Stathmin Family Protein SCG10 Differentially Regulates the Plus and Minus End Dynamics of Microtubules at Steady State *in Vitro*: Implications for Its Role in Neurite Outgrowth†

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ABSTRACT: SCG10 (superior cervical ganglia neural-specific 10 protein) is a neuron specific member of the stathmin family of microtubule regulatory proteins that like stathmin can bind to soluble tubulin and depolymerize microtubules. The direct actions of SCG10 on microtubules themselves and on their dynamics have not been investigated previously. Here, we analyzed the effects of SCG10 on the dynamic instability behavior of microtubules *in vitro*, both at steady state and early during microtubule polymerization. In contrast to stathmin, whose major action on dynamics is to destabilize microtubules by increasing the switching frequency from growth to shortening (the catastrophe frequency) at microtubule ends, SCG10 stabilized the plus ends both at steady state and early during polymerization by increasing the rate and extent of growth. For example, early during polymerization at high initial tubulin concentrations (20 μM), a low molar ratio of SCG10 to tubulin of 1:30 increased the growth rate by ∼50%. In contrast to its effects at plus ends, SCG10 destabilized minus ends by increasing the shortening rate, the length shortened during shortening events, and the catastrophe frequency. Consistent with its ability to modulate microtubule dynamics at steady state, SCG10 bound to purified microtubules along their lengths. The dual activity of SCG10 at opposite microtubule ends may be important for its role in regulating growth cone microtubule dynamics. SCG10's ability to promote plus end growth may facilitate microtubule extension into filopodia, and its ability to destabilize minus ends could provide soluble tubulin for net plus end elongation.

Microtubules in neurons mediate many important processes including development and maintenance of neuritic processes and growth cone steering and forward movement (*1*−*8*). As in non-neuronal cells, neuronal microtubules can be highly dynamic and are especially so in growth cones, where their plus ends switch stochastically between growth and shortening states by the process of dynamic instability (*5*−*8*). Dynamic instability occurs at both plus and minus ends of purified microtubules *in vitro*, with the dynamics at plus ends being more robust than those at minus ends (*9*, *10*). However, minus ends in cells do not appear to grow and are either stable or persistently shorten (*11*, *12*).

A substantial number of proteins are now known that regulate microtubule dynamics at their plus ends (*5*, *6*), but only a small number of factors, such as some members of the kinesin I family and the microtubule regulatory phosphoprotein stathmin, have thus far been identified that might regulate minus end disassembly (*13*, *14*). Stathmin and its protein family including the neuron-specific protein SCG10[1] play important roles in neuronal processes such as neuronal differentiation (*15*, *16*). For example, decreasing stathmin expression in PC12 cells blocks nerve growth factor-stimulated differentiation (*16*). SCG10 has ∼70% sequence identity to stathmin (Figure 1A and B) and has recently been identified as a microtubule regulatory/destabilizing factor (*17*). SCG10 is associated with the Golgi complex in neuronal cell bodies from where it is sorted to vesicles that are transported along the neurites down to the growth cones where the protein accumulates. Although the transport of SCG10 to growth cones is dependent on its N-terminal domain that contains two palmitoylation sites, this domain is not important for the microtubule-destabilizing activity of SCG10 (*15*, *17*−*21*). Because of its localized distribution in advancing growth cones, specifically in the central (C) domain where microtubules are highly dynamic, SCG10 is thought to play an important role in regulating growth cone microtubule dynamics (*15*). For example, overexpression of SCG10 in neuronal cells enhances neurite outgrowth, a phenomenon that is believed to be dependent on the assembly−disassembly dynamics of microtubules (*17*). This idea is further supported by the recent observation that

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[1] Abbreviations: SCG10, superior cervical ganglia neural-specific 10 protein; MAPs, microtubule-associated proteins; PMME buffer, 87 mM Pipes, 36 mM Mes, 1.4 mM $MgCl_2$, and 1 mM EGTA at pH 7.2; PEM buffer, 50 mM Pipes, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 7.2; P domain, actin-rich peripheral domain of a neuronal growth cone; C domain, microtubule-rich central domain of a neuronal growth cone.

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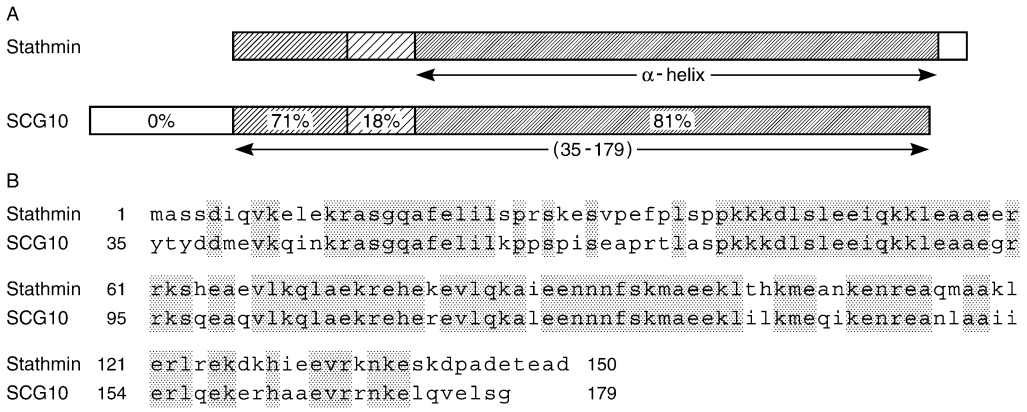

FIGURE 1: (A) Schematic comparison of stathmin and SCG10. The shaded and hatched regions constitute the stathmin-like domains. The percentage of amino acid identity to stathmin in the various segments is shown in the SCG10 structure. The arrow under stathmin denotes the α-helical regions of the two proteins. The membrane-associating N-terminus of SCG10, not present in stathmin, is unshaded. (B) Amino acid sequences of the stathmin like domains of SCG10 and stathmin. The identical residues in the two proteins are highlighted.

blocking SCG10 function specifically in cultured neuronal growth cones leads to an arrest of the forward elongation of the growth cones (*22*).

