Developmental Cell
Article

Kinesin-13 and Tubulin Posttranslational Modifications Regulate Microtubule Growth in Axon Regeneration

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http://dx.doi.org/10.1016/j.devcel.2012.08.010

SUMMARY

The microtubule (MT) cytoskeleton of a mature axon is maintained in a stabilized steady state, yet after axonal injury it can be transformed into a dynamic structure capable of supporting axon regrowth. Using *Caenorhabditis elegans* mechanosensory axons and in vivo imaging, we find that, in mature axons, the growth of MTs is restricted in the steady state by the depolymerizing kinesin-13 family member KLP-7. After axon injury, we observe a two-phase process of MT growth upregulation. First, the number of growing MTs increases at the injury site, concomitant with local downregulation of KLP-7. A second phase of persistent MT growth requires the cytosolic carboxypeptidase CCPP-6, which promotes Δ2 modification of α-tubulin. Both phases of MT growth are coordinated by the DLK-1 MAP kinase cascade. Our results define how the stable MT cytoskeleton of a mature neuron is converted into the dynamically growing MT cytoskeleton of a regrowing axon.

INTRODUCTION

Microtubules (MTs) define and maintain neuronal polarity and act as highways for transport of proteins and organelles to cell compartments distant from the neuronal cell body (Witte and Bradke, 2008). MTs are intrinsically unstable, exhibiting dynamic instability in vitro (Mitchison and Kirschner, 1984) and in vivo (Cassimeris et al., 1988). Dynamic instability is a hallmark of MTs in cells or compartments that undergo rapid morphological changes, such as dividing cells or axonal growth cones (Suter et al., 2004). In contrast, long-lived cellular compartments such as axons or dendrites are enriched in stabilized MTs (Baas et al., 1993). In early neuronal development, MT stabilization plays an instructive role in selection of a single neurite as the future axon (Witte et al., 2008). Subsequently, dynamic MTs are spatially and temporally regulated such that they are confined to growth cones or sites of branch formation (Yu et al., 1994). Finally, the integrity of mature neurons in a functional circuit requires a stabilized axonal and dendritic MT cytoskeleton, although such neurons retain latent plasticity in their MT arrays (Bradke and Dotti, 2000; Gomis-Rüth et al., 2008).

The MT cytoskeleton of mature axons is composed of two types of MT, stable and labile (Ahmad et al., 1993). Neurons are rich in microtubule-associated proteins that stabilize the axonal MT array, including tau, MAP1B, and STOP (Dehmelt et al., 2003; Guillaud et al., 1998). Such factors counteract the effects of MT-severing enzymes such as spastin or katanin (Baas and Qiang, 2005). Recent work has also revealed the critical roles of tubulin posttranslational modifications in regulating MT stability (Janke and Kneussel, 2010). α-tubulins are deglutamylated at their C termini by cytosolic carboxypeptidases, or CCPs (Rogowski et al., 2010). α-tubulins lacking C-terminal tyrosine and glutamate, also known as Δ2-tubulin, form extremely stable microtubules (Paturle-Lafanechère et al., 1994). Lack of the CCP Nna1 leads to late-onset neuronal degeneration (Fernandez-Gonzalez et al., 2002), suggesting that stable MTs maintain axonal integrity. Detyrosination, but not Δ2 formation, can be reversed by tubulin tyrosine ligases (TTLs); MTs enriched in tyrosinated α-tubulin are less stable as they are more sensitive to MT depolymerizing enzymes such as the kinesin-13 family member MCAK (Peris et al., 2009). At present, little is known about how such MT regulatory factors are regulated in mature neurons.

Mature axons are frequently capable of remarkable regenerative growth after injury (Ramon y Cajal, 1928). Such regrowth often involves the regeneration of a severed axon stump into a motile growth cone resembling those of developmental outgrowth (Bradke et al., 2012). The ability of axons to regenerate after injury must therefore involve local conversion of a stable MT cytoskeleton into the growing MT cytoskeleton of a motile growth cone (Erez and Spira, 2008). Indeed, an early axonal response to injury is the local disassembly or severing of axonal MTs, potentially creating free plus ends that allow new MT polymerization. Conversely, moderate stabilization of axonal MTs by Taxol can promote axon regrowth in the inhibitory microenvironment of the mammalian central nervous system (CNS) (Hellal et al., 2011; Sengottuvel et al., 2011). Drugs that promote MT growth can also promote axon regeneration on inhibitory substrates in vitro (Usher et al., 2010). Once new dynamic MTs have been generated, they must undergo persistent growth to permit axon elongation. However, the mechanisms by which the MT cytoskeleton is remodeled after injury are unclear. A better understanding of MT remodeling mechanisms will provide insights into the initial stages of axon regrowth.

Caenorhabditis elegans is a tractable model for studies of axon regrowth after injury (Chen and Chisholm, 2011; Gabel et al., 2008; Samara et al., 2010; Wu et al., 2007). Many *C. elegans* axons can regrow after laser axotomy, which triggers second messenger cascades related to those involved in regrowth in other organisms (Ghosh-Roy et al., 2010). Genetic screens have identified many genes and pathways specifically required for adult axon regrowth (Chen et al., 2011; El Bejjani and Hammarlund, 2012; Pinan-Lucarre et al., 2012). Among these, the DLK-1 MAPK cascade is essential for early stages of regrowth (Hammarlund et al., 2009; Yan et al., 2009), a function conserved in insects (Xiong et al., 2010) and in mammals (Itoh et al., 2009; Shin et al., 2012). One effector of the DLK-1 pathway in *C. elegans* is the bZip protein CEBP-1 (Yan et al., 2009), but the DLK-1 pathway likely has additional targets. A second MAPK cascade, the MLK-1/KGB pathway, acts in parallel to the DLK pathway in regrowth (Nix et al., 2011).

