Regulation of Microtubule Dynamic Instability in Vitro by Differentially Phosphorylated Stathmin*

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Stathmin is an important regulator of microtubule polymerization and dynamics. When unphosphorylated it destabilizes microtubules in two ways, by reducing the microtubule polymer mass through sequestration of soluble tubulin into an assembly-incompetent T2S complex (two αβ tubulin dimers per molecule of stathmin), and by increasing the switching frequency (catastrophe frequency) from growth to shortening at plus and minus ends by binding directly to the microtubules. Phosphorylation of stathmin on one or more of its four serine residues (Ser16, Ser25, Ser38, and Ser63) reduces its microtubule-destabilizing activity. However, the effects of phosphorylation of the individual serine residues of stathmin on microtubule dynamic instability have not been investigated systematically. Here we analyzed the effects of stathmin singly phosphorylated at Ser16 or Ser63, and doubly phosphorylated at Ser25 and Ser38, on its ability to modulate microtubule dynamic instability in steady-state in vitro. Phosphorylation at either Ser16 or Ser63 strongly reduced or abolished the ability of stathmin to bind to and sequester soluble tubulin and its ability to act as a catastrophe factor by directly binding to the microtubules. In contrast, double phosphorylation of Ser25 and Ser38 did not affect the binding of stathmin to tubulin or microtubules or its catastrophe-promoting activity. Our results indicate that the effects of stathmin on dynamic instability are strongly but differently attenuated by phosphorylation at Ser16 and Ser63 and support the hypothesis that selective targeting by Ser16-specific or Ser63-specific kinases provides complimentary mechanisms for regulating microtubule function.

Stathmin is an 18-kDa ubiquitously expressed microtubule-destabilizing phosphoprotein whose activity is modulated by phosphorylation of its four serine residues, Ser16, Ser25, Ser38, and Ser63 (1–7). Several classes of kinases have been identified that phosphorylate stathmin, including kinases associated with cell growth and differentiation such as members of the mitogen-activated protein kinase (MAPK)2 family, cAMP-dependent protein kinase (1–5, 8–11), and kinases associated with cell cycle regulation such as cyclin-dependent kinase 1 (3, 12–14). Phosphorylation of stathmin is required for cell cycle progression through mitosis and for proper assembly/function of the mitotic spindle (3, 13–16). Inhibition of stathmin phosphorylation produces strong mitotic phenotypes characterized by disassembly and disorganization of mitotic spindles and abnormal chromosome distributions (3, 13–14).

Stathmin is known to destabilize microtubules in two ways. One is by binding to soluble tubulin and forming a stable complex that cannot polymerize into microtubules, consisting of one molecule of stathmin and two molecules of tubulin (T2S complex) (17–24). Addition of stathmin to microtubules in equilibrium with soluble tubulin results in sequestration of the tubulin and a reduction in the level of microtubule polymer (17–18, 22, 25–28). In addition to reducing the amount of assembled polymer, tubulin sequestration by stathmin has been shown to increase the switching frequency at microtubule plus ends from growth to shortening (called the catastrophe frequency) as the microtubules relax to a new steady state (17, 29). The second way is by binding directly to microtubules (27–30). The direct binding of stathmin to microtubules increases the catastrophe frequency at both ends of the microtubules and considerably more strongly at minus ends than at plus ends (27). Consistent with its strong catastrophe-promoting activity at minus ends, stathmin increases the treadmilling rate of steady-state microtubules in vitro (27). These results have led to the suggestion that stathmin might be an important cellular regulator of minus-end microtubule dynamics (27).

Phosphorylation of stathmin diminishes its ability to regulate microtubule polymerization (3, 14, 25–26). Phosphorylation of Ser16 or Ser63 appears to be more critical than phosphorylation of Ser25 and Ser38 for the ability of stathmin to bind to soluble tubulin and to inhibit microtubule assembly in vitro (3, 25). Inhibition of stathmin phosphorylation induces defects in spindle assembly and organization (3, 14) suggesting that not only soluble tubulin-microtubule levels are regulated by phosphorylation of stathmin, but the dynamics of microtubules could also be regulated in a phosphorylation-dependent manner.

