Role of Tropomyosin in Formin-mediated Contractile Ring Assembly in Fission Yeast

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Like animal cells, fission yeast divides by assembling actin filaments into a contractile ring. In addition to formin Cdc12p and profilin, the single tropomyosin isoform SpTm is required for contractile ring assembly. Cdc12p nucleates actin filaments and remains processively associated with the elongating barbed end while driving the addition of profilin-actin. SpTm is thought to stabilize mature filaments, but it is not known how SpTm localizes to the contractile ring and whether SpTm plays a direct role in Cdc12p-mediated actin polymerization. Using “bulk” and single actin filament assays, we discovered that Cdc12p can recruit SpTm to actin filaments and that SpTm has diverse effects on Cdc12p-mediated actin assembly. On its own, SpTm inhibits actin filament elongation and depolymerization. However, Cdc12p completely overcomes the combined inhibition of actin nucleation and barbed end elongation by profilin and SpTm. Furthermore, SpTm increases the length of Cdc12p-nucleated actin filaments by enhancing the elongation rate twofold and by allowing them to anneal end to end. In contrast, SpTm ultimately turns off Cdc12p-mediated elongation by “trapping” Cdc12p within annealed filaments or by dissociating Cdc12p from the barbed end. Therefore, SpTm makes multiple contributions to contractile ring assembly during and after actin polymerization.

INTRODUCTION

Actin filament organization and dynamics must be spatially and temporally regulated to drive fundamental cellular processes, including division, motility, intracellular transport, polarity establishment, adhesion, and morphological changes (Pollard *et al.*, 2000; Pollard and Borisy, 2003; Chhabra and Higgs, 2007). Diverse actin filament structures are built through the coordinated action of numerous actin-regulatory proteins with complementary properties from nucleating to severing and depolymerizing (Pollard *et al.*, 2000; Pollard and Borisy, 2003; Chhabra and Higgs, 2007). A major challenge is to determine how subsets of actin regulatory proteins localize to specific structures and collectively influence actin filament dynamics, architecture, and function.

The actin filament side-binding protein tropomyosin provides an excellent example of spatial, temporal, and functional diversity (reviewed in Pittenger *et al.*, 1994; Gunning *et al.*, 2005, 2008). Through the alternative splicing of four genes, vertebrates express >40 tropomyosin isoforms that are differentially expressed during development (Gunning *et al.*, 2005, 2008). Furthermore, individual cells express multiple isoforms that localize to different actin filament structures and exhibit distinct biochemical properties (Gunning *et al.*, 2008). Tropomyosins form coiled-coil dimers that assemble end to end along an actin filament (Gunning *et al.*, 2008). In skeletal muscle, tropomyosin mediates muscle contraction by regulating access of myosin motor to the actin filament (Perry,

2001). In vitro data suggest that some nonmuscle tropomyosin isoforms regulate myosin function (Fanning *et al.*, 1994) or play a role in stabilizing filaments from the depolymerization and/or severing activities of cofilin and gelsolin (Fattoum *et al.*, 1983; Bernstein and Bamburg, 1992; Cooper, 2002; Ono and Ono, 2002; Fan *et al.*, 2008). Tropomyosin's role in filament stabilization has been best shown in budding yeast where conditional loss of tropomyosin function causes actin cables to disappear within a minute (Pruyne *et al.*, 1998). Tropomyosin also inhibits the Arp2/3 complex from nucleating branched actin filaments (Blanchoin *et al.*, 2001). However, the mechanisms by which tropomyosin localizes to specific cellular structures, and then in combination with other regulatory proteins influences actin filament diversity remains poorly understood.

