BL21-CodonPlus (DE3)-RIPL *E. coli* (Stratagene). Cultures were grown in LB supplemented with carbenicillin, chloramphenicol, and streptomycin to an OD of 1.0 and induced overnight at 30°C by adding IPTG to 0.5 mM. Cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 1 mM ATP, and EDTA-free protease inhibitor tablet [Roche]) per 20 ml culture using a high-pressure homogenizer (Emulsiflex C-5; Avestin). Triton X-100 was added to 1% to the lysate at this stage. Detergent was not kept in the buffer during purification. Lysates were cleared by centrifugation (Ti60 rotor [Beckman Coulter]; 45,000 rpm, 45 min, 4°C) and pumped over a 1- or 5-ml glutathione-Sepharose column (GE Healthcare). The column was washed with 10 column volumes of lysis buffer with 500 mM NaCl and 1 mM DTT (no ATP) followed by 10 column volumes of lysis buffer with 150 mM NaCl and 1 mM DTT (no ATP). Protein was eluted using a gradient of 1–50 mM glutathione in lysis buffer with 1 mM DTT and no ATP. For cleavage of the GST moiety, protein was desalted (PD10 column; GE Healthcare) into enzyme buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, and 1 mM DTT) and cut overnight at 4°C with PreScission Protease (GE Healthcare). Protease and GST were removed using glutathione-Sepharose beads (GE Healthcare). ATPase assays of Spastin activity by the malachite green method are as described previously (Evans et al., 2005). K_m and V_{max} were calculated by nonlinear regression analysis using the Prism 4 software package.

Gel filtration chromatography

Gel filtration chromatography was performed using a Superdex 200 column (bed volume = 24 ml) attached to an FPLC (GE Healthcare) system that was run at 0.4 ml/min. Typically, 50–100 μ g Spastin in a volume of 100 μ l was centrifuged (TL100 rotor [Beckman Coulter]; 50,000 rpm, 10 min, 4°C) immediately before analysis. 0.4-ml fractions were collected. The column was calibrated with the size standards indicated in the figures. Typically, the buffer composition was 50 mM Hepes, pH 7.3, 100 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. Where indicated in the figures, the column buffer and the sample contained 1 mM ATP or 0.1 mM ATP γ S. Sometimes Spastin was concentrated before chromatography using Amicon concentrators blocked with 5% Tween-20.

Analytical ultracentrifugation

Sedimentation velocity analysis was performed with an Optima XL-I (Beckman Coulter) in aluminum-filled epon double-sector cells at 20°C and 50,000 rpm. Interference scans were collected at 45-s intervals. Four dilutions of E442Q Spastin, ranging from 0.94 to 0.08 mg/ml, were prepared in 50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 1 mM ATP.

The sedimentation of macromolecules in a centrifugal field is governed by the Lamm equation:

$$\frac{dc}{dt} = \frac{1}{r} \frac{d}{dr} \left(rD \frac{dc}{dr} - s\omega^2 r^2 c \right),$$

where c is the concentration, t is time, r is the radial distance, D is the translation diffusion constant, s is the sedimentation coefficient, and ω is the rotation velocity. The sedimentation coefficient and translation diffusion constant were obtained by analysis sedimentation velocity data. These parameters were then used to obtain the molecular weight using the Svedberg equation:

$$M = \frac{sRT}{D(1 - \bar{v}\rho)},$$

where M is the molecular weight, R is the gas constant, T is the temperature, \bar{v} is the partial specific volume of the macromolecule, and ρ is the solvent density.

We used the program DcDt+ to analyze the association state of Spastin. This program computes the apparent sedimentation coefficient

distribution function $g(s^*)$, where the peak of the function is related to the sedimentation coefficient and the width of the function is related to the diffusion constant. These parameters are obtained by nonlinear least squares fits to the $g(s^*)$ functions, and the molecular masses are then computed using the Svedberg equation. The data were analyzed using the time-derivative method (Stafford, 1992) to obtain normalized $g(s^*)$ profiles with the DcDt+ analysis software (Philo, 2000, 2006). The buffer density and the protein mass and partial specific volume were calculated using the SEDNTERP program (Laue et al., 1992).

Videomicroscopy, cell culture, transfections, and immunofluorescence

Videomicroscopy, cell culture, transfections, and immunofluorescence were performed as described previously (Evans et al., 2005). Cos-7 cells were transfected with Polyfect (QIAGEN) for 36–48 h before being fixed in methanol. Tubulin was stained with YL1/2 antibody. In general, Spastin was visualized as a YFP fusion, and tubulin was visualized using a Cy5-labeled secondary antibody. All images were obtained with a microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) using a 63× 1.6 NA plan-apochromat oil-immersion lens. Images were acquired with a CE camera (Roper Scientific) using MetaMorph software (Universal Imaging Corp.) and processed with Photoshop (Adobe). Site-directed mutagenesis of the YFP-Spastin plasmid was achieved using the QuikChange kit.

Spin-down assay for MT severing

The centrifugation or spin-down assay for MT severing is essentially as published previously (Evans et al., 2005). For the tubulin tail competition experiment, 25- μ l assays contained 0.5 μ g Spastin, 1.0 μ g taxol-stabilized MTs, and 8.6 μ g GST-tubulin tail peptide. The GST-tubulin tail construct encodes GST fused to the C-terminal 104 amino acids of murine α -tubulin. Recombinant protein production was essentially as described for GST-Spastin. Although this protein has Tyr as the ultimate amino acid, we also produced a similar peptide corresponding to detyrosinated tubulin that ends in Glu. These plasmids were the gift of G. Gundersen (Columbia University, New York, NY).

GST-tubulin tail binding assay

8.6 μ g of Spastin was added to the aforementioned GST-tubulin tail peptides (6.0 μ g) immobilized on glutathione-Sepharose in a total volume of 100 μ l (50 mM Hepes, pH 7.3, 150 mM NaCl, 5 mM MgCl₂, and 1 mM DTT) with or without 1 mM ATP, as indicated in the figures. After 30 min at 25°C, beads were washed three times with buffer supplemented with 0.1% Triton X-100 and either lacking or containing ATP. Next, SDS-PAGE sample buffer was added, and proteins were visualized by Coomassie staining after SDS-PAGE.

EDC cross-linking

EDC (Pierce Chemical Co.) was added to mixtures of proteins to 1–5 mM final from a 50 mM stock. Reaction buffer was 50 mM Hepes, pH 7.4, 150 mM NaCl, and 5 mM MgCl₂. After 15 min at room temperature, reactions were quenched by the addition of SDS-PAGE sample buffer. Samples were analyzed by Coomassie blue staining after SDS-PAGE.

Online supplemental material

Fig. S1 shows EDC cross-linking between Spastin and MTs. Fig. S2 shows a detailed deletion analysis of the N-terminal MTBD. Fig. S3 shows that the C-terminal tail of tubulin is partially protease protected by the Spastin hexamer. Fig. S4 shows the biochemical characterization of Spastin lacking the MTBD. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200610072/DC1>.

Funding was provided by the National Institutes of Health/National Institute of Neurological Disorders and Stroke grants R01 NS043298 and KO2 NS046472 (B. Lauring) and by the Spastic Paraplegia Foundation (B. Lauring). K.J. Evans was supported in part by training grant NIH T32 MH15174-28.

Submitted: 16 October 2006

Accepted: 13 February 2007

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