It is not known how phosphorylation at any of the four serine residues of stathmin affects its ability to regulate microtubule dynamics and, specifically, its ability to increase the catastrophe frequency at plus and minus ends due to its direct interaction with microtubules. Thus, we determined the effects of stathmin individually phosphorylated at either Ser16 or Ser63 and doubly phosphorylated at both Ser25 and Ser38 on dynamic instability at plus and minus ends in vitro at microtubule polymer steady.
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state and physiological pH (pH 7.2). We find that phosphorylation of Ser\textsuperscript{16} strongly reduces the direct catastrophe-promoting activity of stathmin at plus ends and abolishes it at minus ends, whereas phosphorylation of Ser\textsuperscript{63} abolishes the activity at both ends. The effects of phosphorylation of individual serines correlated well with stathmin’s reduced abilities to form stable T2S complexes, to inhibit microtubule polymerization, and to bind to microtubules. In contrast, double phosphorylation of Ser\textsuperscript{25} and Ser\textsuperscript{48} did not alter the ability of stathmin to modulate dynamic instability at the microtubule ends, its ability to form a stable T2S complex, or its ability to bind to microtubules. The data further support the hypotheses that phosphorylation of stathmin on either Ser\textsuperscript{16} or Ser\textsuperscript{63} plays a critical role in regulating microtubule polymerization and dynamics in cells.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—For the production of specifically phosphorylated forms of stathmin, the serine residues to remain unphosphorylated were first converted to alanines. Specifically, Ser\textsuperscript{25}, Ser\textsuperscript{38}, and Ser\textsuperscript{63} were mutated to Ala\textsuperscript{25}, Ala\textsuperscript{38}, and Ala\textsuperscript{63} for p16 stathmin; Ser\textsuperscript{16}, Ser\textsuperscript{25}, and Ser\textsuperscript{38} were changed to Ala\textsuperscript{16}, Ala\textsuperscript{25}, and Ala\textsuperscript{38} to prepare p63 stathmin; Ser\textsuperscript{16} and Ser\textsuperscript{63} were changed to Ala\textsuperscript{16} and Ala\textsuperscript{63} to prepare p25 and 38 stathmin; and Ser\textsuperscript{16}, Ser\textsuperscript{25}, Ser\textsuperscript{38} and Ser\textsuperscript{63} were all changed to Ala to create 4A stathmin (25). Recombinant stathmin proteins were bacterially expressed and purified as previously described (23, 25). Phosphorylation of the stathmin constructs was then carried out as described previously (25).

Briefly, protein kinase A (cAMP-dependent protein kinase) was used to phosphorylate Ser\textsuperscript{16} and Ser\textsuperscript{25} and a mixture of mitogen-activated protein kinase (MAPK) and Cdc2 was used to phosphorylate Ser\textsuperscript{25} and Ser\textsuperscript{38}. Proteins were incubated with the kinases (2.8, 2.0, and 0.5 units of cAMP-dependent protein kinase, MAPK, and/or Cdc2, respectively, per microgram of stathmin) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 5 mM EGTA, 2 mM dithiothreitol, 500 mM ATP for 6–8 h at 30 °C. Product formation was assessed by native-PAGE. Kinases were heat-inactivated at 75 °C for 10 min; because stathmin displays a fully reversible thermal unfolding/refolding (21), this heat-inactivation step does not impair the activity of stathmin. Phosphoisoforms (>93% phosphorylation) were purified to homogeneity by anion exchange chromatography. Identities of the stathmin proteins were assessed by mass spectral analyses. We have previously demonstrated that both the tubulin-binding properties and the tubulin-sequestering activities of p16 stathmin and p63 stathmin are very similar to p16,p25,p38 and p25,p38,p63 stathmins, respectively (25). We thus decided to work with the single phosphorylated versions of p16 and p63 stathmin. It should also be noted that high quality production of large amounts of triply phosphorylated stathmin is much more difficult compared with single phosphorylation. Purified bovine brain tubulin was prepared as described previously (27). Protein concentrations throughout this work were determined by the method of Bradford with bovine serum albumin as the standard (31).

**Analysis of Microtubule Dynamics**—Purified tubulin (20 μM) was assembled onto the ends of sea urchin (Strongylocentrotus purpuratus) axoneme seeds at 30 °C in PMME buffer (87 mM Pipes, 36 mM Mes, 1.4 mM MgCl\textsubscript{2}, 1 mM EGTA) at pH 7.2 in the presence or absence of unphosphorylated or phosphorylated stathmin isoforms and incubated at 30 °C for 40 min to reach steady state (confirmed by light scattering at 350 nm). Analysis of dynamic instability at plus and minus ends was carried out by video-enhanced differential interference contrast microscopy as previously described (27, 32). Briefly, reaction mixtures containing tubulin and stathmin proteins at the desired concentrations were injected into a chamber made of a glass coverslip on a glass slide that was pre-saturated with axoneme seeds in PMME buffer. Microtubules were polymerized to steady state at both ends of the axoneme seeds, and real-time images of the microtubules were recorded. The plus ends of microtubules were identified and distinguished from minus ends on the basis of their fast growth rates, the number of microtubules that grew at the ends, and the relative lengths of the microtubules. The growth rates, shortening rates, and transition frequencies were determined as previously described (27). We considered microtubules to be growing if they increased in length >0.3 μm at a rate >0.3 μm/min. Shortening events were identified by a >1 μm length change at a rate of >2 μm/min. Microtubules that changed <0.3 μm/min over a duration of 4 data points were considered to be in an attenuated (paused) state. Between 30 and 50 microtubules were analyzed for each condition. Because microtubule minus ends are relatively stable and rarely undergo shortening or growth events and only a few microtubules out of many that are tracked undergo catastrophe or rescue events, the number of events recordable at the minus ends was lower than at the plus ends.