Here, using in vivo live imaging in *C. elegans* axons, we show that mature axons contain a small population of growing MTs that are maintained in an unstable state by KLP-7, a member of the kinesin-13 family of MT-depolymerizing kinesins. Axonal injury triggers local downregulation of KLP-7 and upregulation of growing MTs at the injured axon tip. MTs then enter a second phase of persistent growth, dependent on cytosolic carboxypeptidases that promote the Δ2 posttranslational modification of tubulin. We show that the DLK-1 pathway promotes both MT upregulation and growth persistency. Our results elucidate the mechanisms that convert the stable MT axon cytoskeleton into a dynamically growing state.

RESULTS

Two Phases of MT Growth Regulation Are Triggered by Axon Injury

To visualize the effects of axotomy on microtubules in vivo, we used green fluorescent protein (GFP)-tagged MT plus-end binding proteins (EBP-1, EBP-2), established markers of the plus ends of growing MTs in neurons (Stepanova et al., 2003). We expressed EBP-1::GFP or EBP-2::GFP in posterior lateral microtubule (PLM) touch neurons and imaged them in immobilized animals using spinning disc confocal microscopy (Figures 1A and S1A available online). We divided touch neuron axons into adjacent 40-μm regions of interest (ROIs) to define the local behavior of MTs (Figure 1A). For each ROI, we imaged EBP-2::GFP dynamics (see Experimental Procedures; Movie S1) and analyzed the results in kymographs (Figure 1B). We observed similar patterns using EBP-1::GFP (Figures S1A–S1C), and refer collectively to these markers as EBP-GFP. We quantitated total numbers of EBP-GFP "comets" (tracks in kymographs) as well as their growth duration, length, and velocity. In the steady state, EBP-GFP comets were most abundant in or close to the PLM soma; axons contained comparatively few comets that grew for short periods (5.9 ± 0.2 s in ROI-A; red arrow in Figures 1A–1C). More distal axon regions also had few growing MTs that underwent frequent catastrophe (data not shown). Almost all EBP-GFP comets grew away from the cell body, suggesting that MT polarity in the PLM process is plus-end out, exhibiting axonal rather than dendritic MT polarity. EBP-GFP-based assays do not detect stable MTs, but, based on EM studies, the PLM axon typically contains 20–50 MTs at any one position (Chalfie and Thomson, 1979) (Figure S1). The steady-state pool of growing MTs is therefore small compared to the stable MT pool. Although MTs are particularly abundant in touch neurons, the EBP-GFP dynamics parameters reported here appear similar to those observed in other *C. elegans* neurons (Goodwin et al., 2012; Hao et al., 2011; Maniar et al., 2012). EBP-GFP dynamics parameters reported here are summarized in Table S1.

To address how axonal injury affected MT dynamics we severed PLM axons 100 μm from the cell body by laser microsurgery (see Experimental Procedures; Figure 1D′). At 3 hr after axotomy, the number of EBP-GFP tracks increased over 3-fold near the cut site (ROI-A; purple traces in Figures 1D and 1E; Movie S2) compared to uninjured axons (2.9 ± 0.2 at 0 hr versus 10.3 ± 1.1 at 3 hr). At times before 3 hr, this upregulation was not statistically significant (data not shown). The length and duration of EBP-GFP tracks at 3 hr was not significantly different from that in uninjured axons. The number of EBP-GFP tracks continued to rise at later time points, although the increase was most significant from 0 to 3 hr (Figure 1E). Further away from the injury site (ROI-B), the number of EBP-GFP tracks remained low (Figure 1E). We found similar local upregulation of growing MTs after axotomy at 60 μm from the cell body, the position used in our standard axon regrowth assay (Figures S1D and S1E). In summary, an early response to injury of the PLM axon is a local upregulation of growing MTs close to the axon stump.

EBP-GFP comets at the severed axon tip at 3 hr had a similar catastrophe frequency (Figure 1F) as comets in uninjured axons. Between 3 and 6 hr, EBP-GFP tracks in ROI-A decreased over 2-fold in catastrophe frequency (Cassimeris et al., 1988) and doubled in length (Figures 1F and 1G), indicating an increase in persistent MT growth. The number and length of EBP-GFP tracks did not significantly change away from the axon tip (in ROI-B; Figure 1E; length data not shown). Injured PLM axons form a growth cone-like structure and begin extending by 4.5–6 hr after injury (Wu et al., 2007). The transition to more persistent MT growth therefore correlates with reformation of a growth cone and the beginning of axon extension. The number and length of EBP-GFP comets continued to increase; a notable change at 10.5 hr was a significant increase in EBP-GFP comet growth velocity (Figure 1H). With this analysis of the normal response to injury in hand, we investigated which regulators of MT dynamics affect axon MT remodeling.

The Kinesin-13 KLP-7 Maintains Steady-State Growing Microtubules and Inhibits MT Upregulation in Early Regrowth

Proteins that induce MT depolymerization, known as "catastrophe factors," are central regulators of MT dynamics. As *C. elegans* lacks orthologs of catastrophe factors such as stathmins (Cassimeris, 2002), we focused on the conserved kinesin-13 family of depolymerizing kinesins (Howard and Hyman, 2007). Kinesin-13 family members such as KIF2B or KIF2C/MCAK diffuse along the MT lattice until they reach plus ends, where they depolymerize the stable GTP-containing cap of a growing MT (Helenius et al., 2006). All kinesin-13 family members tested display similar catastrophe-inducing activity