The mechanisms by which SCG10 and the other stathmin family members regulate microtubule polymerization and dynamics have been the focus of a number of recent studies (*14*, *17*, *23*, *24*). All of the stathmin family proteins can bind to tubulin and can sequester the tubulin into a ternary "T2S" complex consisting of 2 molecules of the tubulin dimer and one molecule of stathmin family protein (*25*−*27*). Stathmin itself can strongly sequester tubulin, especially at low pH, which reduces the concentration of soluble tubulin available for assembly into microtubules. Thus, one important potential action of stathmin and its family members on microtubules is that they might control the microtubule polymer mass (*27*). Apart from the ability to destabilize microtubules through the sequestration of soluble tubulin, stathmin can also destabilize microtubules by increasing the catastrophe frequency at microtubule ends through a direct action on the microtubules themselves (*14*, *24*). Interestingly, its destabilizing effects at minus ends are considerably stronger than those at the plus ends, suggesting that one of its important roles in cells is to regulate minus end disassembly (*14*).

Like stathmin, SCG10 sequesters soluble tubulin into a T2S complex (*27*) and has been shown to inhibit the assembly of purified brain microtubules and to depolymerize pre-assembled microtubules (*17*, *23*). SCG10 has also been found to interact with purified microtubules (*17*). Specifically, it co-purified with microtubules isolated by cycles of assembly and disassembly. However, the mechanisms by which SCG10 modulates microtubule dynamics have not been previously investigated. In this work, we analyzed the effects of SCG10 on microtubule dynamic instability at both plus and minus ends *in vitro* at polymer mass steady state and at plus ends during pre-steady-state net elongation conditions when the polymer mass was increasing. Analysis at steady state conditions in which the soluble tubulin concentration remained constant allowed us to determine the effects of SCG10 on dynamic instability occurring through a direct action on the microtubules, independent of its effects associated with the sequestration of soluble tubulin. We find that in contrast to stathmin, which destabilized plus ends and exerted little or no effect on plus end growth, SCG10 significantly increased the rate and extent of growth at these ends. In further contrast to stathmin, SCG10 only weakly increased the plus end catastrophe frequency. However, like stathmin, SCG10 strongly destabilized minus ends, but it did so in a manner different from that of stathmin. The results are consistent with the hypothesis that an important function of SCG10 in advancing growth cones is to regulate microtubule plus end elongation. The results also suggest that SCG10's effect on minus end disassembly could play an important indirect role in controlling the forward movement of growth cones because soluble tubulin derived from microtubule minus end disassembly could facilitate plus end growth near the growth cone tips (*12*, *15*).

MATERIALS AND METHODS

Tubulin, Stathmin, and SCG10 Constructs. Bovine brain microtubule protein consisting of ∼70% tubulin and 30% microtubule associated proteins was purified in the absence of glycerol by two cycles of polymerization and depolymerization as described elsewhere (*28*, *29*). Tubulin was purified from the microtubule protein as described previously by elution through a phosphocellulose column (Whatman p-11) equilibrated in 50 mM Pipes, 1 mM $MgS0_4$, 1 mM EGTA, and 0.1 mM GTP at pH 6.8. It was drop frozen in liquid nitrogen and stored at −70 °C (*29*). The cloning, expression, and purification of human recombinant stathmin have been previously described in detail (*30*). The purification of recombinant full-length SCG10 and N-terminally truncated SCG10 (retaining residues 35−179) have also been described previously (*31*, *32*). Recombinant full-length SCG10 has poor solubility and forms aggregates because of the high hydrophobicity of the N-terminal domain of 34 amino acids (*32*). Because these forms of SCG10 readily degraded into a shorter cleavage product (48−179) (*31*, *32*), an N-terminally His-tagged soluble form of SCG10, referred to here as SCG10 (35−179) or simply SCG10, which does not have the membrane attachment domain, was used in this work. This construct was obtained by PCR amplification and cloning into pQE30 (Qiagen). The recombinant protein was expressed and purified under native conditions using Ni^{2+}-NTA agarose columns (Qiagen) according to the manufacturer's instructions. The bacterial cultures were grown to an optical density of 0.6 at 600 nm (37 °C) and expression was

induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h. Cells were pelleted and resuspended in lysis buffer containing 10 mM imidazole. After sonication, DNAse I and RNAse A treatment, and centrifugation, the supernatant was mixed with Ni-NTA slurry and loaded to a column. Column flow-through was collected, followed by a first wash in buffer containing 20 mM imidazole and a second wash in buffer containing 50 mM imidazole. The protein was then eluted with elution buffer containing 250 mM imidazole, and all fractions were analyzed by 14% SDS−PAGE to ensure the size and purity of the recombinant protein. The eluted SCG10, which was at least 95% pure, was dialyzed into PEM Buffer (80 mM Pipes, 1 mM EGTA, and 4 mM $MgCl_2$ at pH 6.85). SCG10 protein concentration was determined using a protein assay reagent (catalog number ADV02) obtained from Cytoskeleton, Inc. (Denver, CO). The protein was then frozen in aliquots and stored at −80 °C.

The microtubule depolymerizing activity of the His-tagged SCG10 (35−179) protein *in vitro* was verified at pH 6.85 using a light scattering assay as described by Bondallaz et al. (*33*). Specifically, the microtubule assembly inhibitory activity of SCG10 (35−179) was found to be identical to that of the non-His-tagged protein, that is, it completely blocked microtubule polymerization when used at a concentration of 13 μM together with 25 μM purified tubulin at this pH. The ability of SCG10 (35−179) to depolymerize microtubules was also verified in cells by transfecting the corresponding pcDNA3 construct in cultured COS-7 cells and analyzing the microtubule content of the cells (*34*); its depolymerizing ability was clearly independent of the presence of the N-terminal domain.