**Gel Filtration**—Mixtures of tubulin (25 μM) and unphosphorylated or phosphorylated stathmin (5 μM) were incubated for 20 min at room temperature and then loaded into a Superose 12 GF 24 × 1.0 cm fast-protein liquid chromatography column. The elution profiles of unbound and stathmin-bound tubulin (T2S complex) were obtained by monitoring absorbance at 280 nm as described (18). The column was equilibrated with PEM buffer (100 mM Pipes, 1 mM EGTA, and 0.5 mM MgCl\textsubscript{2}) at pH 7.2 containing 2 mM GTP and developed at a flow rate of 0.3 ml/min. Total volume (V\text{c}) of the gel bed and the void volume (V\text{o}) were determined as previously described (32). The column was calibrated using a gel filtration molecular weight calibration kit (Amersham Biosciences).

**Determination of Microtubule Polymer Mass**—Microtubule suspensions containing 20 μM tubulin and unphosphorylated or phosphorylated stathmin isoforms (4 μM) were polymerized at 37 °C for 1 h in PMME buffer (pH 7.2) with 1.5 mM GTP in the presence of 1% glycerol-stabilized nucleating microtubule seeds (32, 33). Seeds were prepared by assembling purified tubulin (2.0 mg/ml) in PEM buffer, pH 6.8, plus 10% glycerol and 1 mM GTP, then shearing the microtubules six times through a 25-gauge needle. Seed suspensions were used at a 1:10 dilution. The amount of protein in microtubule pellets was determined after sedimenting and re-suspending the microtubules in ice-cold buffer (32). The final amounts of protein polymerized into microtubules were determined by subtracting the amount of protein present in the control seeds (10% v/v) from the total amount of protein in the sedimented pellets.
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Binding of Stathmin Isoforms to Pre-assembled Microtubules—
We analyzed the binding of stathmin to microtubules by first polymerizing purified tubulin to steady state (40 μM) with 4% glycerol-induced microtubule seeds for 1 h at 37 °C. Stathmin isoforms (4A stathmin, p63 stathmin, p16 stathmin, or p25,38 stathmin) were added to the preassembled microtubules, and incubation continued for an additional 30 min. Microtubules were sedimented onto coverslips through 30% sucrose cushions containing 1 μM taxol and treated with a mixture of primary antibodies against α-tubulin (DM1A mouse monoclonal (1:500), Sigma) and stathmin (rabbit antiserum (1:200), Calbiochem) followed by addition of secondary antibodies (donkey anti-mouse Cy3 and goat-anti-rabbit fluorescein) (27). We confirmed by Western blot analysis that the primary antibody against stathmin, which was specific to the C-terminal region of stathmin, has similar affinities for unphosphorylated wild-type stathmin, 4A stathmin, and the phosphorylated stathmin isoforms (not shown). Images were obtained with a Nikon Eclipse E800 Immunofluorescence microscope with Metamorph 4.6 software.

RESULTS

Effect of Phosphorylation of Ser16 and Ser63 of Stathmin on Microtubule Polymerization and on Sequestration of Soluble Tubulin into Inactive T2S Complexes—Specifically phosphorylated stathmin isoforms were constructed by mutating all serine residues into alanines except for the specific phosphoserine to be studied (“Experimental Procedures”). As a control for the possibility that conversion of serines to alanines at any of the four positions might modulate the activity of stathmin, we also prepared and analyzed the activity of stathmin with all four serines converted to alanines (4A stathmin). Mixtures of the unphosphorylated and 4A stathmin at a molar ratio of stathmin isoform to tubulin (20 μM) of 1:5 were assembled to steady state at 37 °C by addition of nucleating microtubule seeds, and the quantity of polymer was determined in the microtubule pellets after sedimentation (“Experimental Procedures”). As shown in Fig. 1A, unphosphorylated stathmin (un-phos) reduced the percent tubulin in microtubules by ~53%, and 4A stathmin reduced polymerization by a similar extent of ~57%. Stathmin reduces the amount of assembled microtubules by sequestering soluble tubulin dimers into a T2S complex consisting of one molecule of stathmin stably bound to 2 molecules of tubulin dimer (17–24, 25–26).

Next we wanted to ensure that 4A stathmin sequestered tubulin as well as unphosphorylated stathmin. Mixture of tubulin (25 μM) and wild-type stathmin were incubated for 20 min at room temperature, and the amount of T2S complex was determined after separating the complex from unbound tubulin by size exclusion chromatography (“Experimental Procedures”). Consistent with previous studies (17–23, 32), wild-type stathmin formed a stable T2S complex as visualized by the ratio of the quantity of protein in the high molecular weight peak at 10.4 ml to that in the lower molecular weight peak (11.6 ml) which corresponds to free tubulin dimers (32) (Fig. 1B). Similarly, 4A stathmin formed a similar ratio of T2S complex with free tubulin (Fig. 1B). The slight difference in the relative absorbance between unphosphorylated and 4A stathmin could be due to differences in hydrophobicity. Conversion of all four serines to alanines did not affect the ability of stathmin to inhibit polymerization or to sequester tubulin, consistent with previous studies (25). Thus, any effects of the specific phosphoisoforms on microtubule polymerization could not be due to mutation of the serine residues to alanines.