The vertebrate cytoskeleton is impressively complex because actin filaments assemble into at least 15 distinct structures (Chhabra and Higgs, 2007). A great deal of work is required to determine which specific proteins are necessary for particular processes before a rigorous investigation of their coordinated effect on actin can be engaged. Conversely, the fission yeast *Schizosaccharomyces pombe* assembles actin into just four distinct structures that govern diverse processes throughout the cell cycle: actin cables (polar growth), actin patches (endocytosis), contractile ring (division), and the mating projection tip (cell fusion) (Marks and Hyams, 1985; Petersen *et al.*, 1998a; La Carbona *et al.*, 2006). A core set of fundamental actin-binding proteins are differentially required for the various fission yeast actin structures (La Carbona *et al.*, 2006). These include monomer-binding (profilin; Balasubramanian *et al.*, 1994), nucleation factors (Arp2/3 complex and formin; Balasubramanian *et al.*, 1996; McCollum *et al.*, 1996; Chang *et al.*, 1997; Petersen *et al.*, 1998b; Feierbach and Chang, 2001), side binding (tropomyosin, fimbrin, α -actinin; Balasubramanian *et al.*, 1992; Wu *et al.*, 2001), capping

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Abbreviations used: FH, formin homology; SpTm, fission yeast tropomyosin Cdc8p; TIRF, total internal reflection fluorescence.

(actin capping protein; Nakano *et al.*, 2001; Kovar *et al.*, 2005), and severing (cofilin; Nakano and Mabuchi, 2006).

Of particular importance is determining how cells coordinate contractile ring assembly for cell division (Glotzer, 2005). Actin filaments within the fission yeast contractile ring are assembled by the formin Cdc12p, which nucleates the rapid polymerization of actin bound to profilin Cdc3p (Pelham and Chang, 2002; Kovar *et al.*, 2003; Yonetani *et al.*, 2008). The single fission yeast tropomyosin isoform Cdc8p (SpTm) also localizes to the contractile ring and is required for contractile ring assembly (Balasubramanian *et al.*, 1992), but its precise role is less clear. In part, SpTm promotes contractile ring stability by antagonizing cofilin-mediated severing (Nakano and Mabuchi, 2006) and may regulate myosin-II binding to actin filaments (Skoumpla *et al.*, 2007). However, it is not known how SpTm sorts to the contractile ring, and whether SpTm affects Cdc12p-mediated actin filament assembly.

We discovered that SpTm preferentially binds Cdc12p-associated filaments and has diverse effects on Cdc12p-mediated actin assembly. Simultaneous overexpression of SpTm and Cdc12p leads to ectopic accumulation of thick actin bundles in cells, SpTm enhances the rate of Cdc12p-mediated actin filament elongation, SpTm allows Cdc12p-nucleated filaments to anneal, and SpTm ultimately limits the duration of Cdc12p-mediated barbed end elongation.

MATERIALS AND METHODS

Strains, Media, and Electroporation

Wild-type *S. pombe* strain FY436 (KV25; *h⁺ his7-366 ura4-D18 leu1-32 ade6-M216*) was grown in Edinburgh minimal medium (EMM) and electroporated with various plasmid constructs by standard procedures (Moreno *et al.*, 1991; Wu *et al.*, 2001). Expression under the *nmt1* promoter was regulated with the presence or absence of 10.0 μ g/ml thiamine (Sigma-Aldrich, St. Louis, MO).

Plasmid Constructs

Fission yeast expression constructs were prepared by amplifying the appropriate gene (iProof; Bio-Rad, Hercules, CA) and cloning by standard procedures into vectors with variable strength *nmt1* repressible promoters and auxotrophic selection: 1) low strength *pREP81-cdc12(882-1390)* (*leu2⁺*) [Cdc12(FH1FH2)p], 2) high-strength *pREP4X-cdc8* (*ura⁺*), 3) medium strength *pREP4X-cdc8* (*ura⁺*), 4) low strength *pREP82X-cdc8* (*ura⁺*), and 5) medium strength *pSGP573-41X-cdc8* (*ura⁺*) with a polyglycine linker between green fluorescent protein (GFP) and SpTm (Wu *et al.*, 2003). Inserts of the recombinant plasmids were sequenced to confirm fidelity of the polymerase chain reaction (PCR) amplification. Bacterial expression constructs have been described previously: 1) Cdc12(FH1FH2)p; *pET21a-cdc12(882-1390)-HIS* (Kovar and Pollard, 2004), 2) Cdc12(FH2)p; *pET21a-cdc12(973-1390)-HIS* (Neidt *et al.*, 2008), and 3) Cdc3p SpPRF; *pMW172-SpPRF* (Lu and Pollard, 2001).