Video Microscopy. Purified tubulin (20 μM) was polymerized onto both the plus and minus ends of sea urchin (*Strongylocentrotus purpuratus*) axoneme seeds at 30 °C in the presence or absence of SCG10 (35−179) in 87 mM Pipes, 36 mM Mes, 1.4 mM $MgCl_2$, and 1 mM EGTA at pH 7.2 (PMME Buffer) containing 2 mM GTP (*10*, *14*). A pH of 7.2 was used in this work (*14*). For steady state measurements, incubation was carried out for 40 min to ensure the attainment of steady state prior to analysis of dynamics (turbidimetrically confirmed at 350 nm). For pre-steady-state conditions, dynamics were analyzed between 2 and 10 min after initiating polymerization onto the seeds. Real time recordings of microtubule growth and shortening dynamics were obtained at 30 °C by video-enhanced differential interference contrast microscopy as previously described (*10*, *14*). Briefly, the dimensions of the chamber were ~10 × 2 × 0.5 mm. Chambers were made by placing thin double sided tapes (~0.5 mm width) in parallel at ~2 mm distance and placing the cover slips on top. After injecting the reaction mixture, the two sides of the chamber were sealed with VALAP. Five to 10 μL of the reaction mixture fills the whole chamber. The glass surface inside the chamber was presaturated with axoneme seeds in PMME buffer. After 5 min, the unattached axonemes were removed by perfusing PMME buffer into the chamber. Immediately after that, mixtures of tubulin, SCG10, and GTP in PMME buffer were injected into the chamber, and the two sides were immediately sealed. The seeds remained attached to the glass. The microtubules that grew onto the seeds were not attached. The ends were designated as plus or minus on the basis of growth rates and percent time spent growing, the number of microtubules that grew at opposite ends of the seeds, and the relative lengths of the microtubules (*10*, *14*). End designation was also confirmed with *Chlamydomonas flagella* axonemes (*14*), which exhibit a bifurcation at their plus ends (*35*). Growth rates, shortening rates, and the transition frequencies from the growing or attenuated state to shortening (the catastrophe frequency) and from shortening to the growing or attenuated state (the rescue frequency) were determined as previously described (*10*, *14*). 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 four data points were considered to be in an attenuated (also called pause) state, neither growing nor shortening detectably. Between 25 and 60 microtubules were analyzed for each condition.

Gel Filtration. Mixtures of tubulin (25 μM) and stathmin (5 μM) or SCG10 (35−179) (5 μM) were loaded into a Superose 12 GF 24 × 1.0 cm FPLC column and the elution profiles of free and SCG10-bound tubulin mixtures were obtained by monitoring absorbance at 280 nm (*36*). The column was pre-equilibrated with PEM buffer (50 mM Pipes, 1 mM EGTA, and 0.5 mM $MgCl_2$ at pH 7.2) at room temperature and developed at a flow rate of 0.3 mL/min. Total volume (V_t) of the gel bed and the void volume (V_o) were determined as previously described (*36*). 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 SCG10 (35−179) at concentrations ranging from 0.5 to 6.7 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 (*14*, *37*). Microtubule nucleating seeds were prepared by assembling purified tubulin (2.0 mg/mL) in PEM buffer at pH 7.2 and 10% glycerol and 1 mM GTP, then shearing the microtubules six times through a 25-gauge needle. The mean length of the microtubule seeds in the seed suspension determined by negative stain electron microscopy (*37*) varied between 11.3 and 15.5 μm, and the seed concentration varied between 5×10^{-11} and 2×10^{-10} seeds per L. The seed suspension was used at a 1:10 dilution. The amount of protein in microtubule pellets was determined after sedimenting the microtubules and resuspending them in ice-cold buffer (*10*, *14*). The amount of total protein in each suspended pellet was determined by the Bradford assay, using bovine serum albumin as the standard (*38*).

SCG10 and Stathmin Binding to Microtubules. For quantitation of SCG10 or stathmin binding to microtubules, tubulin (40 μM) was polymerized in the presence of SCG10 35−179 (ranging from 2 to 8 μM) or stathmin (8 μM) in PMME buffer for 40 min at 37 °C with nucleating seeds prepared as described above. The microtubules were then sedimented through 30% sucrose cushions, and the pellets were re-suspended and subjected to SDS−PAGE followed by Coomassie Blue staining as described previously for stathmin (*14*). The amount of SCG10 or stathmin bound to the microtubules was determined by densitometric quantitative comparison with a known standard SCG10 or stathmin band by using a UVP EPI-CHEM Darkroom as described previously for stathmin (*14*). The amount of total protein in

each pellet was determined prior to electrophoresis by the Bradford protein assay (*38*), and the tubulin concentration was determined after subtracting the quantity of bound SCG10 from the total amount of protein in each pellet. Each experiment was performed three times.

RESULTS

Effects of SCG10 on Microtubule Polymerization and Sequestration of Soluble Tubulin. At low pH (pH 6.4), SCG10 strongly inhibits the polymerization of microtubule-associated protein (MAP)-rich tubulin into microtubules and also depolymerizes pre-assembled microtubules polymerized in the presence of MAPs or with high concentrations of taxol *in vitro* (*17*). Because stathmin sequesters soluble tubulin and inhibits polymerization more strongly at pH 6.8 than at pH 7.2 (*14*), yet still modulates dynamics strongly at pH 7.2, and because pH 7.2 is the mean pH in the cytoplasm of most cells (*14*), we wanted to use pH 7.2 in this work. To ensure adequate numbers and lengths of microtubules so as to be able to analyze the effects of SCG10 on microtubule dynamic instability by video microscopy, we first determined the relationship between SCG10 concentration and inhibition of pure tubulin (20 μM) polymerization into microtubules at the conditions used. As expected (*17, 33*), SCG10 (35−179) inhibited polymerization of the microtubules. However, in contrast to its strong ability to inhibit polymerization at pH 6.8, it relatively weakly inhibited polymerization at pH 7.2. Mixtures of tubulin (20 μM) and SCG10 at concentrations between 1 and 4 μM were polymerized by the addition of nucleating seeds, and the quantity of polymer was determined in microtubule pellets after sedimentation (Materials and Methods). As shown in Figure 2A, SCG10 (35−179) reduced the microtubule polymer mass in a concentration-dependent manner. In the absence of SCG10, ~70% of the total tubulin assembled into microtubules (no inhibition; 100% of control). At the highest SCG10 (35−179) concentration used in this experiment (an SCG10 to tubulin molar ratio of 1:5), the quantity of assembled polymer was reduced only by ~30%. Thus, we reasoned that ratios of SCG10 to tubulin of 1:5 and lower would provide adequate microtubule numbers and lengths for analyzing the effects of SCG10 on dynamics.