We next determined how stathmin specifically phosphorylated at Ser16 (p16 stathmin) or Ser63 (p63 stathmin) modulates the amount of microtubule polymer and the ability of stathmin to form T2S complexes. As shown in Fig. 1A, p16 stathmin reduced polymerization by only ~12% (Fig. 1A). In addition, p16 stathmin sequestered soluble tubulin poorly (Fig. 1B). These data are consistent with those of Honnappa et al. (25) who demonstrated, using high sensitivity isothermal titration calorimetry, that p16 binds to two tubulin subunits although at a lower affinity as compared with unphosphorylated stathmin. Similarly, p63 stathmin did not reduce microtubule polymerization and did not form detectable T2S complexes, indicating that, at physiological pH, phosphorylation of either Ser16 or Ser63 strongly reduces the ability of stathmin to inhibit microtubule polymerization and to sequester soluble tubulin into assembly-incompetent T2S complexes.
Effects of P16 and P63 Stathmin on Dynamic Instability at Polymer Mass Steady State—We wanted to analyze the effects of phosphorylation on the ability of stathmin to modulate microtubule dynamic instability at steady state. Once steady state is achieved, the mass of assembled microtubule polymer and the concentration of assembly-competent soluble tubulin remain constant, so the effects of stathmin on dynamics due to a direct interaction with the microtubules can be analyzed in the absence changes in the level of assembly-competent soluble tubulin (27). We used a pH of 7.2 (the average pH of the cytoplasm in most mammalian cells, 34–44). Although stathmin sequesters soluble tubulin more strongly at pH 6.8 than at pH 7.2 (17, 27, 29), it modulates dynamic instability by a direct action on the microtubules considerably more strongly at pH 7.2 than at pH 6.8 (27, 29). At pH 7.2, unphosphorylated stathmin increases the catastrophe frequency strongly (conversion of a microtubule end from a growing or attenuated state to rapid shortening) with its effects at minus ends considerably stronger than at plus ends (27). In addition, unphosphorylated stathmin does not exert significant effects on other dynamic parameters except for an increase in the percentage of time the microtubules shorten and a slight compensatory increase in the growth rate (27).

To determine how phosphorylation of Ser16 or Ser63 changes the ability of stathmin to regulate microtubule dynamics, we analyzed the effects of p16 and p63 stathmin at three different ratios of stathmin to tubulin at the plus and minus ends of individual steady-state microtubules by video microscopy. As shown in Table 1 and Fig. 2A, phosphorylation of serine 16 essentially abolished the ability of stathmin to increase the catastrophe frequency at plus ends. The apparent increase in the catastrophe frequency from 0.16 per min to 0.22 per min at a 1:5 molar ratio of p16 stathmin to tubulin was not statistically significant (Student’s t test). At the same ratio, p16 stathmin had no detectable effect on the catastrophe frequency at minus ends (Table 1 and Fig. 2B). Consistent with previous results (27), a similar ratio of unphosphorylated stathmin to tubulin increased the catastrophe frequency ~2.5-fold at plus ends and ~8-fold at minus ends (Table 1 and Fig. 2A and B). Unlike the effects of unphosphorylated stathmin on the increased percentage of time that microtubules shortened at plus and minus ends, p16 stathmin did not significantly change these parameters.

**TABLE 1**

Effect of P16 stathmin on steady-state microtubule dynamics at plus and minus ends

|                      | Unphosphorylated stathmin:tubulin | P16 stathmin:tubulin |
|----------------------|-----------------------------------|----------------------|
|                      | Control (0)                        | 1:5                  |
|                      |                                    | 1:20                 |
|                      |                                    | 1:5                  |
| **Plus end**         |                                    |                      |
| Growth rate (μm/min) | 1.2 ± 0.6 (34)                     | 1.7 ± 0.6 (44)       |
| Shortening rate (μm/min) | 24.7 ± 7.0 (25)                  | 26.6 ± 8.7 (41)      |
| Catastrophe frequency (events per min) | 0.16 ± 0.03 (25)           | 0.43 ± 0.12* (41)    |
| Rescue frequency (events per min) | 2.2 ± 0.6 (18)              | 2.8 ± 0.9 (15)       |
| Percentage of time   |                                    |                      |
| Growing              | 53.0                               | 65.6                 |
| Shortening           | 3.6                                | 8.1                  |
| Attenuated           | 43.4                               | 26.3                 |
| Dynamicity (μm/min)  | 1.4                                | 2.2                  |
| **Minus end**        |                                    |                      |
| Growth rate (μm/min) | 0.7 ± 0.26 (19)                    | 0.95 ± 0.3 (35)      |
| Shortening rate (μm/min) | 15.8 ± 4.5 (13)                 | 19.2 ± 6.8 (28)      |
| Catastrophe frequency (events per min) | 0.03 ± 0.01 (13)          | 0.21 ± 0.05 (18)     |
| Rescue frequency (events per min) | 2.8 ± 1.15 (12)            | 1.9 ± 0.6 (20)       |
| Percentage of time   |                                    |                      |
| Growing              | 11.4                               | 29.5                 |
| Shortening           | 0.6                                | 4.5                  |
| Attenuated           | 88.0                               | 66.0                 |
| Dynamicity (μm/min)  | 0.16                               | 1.1                  |

*Significant at \( p < 0.001 \). Values in the parentheses = number of events.

