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Patronin Regulates the Microtubule Network by Protecting Microtubule Minus Ends

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SUMMARY

Tubulin assembles into microtubule polymers that have distinct plus and minus ends. Most microtubule plus ends in living cells are dynamic; the transitions between growth and shrinkage are regulated by assembly-promoting and destabilizing proteins. In contrast, minus ends are generally not dynamic, suggesting their stabilization by some unknown protein. Here, we have identified Patronin (also known as ssp4) as a protein that stabilizes microtubule minus ends in *Drosophila* S2 cells. In the absence of Patronin, minus ends lose subunits through the actions of the Kinesin-13 microtubule depolymerase, leading to a sparse interphase microtubule array and short, disorganized mitotic spindles. In vitro, the selective binding of purified Patronin to microtubule minus ends is sufficient to protect them against Kinesin-13-induced depolymerization. We propose that Patronin caps and stabilizes microtubule minus ends, an activity that serves a critical role in the organization of the microtubule cytoskeleton.

INTRODUCTION

Microtubules are the principle scaffold of the mitotic spindle, serve as tracks for intracellular transport of proteins and mRNAs, and also participate in signaling functions. The repeating subunit of the microtubule is the α/β-tubulin heterodimer, which polymerizes in a head-to-tail fashion to form protofilaments; typically ~13 protofilaments associate laterally to form the microtubules seen in vivo. Due to the head-to-tail assembly, the microtubule is a polar filament, with β-tubulin facing the plus end and α-tubulin at the minus end (Mitchison, 1993). In vitro experiments using purified tubulin first demonstrated that microtubules exhibit an unusual property called "dynamic instability," whereby microtubules undergo prolonged periods of polymerization and depolymerization with transitions between the two states called catastrophe (from polymerization to depolymerization) and rescue (from depolymerization to polymerization) (Desai and Mitchison, 1997). In vitro, plus and minus ends both undergo dynamic instability over the same range of tubulin concentrations but display small quantitative differences.

As a result of interactions with specific binding proteins, the dynamic behavior of microtubules in vivo can differ dramatically from that described in vitro. Many proteins have been identified that bind at microtubule plus ends and regulate their dynamics. For example, MAP215 accelerates tubulin subunit addition at the plus end, EB1 promotes plus end growth and dynamicity, and Clip170 increases rescue frequency (Akhmanova and Steinmetz, 2008). Opposing these growth-promoting proteins are the depolymerizing Kinesin-13 motors, which use ATP hydrolysis to induce a conformational change at plus ends to promote catastrophe (Moores and Milligan, 2006). The antagonistic actions of different +TIP proteins account for the more pronounced dynamic instability of microtubules in vivo compared to microtubules composed of pure tubulin in vitro (Kinoshita et al., 2001).

In contrast to the wealth of information on the microtubule plus end, the regulation of the microtubule minus end in vivo is poorly understood. In many cell types, the minus ends are clustered and anchored at a central microtubule-organizing center (MTOC). This organization has hindered visualization of their dynamics, in contrast with plus ends, which are more easily viewed at the cell periphery by microscopy. Even in organisms and cell types that lack a central MTOC (e.g., *S. pombe*, *D. melanogaster*, *A. thailinia*, neurons, epithelial cells, and myotubes), the microtubule minus ends appear to be embedded in poorly characterized anchoring sites around the cell (Bartolini and Gundersen, 2006; Rusan and Rogers, 2009).

Occasionally, in animal cells, microtubules are released from a MTOC or break due to actomyosin forces, thereby allowing minus ends to be observed free from any nucleating material (Rodionov and Borisy, 1997; Vorobjev et al., 1999; Yvon and Wadsworth, 1997; Waterman-Storer and Salmon, 1997; Keating et al., 1997). The conclusion from these studies is that the vast majority (80%–90%) of free microtubule minus ends are stable, neither visibly growing nor shrinking. A similar stability of minus ends has been observed in cytoplasmic extracts (Rodionov et al., 1999; Vorobjev et al., 1997). Some minus ends, however, transition to rapid depolymerization resulting in the disappearance of the microtubule, and a very small percentage of microtubules treadmill through the cytoplasm (caused by simultaneous minus end shrinkage and plus end growth) (Rodionov and

Borisy, 1997). Microtubule elongation from minus ends has not been reported in vivo. Thus, in contrast to the pronounced dynamic instability of plus ends, minus ends are mostly static and are indeed less dynamic than minus ends composed of pure tubulin in vitro. These results suggest that microtubule minus ends might be capped by some unknown protein(s) that suppresses subunit dynamics.

In a whole-genome RNAi screen for spindle morphology defects in *Drosophila* S2 cells, we identified a previously uncharacterized protein (short spindle phenotype 4 [ssp4]), whose depletion caused short spindles in mitosis and microtubule fragments in interphase (Goshima et al., 2007). Three homologs exist in humans (Baines et al., 2009), one of which localizes at microtubule minus ends located close to adherens junctions in epithelial cells (Meng et al., 2008). In this study, we show that *Drosophila* ssp4, which we have renamed Patronin for the Latin "patronus" (protector), protects microtubule minus ends in vivo against depolymerization by Kinesin-13. In the absence of Patronin, microtubules release from their nucleating sites and treadmill through the cytoplasm, a result of unhindered minus end depolymerization. Purified Patronin selectively binds to and protects minus ends against Kinesin-13-induced depolymerization in vitro, demonstrating that Patronin alone is sufficient to confer minus end stability. We also show that microtubule minus end dynamics are regulated by competing actions of destabilizing and stabilizing proteins, as has been shown previously for the plus end.