SCG10 only weakly inhibited polymerization as compared with stathmin (*14*), suggesting that it might sequester tubulin much less effectively than stathmin. Because we also wanted to compare the actions of stathmin and SCG10 on polymerization and dynamics, we also determined the relative abilities of SCG10 and stathmin to sequester tubulin dimers (*27*). SCG10 (35−179) (5 μM) or stathmin (5 μM) was incubated with soluble tubulin (25 μM) for 30 min at room temperature, and the amount of sequestered tubulin and free tubulin was determined by gel filtration chromatography (Materials and Methods). As shown in Figure 2B, both SCG10 (35−179) and stathmin formed high molecular weight complexes corresponding to 1:2 SCG10−tubulin or stathmin−tubulin T2S complexes (at 10.4 mL) (*14, 27*). The amount of the T2S SCG10−tubulin complex that formed was considerably lower than the amount of the stathmin−tubulin T2S complex formed (Figure 2B). Thus, at the physiological pH and buffer conditions used, SCG10 sequesters soluble tubulin relatively poorly. These data are consistent with the relatively weak ability of SCG10 to inhibit polymerization at the conditions used and further indicate that a substantial amount of free SCG10 would be available for direct interaction with the microtubules.

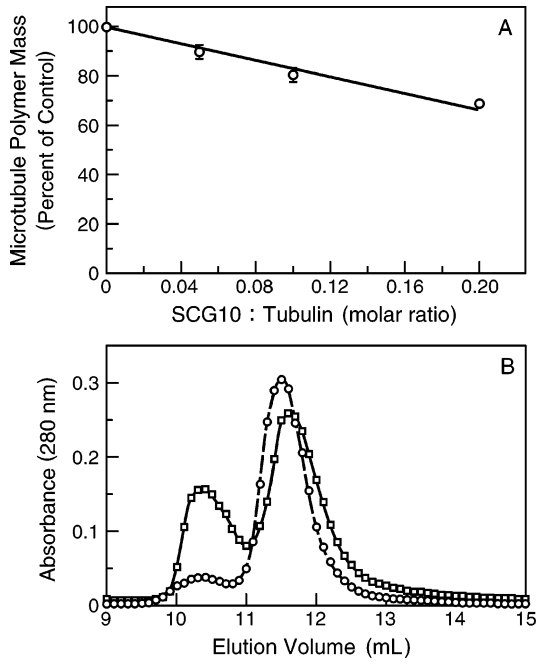

FIGURE 2: Inhibition of tubulin polymerization into microtubules by SCG10 (35−179) and the relative abilities of stathmin and SCG10 to sequester tubulin. (A) Tubulin (20 μM) was polymerized into microtubules in the absence and presence of SCG10, and the amount of protein in microtubule pellets was determined after sedimentation of the microtubules as described in Materials and Methods. Data are mean ± SEM. (B) Tubulin (25 μM) and 5 μM SCG10 (○) or 5 μM stathmin (□) were incubated for 30 min at room temperature and subjected to size-exclusion FPLC column chromatography as described in Materials and Methods. Free tubulin eluted at a volume of ~11.5 mL, and the stathmin and SCG10 complexes eluted at 10.4 mL.

Effects of SCG10 on Dynamic Instability at Microtubule Plus Ends in Vitro. Life history traces of several individual growing and shortening microtubules at their plus ends at steady state in the absence and presence of SCG10 (35−179) (1:5 ratio of SCG10 to tubulin) are shown in Figure 3. By visual inspection, it is readily apparent that SCG10 strongly increased the steady-state growth rate. As shown in Figure 4A, a 0.05 (1:20) molar ratio of SCG10 to tubulin increased the growth rate from 1.2 μm per min to 1.6 μm per min, an increase of ~30%. A further increase in the molar ratio of SCG10 to tubulin to 0.1 (1:10) increased the growth rate by ~50% (~1.6-fold) ($P < 0.001$ by student's *t*-test). Consistent with its ability to increase the plus end growth rate, SCG10 also significantly increased the average length the microtubules grew during individual growth events (Figure 4B). It also increased the fraction of time the microtubules remained in the growth state and thus increased the overall dynamicity and the total extent of growth and shortening per unit time by ~2 fold (Table 1; 1:5 SCG10/tubulin). SCG10 (35−179) had little effect on the plus end shortening rate or the rescue frequency (Table 1). In contrast to stathmin, which has significant catastrophe-promoting activity at plus ends (*14, 24*), SCG10 only minimally increased the steady-state catastrophe frequency at these ends (Table 1).

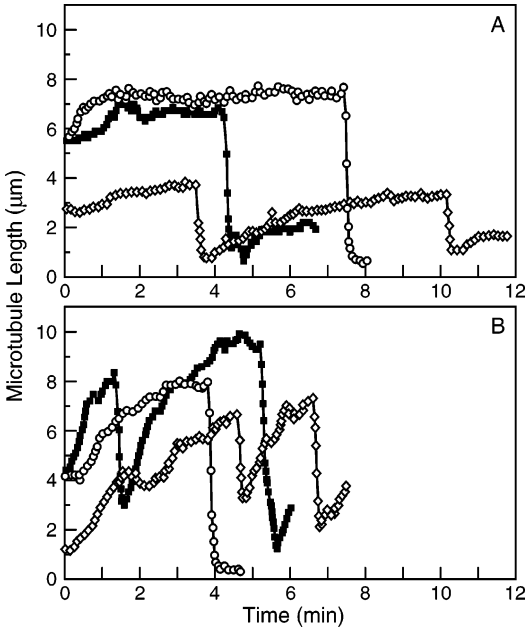

FIGURE 3: Length changes with time at the plus ends of microtubules at steady state in the absence (A) and presence (B) of a 1:5 molar ratio of SCG10 to tubulin. Each trace is an individual microtubule (see Materials and Methods).