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Phosphorylation of stathmin at Ser63 also abolished the ability of stathmin to increase the catastrophe frequency at both ends (Table 2 and Fig. 3, A and B). Like p16 stathmin, p63 stathmin had no significant effect on total shortening time or on the rate of growth or shortening at either end (Table 2). We also prepared a stathmin isoform with all four serine residues, Ser16, Ser25, Ser38, and Ser63, phosphorylated. This isoform, as expected, behaved exactly as p63 stathmin, inducing no detectable change in the catastrophe frequency or any other dynamic instability parameter at either microtubule end (data not shown). To further eliminate any possibility that the inabilities of p16 or p63 stathmin to increase the catastrophe frequency at plus or minus ends were due to mutations of the remaining serines to alanines, we analyzed the ability of 4A stathmin to modulate dynamic instability and found, as expected, that it increased the catastrophe frequency at plus and minus ends in the same way and to the same extent as unphosphorylated stathmin (Fig. 2, A and B).

Effects of Stathmin Doubly Phosphorylated at Ser25 and Ser38 on Microtubule Polymerization, Sequestration of Soluble Tubulin, and Dynamic Instability—Phosphorylation of Ser25 and Ser38 occur through the action of cyclin-dependent kinase 1, a different kinase than that which phosphorylates Ser16 and Ser63 (3, 13, 14). Phosphorylation of these two serine residues has been thought to play a minor role in modulating the microtubule regulatory activity of stathmin in cells (3, 14). Thus, we wanted to determine the effects of stathmin doubly phosphorylated on these two residues (p25p38 stathmin, see “Experimental Procedures”) on microtubule assembly, on formation of T2S complexes, and on steady-state dynamic instability. As shown in Fig. 4A, p25p38 stathmin reduced the steady-state

### TABLE 2
Effect of P63 stathmin on steady-state microtubule dynamics at plus and minus ends

| P63 stathmin:tubulin | 1:20 | 1:5 |
|----------------------|------|-----|
| **Plus end**         |      |     |
| Growth rate (μm/min) | 1.24 ± 0.4 (41) | 1.26 ± 0.5 (42) |
| Shortening rate (μm/min) | 23.0 ± 5.4 (24) | 18.0 ± 5.5 (21) |
| Catastrophe frequency (events per min) | 0.14 ± 0.03 (24) | 0.12 ± 0.03 (21) |
| Rescue frequency (events per min) | 2.6 ± 1.0 (16) | 2.9 ± 1.0 (16) |
| Percentage of time |      |     |
| Growing | 44 | 47.4 |
| Shortening | 3.7 | 3.0 |
| Attenuated | 52.3 | 49.6 |
| Dynamicity (μm/min) | 1.1 | 1.0 |
| **Minus end**        |      |     |
| Growth rate (μm/min) | 0.83 ± 0.3 (24) | 1.1 ± 0.3 (21) |
| Shortening rate (μm/min) | 14.3 ± 3.4 (17) | 11.7 ± 3.2 (20) |
| Catastrophe frequency (events per min) | 0.04 ± 0.01 (17) | 0.04 ± 0.02 (20) |
| Rescue frequency (events per min) | 2.4 ± 1.3 (13) | 2.4 ± 0.8 (14) |
| Percentage of time |      |     |
| Growing | 19.3 | 17 |
| Shortening | 1 | 1.2 |
| Attenuated | 79.7 | 81.8 |
| Dynamicity (μm/min) | 0.3 | 0.22 |

Phosphorylation of stathmin at Ser63 also abolished the ability of stathmin to increase the catastrophe frequency at both ends (Table 2 and Fig. 3, A and B). Like p16 stathmin, p63 stathmin had no significant effect on total shortening time or on the rate of growth or shortening at either end (Table 2). We also prepared a stathmin isoform with all four serine residues, Ser16, Ser25, Ser38, and Ser63, phosphorylated. This isoform, as expected, behaved exactly as p63 stathmin, inducing no detectable change in the catastrophe frequency or any other dynamic instability parameter at either microtubule end (data not shown). To further eliminate any possibility that the inabilities of p16 or p63 stathmin to increase the catastrophe frequency at plus or minus ends were due to mutations of the remaining serines to alanines, we analyzed the ability of 4A stathmin to modulate dynamic instability and found, as expected, that it increased the catastrophe frequency at plus and minus ends in the same way and to the same extent as unphosphorylated stathmin (Fig. 2, A and B).
microtubule polymer mass as efficiently as unphosphorylated and 4A stathmin. Specifically, a 1:5 molar ratio of p25p38 stathmin to tubulin reduced microtubule polymerization by ~50% (Fig. 4A), which is similar to the extent to which 4A stathmin (Fig. 4A), and unphosphorylated stathmin inhibit polymerization (data not shown). Consistent with its strong ability to reduce microtubule polymerization, a 1:5 molar ratio of p25p38 stathmin to tubulin also formed a stable T2S complex as efficiently as 4A stathmin (Fig. 4B) or unphosphorylated wild-type stathmin (data not shown). Thus, phosphorylation at Ser^{25} and Ser^{38} together has no significant effect on the ability of stathmin to sequester soluble tubulin or to inhibit microtubule polymerization.