RESULTS

Depletion of Patronin Results in Free Microtubules that Move through the Cytoplasm

Drosophila S2 cells do not have a central MTOC in interphase but rather generate microtubules from multiple small nucleating sites, with microtubule plus ends generally visible at the cell periphery, whereas minus ends lie more centrally (Rogers et al., 2008; Rusan and Rogers, 2009). In wild-type cells, "free" microtubules (where both the plus and minus ends of the same microtubule are clearly observed) are rarely found in the periphery (Figure 1A). In striking contrast, when Patronin was depleted by RNAi (Figure S1A available online), the interphase microtubule cytoskeleton became less dense (Figure 1A) (45% polymer decrease; Figure S1B) and the majority of cells had >5 free microtubules visible at the cell periphery (Figure 1A, Movie S1). Previously, we speculated that free microtubules might arise from increased severing after RNAi of Patronin (Goshima et al., 2007). However, we did not observe microtubule severing events in Patronin RNAi cells, and RNAi knockdown of microtubule-severing proteins did not suppress the number of free microtubules seen after Patronin RNAi (Figure S1F).

Time-lapse observation of GFP-tubulin in Patronin-depleted cells provided insight into how Patronin affects microtubules. Free microtubules appeared to move in a linear manner within the cytoplasm (Figure 1B, Movie S2). In many cases, we observed microtubules releasing from sites of nucleation and moving toward the cell periphery, which might explain the appearance of free microtubules near the cell boundary (Figures 1A and 1C, Figure S1C, Movie S2). As microtubules are nucleated at their minus ends, these observations indicated that the free microtubules were "moving" with their plus ends leading and their minus ends trailing. This conclusion is further supported by observations of EB1-GFP, which always localized to the leading end of the translocating microtubule in Patronin RNAi cells (Figure 1D, Movie S3).

Free Microtubules Move by Treadmilling in Patronin-Depleted Cells

The movement of microtubules in the cytoplasm of Patronin-depleted cells could result from either (1) transport by an anchored minus end-directed motor protein (e.g., cytoplasmic dynein) or (2) microtubule treadmilling caused by tubulin addition at the plus end at a similar rate as tubulin loss at the minus end. To distinguish between these two mechanisms, we photobleached a section of a free GFP-labeled microtubule and observed how the bleach mark moved relative to the two microtubule ends. If the free microtubule is actively transported, the bleach mark should remain stationary relative to the plus and minus ends of the moving microtubule. Conversely, if the microtubule is treadmilling, the bleach mark should appear to move away from the plus end and get closer to the minus end. In Patronin-depleted cells, we observed the latter result; all plus ends moved away from the bleach mark (3.3 ± 0.3 μm/min; n = 20) (mean ± standard deviation [SD]) whereas the minus ends moved closer (3.2 ± 0.3 μm/min; n = 20) and eventually passed through the bleached area (Figure 2A). These results indicate that microtubules move through the cytoplasm by treadmilling.

We next wanted to determine whether microtubule treadmilling occurs for any free microtubule or if this phenomenon requires the depletion of Patronin. In wild-type cells, it was possible to find an occasional free microtubule, but these did not translocate in the cytoplasm. When we photobleached a free microtubule from a wild-type cell, the bleach mark remained at a constant distance from the minus end (0.01 ± 0.07 μm/min; n = 10), whereas the plus end continued to polymerize (3.25 ± 0.24 μm/min; n = 10) (Figure 2A). This finding suggests that free microtubule minus ends are stable in wild-type cells, as has been observed in other cell types (Dammermann et al., 2003) and that the minus end depolymerization that gives rise to microtubule treadmilling requires the depletion of Patronin. We also examined whether minus end depolymerization occurred after RNAi depletion of γ-tubulin and γ-TuRC and γ-TuSC components, as the γ-TuRC complex has been shown to bind to microtubule minus ends in vitro (Moritz et al., 1995; Zheng et al., 1995; Wiese and Zheng, 2000). However, in these RNAi cells, free microtubules were rare and did not undergo treadmilling (Figure S1D).

To learn more about microtubule behavior after Patronin depletion, we measured the plus and minus end dynamics in wild-type and Patronin-depleted cells. For the microtubule plus end, the rates of growth and shrinkage and the frequencies of catastrophe and rescue were similar under Patronin depletion and wild-type conditions (Table 1). Thus, Patronin appears to have negligible effects on plus end dynamics. In contrast, minus ends displayed very different dynamics after Patronin depletion. In Patronin RNAi cells, minus ends of treadmilling microtubules often depolymerized at a rate of 3.9 ± 0.9 μm/min (mean ± SD),