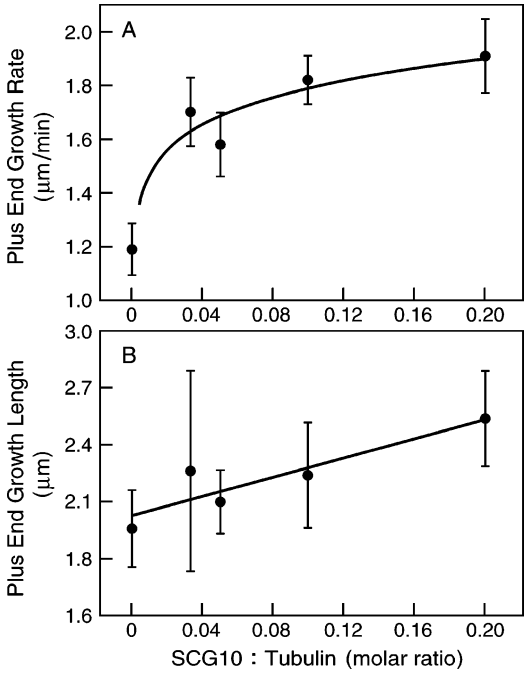

FIGURE 4: Effects of SCG10 on the growth rate (A) and the length grown during growth events (B) at plus ends of microtubules at steady-state *in vitro.* Data are the mean ± SEM.

We also wanted to determine the ability of SCG10 to increase the growth rate at net elongation conditions when the microtubule polymer mass is increasing, as might occur during early neurite extension. Thus, we analyzed the ability of SCG10 (35−179) to increase the rate and extent of growth early during polymerization when the initial tubulin concentration was high (20 μM), and the microtubules were undergoing net elongation and minimal shortening. Under these conditions, only a 1:30 molar ratio of SCG10 to tubulin was sufficient to increase the plus end growth rate by ∼50% and the length the microtubules grew per growth event by ∼20% (Table 2). Thus, both at steady state and during initial polymerization, SCG10 significantly increases the plus end growth rate by an action that must be independent of its ability to sequester soluble tubulin.

Effects of SCG10 (35−179) on Dynamic Instability at Minus Ends in Vitro. The effects of SCG10 on the dynamic instability parameters at minus ends contrasted significantly with its effects at plus ends. As shown in Figure 5A and B, SCG10 (35−179) significantly increased the shortening rate and the catastrophe frequency at minus ends. For example, only a 1:40 molar ratio of SCG10 to tubulin increased the shortening rate by 50% (∼1.5-fold). At a 1:10 molar ratio of SCG10 to tubulin, the shortening rate was increased ∼80% (Figure 5A). Consistent with its ability to increase the shortening rate, SCG10 increased the average length shortened during individual shortening events (Figure 5A) and strongly increased the fraction of time that the minus ends shortened (Table 3). It also decreased somewhat the fraction of time the ends remained in an attenuated state, neither growing nor shortening detectably, and it increased the overall dynamicity by ∼4-fold (Table 3). Consistent with the ability to destabilize minus ends, SCG10 significantly increased the catastrophe frequency and decreased the rescue frequency at these ends (Figure 5B). At a 1:10 molar ratio of SCG10 to tubulin, the steady-state catastrophe frequency was increased by ∼4-fold and the rescue frequency was decreased by ∼40% (Figure 5B). In contrast to its effects at plus ends, SCG10 had little effect on the minus end growth rate (Table 3).

SCG10 Binds along the Microtubule Surface. SCG10 modulated dynamics at microtubule ends at steady state, when the concentration of soluble tubulin remained constant. The results indicate that SCG10 or SCG10−tubulin complexes must modulate the dynamics by directly acting on the microtubules themselves. Strong evidence that SCG10 can indeed bind to microtubules was obtained very recently by Bondallaz et al. (*33*) who have shown by immunoelectron microscopy that SCG10 binds along the surfaces of purified microtubules *in vitro* and microtubules in neuronal growth cones. Here, we measured the binding of SCG10 to microtubules by assembling 40 μM tubulin into microtubules in the presence of 4 or 8 μM SCG10. The amount of bound SCG10 was then determined by SDS−PAGE and densitometric analysis after sedimenting the microtubules through sucrose cushions to ensure the removal of nonspecifically bound SCG10 (Materials and Methods). As shown in Figure 6, SCG10 clearly co-sedimented with the microtubules. Although we did not determine whether any SCG10 may have dissociated during centrifugation through the sucrose cushions, SCG10 binding was high, and high concentrations of sucrose stabilize microtubules and their associated proteins (e.g., see *29*), suggesting that dissociation, if any occurred, was probably minimal. At an initial molar ratio of SCG10 to tubulin of 1:10, 1 mol of SCG10 was bound per 15.7 ± 4.2 mol of tubulin dimers in the microtubules. At an initial molar ratio of SCG10 to tubulin of 1:5, the highest ratio of SCG10 to tubulin used, 1 mol of SCG10 was bound per 6.7 ± 1.5 mol of tubulin dimer. Such a high binding stoichiometry further supports the idea that the effects of SCG10 on dynamics are due to its binding to microtubules. The relatively weak SCG10 band compared with that of tubulin is to be expected, on the basis of the fact that with one

Table 1: Modulation of Dynamic Instability at Plus Microtubule Ends at Steady State *in Vitro* by SCG10[a]

	ratio of SCG10 to Tubulin				
	0 (control)	1:30	1:20	1:10	1:5
growth rate (μm/min)	1.2 ± 0.1 (41)	1.7 ± 0.1 (23)	1.6 ± 0.1 (38)	1.8 ± 0.1 (36)	1.9 ± 0.1** (34)
shortening rate (μm/min)	25.2 ± 1.5 (29)	29.1 ± 1.7 (19)	25.8 ± 2.0 (30)	29.0 ± 1.3 (29)	26.9 ± 2.2 (31)
catastrophe frequency (events per min)	0.17 ± 0.03 (29)	0.27 ± 0.07 (19)	0.27 ± 0.07 (30)	0.29 ± 0.07 (29)	0.28 ± 0.08* (31)
rescue frequency (events per min)	2.2 ± 0.6 (27)	2.8 ± 1.0 (19)	2.8 ± 0.8 (28)	3.0 ± 1.0 (28)	2.6 ± 0.8 (28)
percentage time					
growing	53.8	70.4	77.6	63.3	64.8
shortening	3.7	5.6	6.1	5.9	6.5
attenuated	43.5	23.9	16.2	30.8	28.7
dynamicity (μm/min)	1.4	2.6	2.5	2.6	2.7

[a] Data are the mean ± SEM. *P = < 0.05 (student's t test). **P = 0.01. Tests of significance were not performed on the percentage time or dynamicity, an overall parameter. The values in parentheses = the number of events measured.