We also analyzed the effects of phosphorylation at Ser^{25} and Ser^{38} on the ability of stathmin to modulate dynamic instability at steady state in vitro. As shown in Table 3, p25p38 stathmin increased the catastrophe frequency at both plus and minus ends as strongly as unphosphorylated stathmin. A 1:5 molar ratio of p25p38 stathmin to tubulin increased the plus end catastrophe frequency by ~2.2-fold (Table 3 and Fig. 5A). At minus ends, a similar ratio of p25p38 stathmin to tubulin increased the catastrophe frequency by ~9-fold (Table 3 and Fig. 5B). Similar to the effects of unphosphorylated stathmin, p25p38 stathmin significantly increased the fraction of time the microtubules shortened at both plus and minus ends and moderately increased the growth rates at plus ends (compare Tables 1 and 3).

### TABLE 3

|                | Control (0) | P25,38 stathmin:tubulin |
|----------------|-------------|-------------------------|
|                |             | 1:10                    | 1:5                      |
| **Plus end**   |             |                         |                          |
| Growth rate (μm/min) | 0.86 ± 0.06 (51) | 1.03 ± 0.08 (40) | 1.17 ± 0.4 (31) |
| Shortening rate (μm/min) | 31.9 ± 17.5 (23) | 29.4 ± 13.0 (18) | 31.3 ± 12.0 (21) |
| Catastrophe frequency (events per min) | 0.11 ± 0.02 (23) | 0.17 ± 0.04 (17) | 0.24 ± 0.05* (20) |
| Rescue frequency (events per min) | 3.9 ± 1.2 (11) | 3.9 ± 1.4 (8) | 3.9 ± 1.4 (6) |
| **Minus end**  |             |                         |                          |
| Growth rate (μm/min) | 0.50 ± 0.05 (22) | 0.55 ± 0.03 (46) | 0.57 ± 0.23 (38) |
| Shortening rate (μm/min) | 29.4 ± 0.6 (6) | 30.1 ± 2.3 (18) | 30.6 ± 10.8 (22) |
| Catastrophe frequency (events per min) | 0.01 ± 0.06 (6) | 0.06 ± 0.02 (18) | 0.09 ± 0.02 (22) |
| Rescue frequency (events per min) | 4.8 ± 2.2 (6) | 3.8 ± 1.2 (10) | 3.3 ± 0.04 (10) |

* Significant at p = 0.01. Values in parentheses = number of events.

### FIGURE 5

Effects of p25, 38 stathmin on the catastrophe frequency at microtubule plus (A) and minus (B) ends at steady state. The error bars indicate ± S.D.
tubulin and stathmin antibodies (only data with 4A stathmin are shown (Fig. 6, C and D). However, no binding of p63 stathmin or p16 stathmin to microtubules could be detected as shown by the lack of stathmin staining on the microtubules (Fig. 6, G–J). In contrast, p25p38 stathmin did bind to the microtubules, consistent with its ability to increase the steady-state catastrophe frequency in a manner similar to unphosphorylated stathmin (Fig. 6, E and F).

**DISCUSSION**

In cells, the growth and shortening dynamics of individual microtubules, not just the quantity of microtubule polymer present, is critical for microtubule function, as for example, during mitosis when rapid dynamics are essential for the precise, rapid, and time-sensitive assembly and function of the mitotic spindle (45–47). Stathmin, one of the best known regulators of microtubule dynamics, destabilizes microtubules, functioning as a catastrophe promoting factor (increasing the frequency of switching from growth to shortening at microtubule ends). It acts in two ways, indirectly by sequestering tubulin into an assembly incompetent soluble T2S complex, and directly by binding to the microtubules (17, 27, 29). Sequestration of tubulin into T2S complexes destabilizes microtubules by reducing the quantity of assembly-competent tubulin and by increasing indirectly the catastrophe frequency as the microtubules depolymerize to a new steady state. The main goal of the present work was to determine how phosphorylation of each of stathmin’s four phosphorylatable serine residues, Ser\(^{16}\), Ser\(^{25}\), Ser\(^{38}\), and Ser\(^{63}\), affect the ability of stathmin to regulate dynamic instability through a direct action on the microtubules. We analyzed the effects of stathmin on dynamic instability after the microtubules attained steady state; a condition at which a stable steady-state equilibrium between polymerized microtubules and soluble tubulin is maintained. At such an equilibrium condition, modulation of microtubule dynamic instability will be due to a direct action of either stathmin or of the T2S complex on the microtubules, and not to reduction of the level of assembly-competent tubulin (27, 29). Ser\(^{16}\) and Ser\(^{63}\) are phosphorylated by different kinase systems than are Ser\(^{25}\) and Ser\(^{38}\), indicating that phosphorylation of stathmin at these two residues may have different effects on microtubule dynamics (3, 25). We found that phosphorylation of stathmin individually at either Ser\(^{16}\) or at Ser\(^{63}\) strongly reduced the binding of stathmin to tubulin, and formation of stable T2S complexes (Figs. 1 and 5). Consistent with the loss of tubulin and microtubule-binding ability, phosphorylation of either of these residues strongly reduced the ability of stathmin to increase the catastrophe frequency at plus and minus ends (Tables 1 and 2 and Figs. 2 and 3). In contrast, double phosphorylation of stathmin at Ser\(^{16}\) and Ser\(^{38}\), the two other remaining phosphorylatable serines, had no effect on the binding of stathmin to tubulin, formation of T2S complexes, or on the ability of stathmin to bind directly to microtubules and act as a catastrophe factor (Table 3 and Fig. 4).