Table 2: Modulation of Dynamic Instability by SCG10 at Plus Microtubule Ends *in Vitro* Early during Assembly[a]

	ratio of SCG10 to tubulin		
	0 (control)	1:50	1:30
growth rate (μm per min)	1.6 ± 0.1 (38)	1.9 ± 0.1 (37)	2.3 ± 0.1 (43)
growth length (μm)	3.5 ± 0.4	3.8 ± 0.4	4.3 ± 0.4
percentage time			
growing	74.5	80	87
shortening	0.8	0.9	1.2
attenuated	24.7	19.2	11.8

[a] Data are the mean ± SEM. The values in parentheses = the number of events measured.

molecule of SCG10 (M_r, 16 899) bound per 6.7 molecules of tubulin dimer (M_r, 100,000), the SCG10 band will have only 2.2% of the protein mass of the tubulin band. Interestingly, at the identical molar ratio of SCG10 or stathmin to tubulin of 1:5, SCG10 bound to microtubules with an approximately 2-fold higher stoichiometry than that of stathmin (*14*).

DISCUSSION

We have analyzed the effects of SCG10 (35−179) on dynamic instability at both plus and minus ends of individual microtubules *in vitro*. At plus ends at steady state conditions in which the tubulin concentration is maintained at the critical concentration (~2.6 μM) and the total polymer mass remains constant (*14*), SCG10 significantly increased the growth rate and the average length the microtubules grew during individual growth events while not causing any significant effects on the shortening rate. A 1:5 molar ratio of SCG10 to tubulin increased the growth rate by ~60% (P < 0.001), and the average growing length was increased by ~55% (Figure 4). We also analyzed the effects of SCG10 on plus end growth during early microtubule elongation when the initial tubulin concentration was high (20 μM), and the microtubule polymer mass was increasing, and found under these conditions that a 1:30 ratio of SCG10 to tubulin was sufficient to increase the plus end growth rate by ~50% and the extent of growth by ~20% (Table 2). In contrast to stimulating growth at plus ends, SCG10 destabilized minus ends at steady state by increasing the shortening rate and the average length shortened during individual shortening events (Table 3, Figure 5). Like stathmin, SCG10 increased the catastrophe frequency at minus ends, but it did so less strongly than stathmin. We also found that SCG10 only weakly inhibited microtubule polymerization and that it poorly sequestered soluble tubulin dimers at the physiological pH and conditions used in this work (Figure 2). These data are consistent with the previously advanced hypothesis that the major function of SCG10 in neurons is the regulation of microtubule dynamics (*15*).

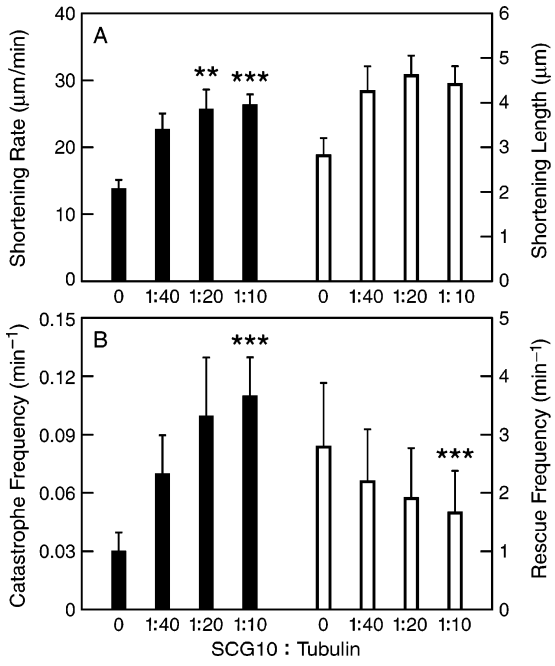

FIGURE 5: Effects of SCG10 on the shortening rate, shortening length (A), and on the catastrophe and rescue frequencies (B) at minus ends of microtubules at steady state *in vitro*. Data are the mean ± SD. **P = < 0.02; ***P = < 0.01 (student's t test).

Because SCG10 modulated the dynamic instability behavior of the microtubules at steady state when the soluble tubulin concentration did not change, the modulation had to be due to a direct action of SCG10 or SCG10−tubulin complexes on the microtubules themselves rather than changes, in this case, a decrease, in the available soluble

Table 3: Modulation of Dynamic Instability at Minus Microtubule Ends at Steady State *in vitro* by SCG10[a]

	ratio of SCG10 to tubulin			
	0 (control)	1:40	1:20	1:10
growth rate (μm/min)	0.68 ± 0.05 (29)	0.89 ± 0.05 (31)	0.82 ± 0.06 (35)	0.82 ± 0.06 (33)
shortening rate (μm/min)	15.8 ± 1.4 (23)	20.4 ± 1.6 (26)	23.5 ± 3.0 (33)	26.7 ± 1.6* (29)
catastrophe frequency (events per min)	0.03 ± 0.01 (23)	0.07 ± 0.02 (26)	0.09 ± 0.02 (33)	0.11 ± 0.02* (29)
rescue frequency (events per min)	2.8 ± 1.2 (21)	2.2 ± 0.9 (24)	1.9 ± 0.9 (26)	1.7 ± 0.7* (27)
percentage time				
growing	11.4	16.0	18.2	21.8
shortening	0.6	1.5	2.0	2.8
attenuated	88.0	82.5	79.8	76.7
dynamicity (μm/min)	0.14	0.40	0.56	0.60

[a] Data are the mean ± SEM. *$P = < 0.01$ (student's *t*-test). Tests of significance were not performed on the percentage time or dynamicity, an overall parameter. The values in parentheses = the number of events measured.

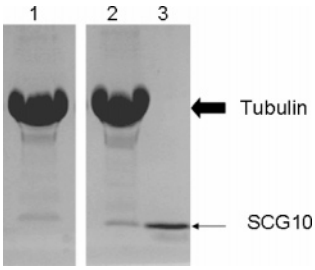

FIGURE 6: SCG10 binding to microtubules *in vitro*. Coomassie Blue stained SDS−PAGE. Tubulin (40 μM) was polymerized into microtubules for 40 min at 30 °C in the presence of 4 μM (lane 1) or 8 μM (lane 2) SCG10. The microtubules were then sedimented through sucrose cushions as described in Materials and Methods. Ten micrograms of an SCG10 standard is shown in lane 3. The positions of the tubulin and SCG10 bands are indicated with arrows. The tubulin band was overloaded on the gel to demonstrate the purity of the tubulin. This is one of three identical experiments.

tubulin concentration (*14*). Consistent with this conclusion, SCG10 bound to the microtubules with a relatively high stoichiometry. At a 1:5 molar ratio of SCG10 to total tubulin (soluble plus assembled), one molecule of SCG10 was bound per 6.7 tubulin dimers in the microtubules, which is ~30% of the maximum number of binding sites predicted to exist in the microtubule lattice along its length. (A stoichiometry of 1 molecule of SCG10 to 2 tubulin dimers is the theoretical maximum based on the structure of the T2S complex.)

Possible Mechanisms of Plus End Stabilization and Minus End Destabilization by SCG10. Because SCG10 modulated dynamics differently at the plus and minus ends, it is reasonable to think that its mechanism of action at opposite microtubule ends must be different because of the different interactions of SCG10 at the opposite ends and/or the intrinsic differences between the ends. However, it is difficult to understand with our present knowledge how SCG10 increases the plus end growth rate while destabilizing the minus ends at the same time. The lateral contacts between adjacent protofilaments at the growing tips of microtubules are suggested to be critical for growth (*39*), and increasing the strength of such lateral contacts by the binding of SCG10 to tubulin at the microtubule surface in the vicinity of a growing plus end tip could increase the growth rate by decreasing the rate constant for tubulin dissociation. However, decreasing the rate constant for tubulin dissociation would be expected to slow the shortening rate because a region of shortening polymer encounters one or several molecules of bound SCG10. Such slowing of the shortening rate did not occur (Table 1). It is hypothesized that tubulin at the growing ends of microtubules is stabilized by a GTP or GDP-Pi cap and is in an unstrained form, whereas tubulin at shortening tips, which is composed of tubulin bound to GDP, is in a strained form (*5*, *39*, *40*). One possible explanation for the discrepancy might be that when a plus end is shortening, the strained conformation of the dissociating tubulin−GDP subunits overcomes the stabilization conferred by bound SCG10. In contrast, when a growing microtubule is stabilized by tubulin−GTP or tubulin−GDP-Pi and is an unstrained conformation, the bound SCG10 may be able to further stabilize the end. Another possibility is that SCG10 might stabilize the chemical form of the cap. However, this possibility seems unlikely because SCG10 did not significantly increase the plus end rescue frequency and actually weakly increased the catastrophe frequency. It also seems unlikely that the tubulin sequestered into a soluble T2S-like complex with SCG10 could bind along the surface of a microtubule.

The differences in the action of SCG10 at plus and minus ends might also be related to the opposite orientation of tubulin dimers at the opposite ends. This will affect the orientation of bound SCG10, and perhaps also the ability of different regions of SCG10 to bind at the opposite ends. β-Tubulin faces the solvent at plus ends, and α-tubulin faces the solvent at minus ends. It seems reasonable to think that SCG10 (35−179) could bind directly to minus ends in a manner similar to the manner it binds to soluble tubulin in a T2S complex (*41*), with its N-terminal region (first ~40 residues) folded around the exposed α-tubulin subunits and perhaps playing a role in destabilizing/straining the end. The binding of the stathmin-like domains of the stathmin family proteins to two tubulin dimers appears to induce a curved conformation of the two bound dimers (*41*), perhaps similar to the curving of protofilaments observed at depolymerizing microtubule ends (*39*). Thus, the formation of similar curvature due to SCG10 binding at minus ends may produce a conformational strain and facilitate depolymerization at these ends. In support of this idea (Figure 5, Table 3), we found that SCG10 not only increased the catastrophe frequency at minus ends, but it also increased the rate and extent of shortening. The N-terminal region of SCG10 could play an important role in destabilizing minus ends, which is consistent with the idea that the N-terminal region of the stathmin-like domain functions predominantly as a microtubule regulatory domain (*24*, *42*). For example, the phosphorylation of serines 16 and 25 of stathmin, which are located in stathmin's N-terminal region, decreases both its microtubule-depolymerizing activity (*14*, *43*, *44*) and its minus end catastrophe-promoting activity (Manna, T., Honnappa, S., Steinmetz, M. O., and Wilson, L., unpublished work). The N-terminal region of SCG10 would not play a destabilizing role at plus ends because when SCG10 is bound to tubulin along the microtubule surface, access of the N-terminus to the tip region of α-tubulin should be sterically

blocked by the β-tubulin subunit of the adjacent tubulin dimer in the lattice.