**How Might Phosphorylation of Stathmin at Ser\(^{16}\) or Ser\(^{63}\) Abolish the Catastrophe-promoting Activity of Stathmin?**—The inability of p16 stathmin to increase the steady-state catastrophe frequency at either microtubule end together with the lack

**FIGURE 6.** Ser\(^{16}\) or Ser\(^{63}\) phosphorylation, but not Ser\(^{25}\) and Ser\(^{38}\) phosphorylation, inhibits stathmin binding to microtubules. Tubulin (40 μM) was polymerized into microtubules in the absence or presence of 4A stathmin, p16 stathmin, p63 stathmin, or p25,38 stathmin (8 μM each) at pH 7.2. Microtubules were sedimented onto coverslips by passing through sucrose cushions and stained with anti-tubulin (A, C, E, G, and I) and anti-stathmin antibodies (B, D, F, H, and J). p25,38 stathmin (E and F) and 4A stathmin (C and D) bind similarly to microtubules. Neither p63 stathmin (G and H) nor p16 stathmin (I and J) bind to microtubules. Control microtubules polymerized in the absence of stathmin are shown in A and B. The length bar is 10 μm.
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of detectable binding to the microtubules indicates that phosphorylation of Ser\textsuperscript{16} impairs the interaction of stathmin with tubulin in microtubules. As described in detail in Fig. 5A of Honnappa et al. (25) (see also Fig. 7), Ser\textsuperscript{16} of stathmin is located within the tight turn between two β strands that make a β-hairpin structure, the only structural part of the N terminus of the T2S complex. α-Tubulin is exposed at the minus ends of microtubules, and if stathmin binds tubulin at minus ends in a manner similar to the way it binds to tubulin in the T2S complex, it is reasonable to conclude that Ser\textsuperscript{16} of stathmin plays a critical role in regulating the catastrophe frequency at these ends (Fig. 1 and Table 1). It is possible that phosphorylation of Ser\textsuperscript{16} disfavors either the formation of the tight hairpin “cap” structure (21, 25, 48) or inhibits interaction of that structural region with the α1 tubulin at minus ends. Unlike unphosphorylated Ser\textsuperscript{16}, whose side chain points toward the α1-tubulin surface in the T2S complex (25), phosphorylation of Ser\textsuperscript{16} is expected to cause a steric problem and thus, the interaction of the β-hairpin at stathmin’s N terminus with the exposed α-tubulin subunit at the minus end would be disrupted.

Stathmin binds to the walls of microtubules along their entire lengths (Fig. 6) (27). It is not known whether its binding along the lengths of the microtubules increases the catastrophe-promoting activity at the ends. It is possible that binding to tubulin in the walls might produce strain within the microtubule lattice causing an increased catastrophe frequency at the ends. Phosphorylation of Ser\textsuperscript{16}, however, reduced the binding of stathmin along the length of microtubules to an undetectable level by immunofluorescence microscopy (Fig. 6), suggesting that the phosphoryl group at this position specifically hinders the binding of stathmin to tubulin along the entire microtubule surface including at or near the ends. Interestingly, phosphorylation of Ser\textsuperscript{16} strongly reduced but did not completely abolish stathmin’s steady-state plus end catastrophe frequency (Table 1 and Fig. 2). It is possible that p16 stathmin interacts with tubulin at plus ends with a very low affinity and thus is undetectable by immunofluorescence microscopy. The ability of p16 stathmin to weakly sequester free tubulin dimers supports this possibility (Fig. 1B). The N terminus of stathmin has been implicated as being involved in stimulating the catastrophe frequency at plus ends (29), although the mechanism for how it could do so is still unclear. A reasonable way stathmin might increase the catastrophe frequency at plus ends is that its N terminus may somehow nestle between the two tubulin dimers (between the α1 subunit of outer exposed tubulin dimer and the β2 subunit of the adjacent tubulin dimer) along individual protofilaments to attain more structural stability or a more energetically favorable conformation, thus breaking the tubulin-tubulin dimer contacts at the ends. Because Ser\textsuperscript{16} phosphorylation interferes with tubulin binding (24, 25), the alternation may reduce its ability to nestle between the adjacent tubulin dimers thus reducing its catastrophe-promoting activity.