Differential Abilities of SCG10 and Stathmin to Regulate Microtubule Dynamics. It is interesting considering their high sequence homology that SCG10 modulates the dynamic instability behaviors at microtubule ends *in vitro* in a manner different from that of stathmin. Specifically, at plus ends, stathmin increases the catastrophe frequency without significantly changing the growth rate (*14*). In contrast, SCG10 very weakly increases the catastrophe frequency, if at all, and it significantly increases the growth rate (Figure 4, Table 1). At minus ends, stathmin strongly increases the catastrophe frequency without significantly changing the shortening rate (*14*), whereas SCG10 increases the catastrophe frequency less strongly than stathmin, but also, in contrast to stathmin, it significantly increases the shortening rate (Figure 5, Table 3). SCG10 also differs from stathmin in its ability to bind soluble tubulin. Under the conditions used (specifically pH 7.2), SCG10 sequesters tubulin dimers ~3-fold less effectively than stathmin. Because SCG10 has a high (~70%) sequence identity to stathmin (Figure 1) and these two proteins are likely to bind to similar regions along the microtubule surface, one obvious question is how do SCG10 and stathmin modulate microtubule dynamics so differently? While this question remains to be answered, there are several possible mechanisms. For example, despite their high sequence homology, there are significant differences and critical amino acid residues involved in the interaction between SCG10 and tubulin along the microtubule surface in regions involved in controlling dynamics may be different or positioned differently than those in stathmin. Because of the central role microtubule dynamics play in cellular processes such as mitosis and neuronal growth cone function (*5*, *6*, *15*), it is not surprising that proteins that bind to microtubule surfaces at similar or overlapping sites modulate specific dynamic parameters in different ways. For example, the three- and four-repeat isoforms of the neuronal microtubule-associated protein tau as well as a number of tau isoforms carrying only single base pair changes have been shown to modulate individual dynamics parameters very differently (*45*, *46*). Similarly, drug molecules that bind to similar binding regions of tubulin in microtubules, such as the Vinca alkaloids, modulate individual dynamics parameters differently (*47*).

Both stathmin and SCG10 are highly expressed in developing neurons. However, their levels of expression and distribution patterns are different (*18*−*20*), further supporting the notion that they have distinct regulatory functions. An examination of the sub-cellular distribution of stathmin in cortical neurons shows its presence in all neuronal compartments except the nucleus (*48*). It is highly expressed (0.25% of total protein) and uniformly distributed. In contrast, the expression of SCG10 is several-fold lower than that of stathmin. SCG10 is predominantly found associated with Golgi vesicles and in the central domains of growth cones (*18*). Stathmin and SCG10 also differ in their phosphorylation patterns with the exception of Ser16, which is conserved in all stathmin family proteins (*23*, *49*).

Possible Regulation of Microtubule Dynamics by SCG10 in Neuronal Growth Cones. Microtubules are mainly found in the central (C) region of advancing growth cones, whereas actin filaments are concentrated in the peripheral (P) region in association with filopodia and lamellipodia. Although many studies have documented the involvement of actin cytoskeleton regulation in growth cone motility, including the extension and retraction of filopodia and the response to axon guidance cues, it has become evident that microtubules are also required for important aspects of axonal motility, including proper growth cone steering and forward movement (*6*−*8*). Microtubules in growth cones are oriented with their plus ends pointing toward the actin-rich filopodia, and these ends dynamically grow and shorten as the microtubules elongate toward and into the actin filament-rich P domain. These microtubules are highly dynamic as demonstrated by treating growth cones with low concentrations of microtubule-targeted drugs such as taxol and vinblastine, which suppress the dynamics but do not depolymerize the microtubules. Such drug treatment inhibits forward and persistent movement of the growth cone, demonstrating the importance of microtubule dynamics in growth cone movement (*1*, *3*, *5*, *50*).

A potentially important attribute of SCG10 is that it can promote plus end growth but does not affect plus end shortening and may even increase the plus end catastrophe frequency. The ability to increase growth, while permitting normal or increased transition to rapid shortening, is an ideal behavior to facilitate growth cone advance and turning because although growth is promoted, the transition to shortening and the shortening itself required for microtubules to respond to signals and probe into the actin filament-rich P domain are not impaired. A similar situation exists with the 3-repeat form of tau, which is the predominant tau isoform expressed in embryonic neurons. Unlike the adult four-repeat tau isoforms, which strongly suppress plus end shortening (*45*, *51*), three repeat tau can promote plus end growth but does not appreciably suppress plus end shortening, either *in vitro* (*45*) or in living cells (*51*). Thus, both SCG10 and three-repeat tau, perhaps in coordinated fashion, could promote plus end growth in advancing growth cones during early embryonic development.

The factors regulating microtubule plus end growth and dynamics in advancing growth cones are largely unknown but most likely involve the concerted actions of multiple microtubule regulatory proteins (*6*, *8*, *15*) including stabilizing MAPs such as MAP1B, which is abundant in growth cones and known to be associated with growth cone microtubules. The high dynamicity of growth cone microtubules has led to the idea that in addition to stabilizing MAPs, neurons must contain regulatory factors that increase microtubule dynamics (*15*). SCG10 appears to be one such regulator (*15*). It is known that overexpression of SCG10 in stably transfected PC12 cells (*17*) and inhibition of SCG10 function by antibody blockade in growth cones (*22*) mirror the effects of drugs that suppress microtubule dynamics. These effects include the cessation of forward elongation and altered microtubule organization in paused growth cones (*22*). Such results suggest that SCG10 is essential for the dynamics of growth cone microtubules and further suggest that SCG10 may antagonize the actions of one or more of the stabilizing MAPs and thus contribute to the regulation of microtubule growth dynamics during axonal elongation and pathfinding.

How Might SCG10 Regulate Dynamics at the Minus Ends of Growth Cone Microtubules? In order to maintain a

constant distance from the leading edge, dynamically unstable microtubules in growing axons undergo a steady net growth at their plus ends. SCG10 may regulate such growth. To conserve the constancy of polymer mass, net growth at the plus ends must be compensated for by net microtubule shortening elsewhere (*52*). A good possibility is that a significant fraction of the microtubules in the C region of growth cones shorten at their minus ends. Although the minus ends of many microtubules in non-neuronal cells are attached to centrosomes and appear to be blocked (*11*, *12*), the minus ends of growth cone microtubules are not attached to centrosomes. These ends are probably dynamic, and molecules such as SCG10 may regulate their shortening dynamics. In the filopodia of growth cones, a retrograde transport of actin filaments occurs, which is thought to facilitate the retrograde transport of microtubules (*50*). These microtubules are under mechanical stress; they form loops and break, which creates new minus ends that rapidly depolymerize. The new plus ends re-grow into filopodia. Thus, SCG10 may have a dual function in growth cones. At plus ends, it may directly favor growth into the filopodia while maintaining the shortening requirements of dynamic instability, and at minus ends, it could facilitate microtubule turnover by promoting disassembly.

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