The complete loss of the tubulin binding and sequestering activity of stathmin (Fig. 1) and its steady-state catastrophe-promoting activity at both microtubule ends, together with the loss of its ability to bind to microtubules when Ser\textsuperscript{63} is phosphorylated (Fig. 6), indicates that phosphorylation of Ser\textsuperscript{63} completely abolishes the functional interactions of stathmin both with soluble tubulin and with tubulin in microtubules. These results are consistent with studies indicating that phosphorylation of Ser\textsuperscript{63} significantly changes the secondary structure of stathmin, reducing the overall structural organization of its helical backbone and thus hindering alignment of the neighboring amino acid residues that maintain the tight interaction of stathmin with α1 tubulin in the T2S complex (Fig. 7) (21, 29, 49).

Possible Role of Ser\textsuperscript{25} and Ser\textsuperscript{38} Phosphorylation on Stathmin Activity—p25p38 stathmin increases the steady-state catastrophe frequency at both ends as efficiently as unphosphorylated stathmin (Table 3 and Fig. 4). It also binds to the microtubules (Fig. 6). These data indicate that Ser\textsuperscript{25} and Ser\textsuperscript{38} by themselves are minimally involved in the interaction of stathmin with soluble tubulin or with tubulin in microtubules. Analysis of the structure of the T2S complex indicates that Ser\textsuperscript{25} and Ser\textsuperscript{38} are located at a poorly ordered loop region of the complex and are not in contact with tubulin (Fig. 7) (19, 20, 25). Phosphorylation of Ser\textsuperscript{25} or Ser\textsuperscript{38} also does not significantly alter the secondary structure of stathmin as determined by CD spectroscopy (25). This raises the question of how phosphorylation of Ser\textsuperscript{25} or Ser\textsuperscript{38} might be involved in controlling cell cycle progression through an action on microtubules without affecting the microtubule destabilizing activity of stathmin? Phosphorylation of Ser\textsuperscript{25} and Ser\textsuperscript{38} appears to be a prerequisite for phosphorylation of Ser\textsuperscript{16} and Ser\textsuperscript{63} during mitosis (3, 14), and one possibility in the case of mitosis is that phosphorylation of these residues may facilitate the activity of kinases that phosphorylate Ser\textsuperscript{16} and Ser\textsuperscript{63}.

Implications for Regulation of Minus End Dynamics by Stathmin—Stathmin increases the catastrophe frequency considerably more strongly at minus ends than at plus ends (27). Minus ends do not grow in cells, but rather, they either remain stable or they depolymerize (50–53). For example, during metaphase and anaphase of mitosis, spindle microtubules in vertebrate cells depolymerize from their minus ends while remaining tethered at the spindle poles in a process called poleward flux that appears to be crucial for chromosome segregation and separation of the spindle poles (53–55). Cellular factors that modulate microtubule shortening at minus ends have been suggested to regulate poleward flux (27, 53–57). Stathmin localizes at the minus ends of spindle microtubules at spindle poles in mitotic HeLa cells (6). Thus, stathmin might increase the flux rate by destabilizing minus...
ends at the poles. Although stathmin is predominantly phosphorylated during mitosis, a fraction of stathmin remains unphosphorylated even in cells blocked in mitosis for prolonged periods (58). The small amount of remaining unphosphorylated stathmin could represent a pool of active stathmin that can bind directly to microtubule minus ends to exquisitely regulate minus-end dynamics. Stathmin might even facilitate flux at minus ends when Ser\textsuperscript{25} and Ser\textsuperscript{38} are phosphorylated, because phosphorylation of these serine residues does not diminish its minus-end catastrophe-promoting activity (Table 3 and Fig. 5B).

**Regulation of Microtubule Dynamics by Stathmin Phosphorylation**—It is not yet understood how stathmin’s abilities to destabilize microtubules and to function as a catastrophe-promoting factor are regulated by phosphorylation at its different kinase-specific phosphorylation sites in cells. Our data indicate that the catastrophe-promoting activity of stathmin can be inactivated in similar fashion by phosphorylation of either Ser\textsuperscript{16} or Ser\textsuperscript{63}, and that its catastrophe-promoting activity is not regulated in the absence of other factors by phosphorylation of Ser\textsuperscript{25} and Ser\textsuperscript{38}. Based upon our data, it is reasonable to hypothesize that in cells, the catastrophe-promoting activity of stathmin can be controlled in a similar fashion by phosphorylation of either Ser\textsuperscript{16} or Ser\textsuperscript{63}. Because the kinases acting at these residues are distinct and might function through different pathways, it is reasonable to think that a similar loss of function can be achieved by multiple pathways, thus providing the cell with a finely tunable mechanism for controlling microtubule assembly and dynamics in relation to its needs.
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