Cell Microscopy

Cell morphology was observed by differential interference contrast (DIC) and epifluorescence microscopy. Images were collected on an Orca-ER camera (Hamamatsu, Bridgewater, NJ) on an IX-81 microscope (Olympus, Tokyo, Japan), with a 60 $\times$ 1.4 numerical aperture Plan-apo objective. Nuclei, septa and actin filaments were visualized with 4,6-diamidino-2-phenylindole (nuclei), Calcofluor (septae), Hoechst (nuclei and septae), and rhodamine-phalloidin (actin filaments) as described previously (Kovar *et al.*, 2003). We quantified the effects of formin and tropomyosin overexpression on at least 200 cells for each time point as described previously (Kovar *et al.*, 2003, 2005).

Protein Purification

Cdc12(FH1FH2)p, Cdc12(FH2)p, profilin, and mouse capping protein were purified from bacteria (Lu and Pollard, 2001; Palmgren *et al.*, 2001; Kovar and Pollard, 2004).

Recombinant fission yeast tropomyosin SpTm was expressed and purified from fission yeast cells. Cells containing the plasmid *pREP4X-cdc8* were grown in minimal media without thiamine to induce expression for 24 h. Cells were harvested by centrifugation, washed with H₂O and extraction buffer (0.3 M KCl, 5 mM MgCl₂, and 50 mM imidazole-HCl, pH 6.9), sedimented, and stored at -80°C . Pellets were resuspended in extraction buffer supplemented with 0.3 mM phenylmethylsulfonyl fluoride and protease inhibitors, boiled

for 10 min, and clarified at 30,000 and 50,000 $\times g$ for 20 min each. SpTm in the supernatant was precipitated by a 30–70% ammonium sulfate cut and dialyzed overnight versus Mono Q Buffer A (50 mM KCl, 10 mM KH₂PO₄, 10 mM K₂HPO₄, and 1 mM dithiothreitol [DTT], pH 7.0). Dialyzed SpTm was loaded onto a 1.0 ml Mono Q column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) and eluted with a linear gradient from 250 to 500 mM KCl. Pure SpTm was dialyzed into tropomyosin buffer (50 mM KCl, 0.5 mM MgCl₂, 10 mM Tris, pH 7.5, and 0.5 mM DTT), flash frozen in liquid nitrogen, and stored at -80°C .

Ca-ATP actin was purified from chicken skeletal muscle (Neidt *et al.*, 2008). Gel-filtered actin was labeled on Cys-374 with pyrenyl iodoacetamide, Oregon Green 488 iodoacetamide (Invitrogen, Carlsbad, CA), or tetramethylrhodamine iodoacetamide (Invitrogen) (Kovar *et al.*, 2003; Kuhn and Pollard, 2005). Immediately before each experiment, Ca-ATP actin was converted to Mg-ATP actin by adding 0.1 volume of 2 mM EGTA and 0.5 mM MgCl₂ for 2 min at 25°C. Mg-ATP actin was converted to ADP actin with 20 U/ml hexokinase (Sigma-Aldrich) and 1 mM glucose for 3 h at 4°C, and clarified for 1 h at 125,000 $\times g$ (Pollard, 1986).

Protein concentrations were determined with extinction coefficients (Neidt *et al.*, 2008). An extinction coefficient for SpTm was estimated with ProtParam (<http://us.expasy.org/tools/>) from the amino acid composition, $A_{280} = 2980 \text{ M}^{-1} \text{ cm}^{-1}$.

High-Speed Sedimentation

The affinity of SpTm for actin filaments was determined from a high-speed cosedimentation assay. Mg-actin (15 μ M) was preassembled in the absence or presence of 750 nM Cdc12(FH1FH2)p and 15 μ M profilin for 1.0 h at 25°C. ADP-Pi actin filaments were prepared by assembling ADP-actin for 5.5 h in the presence of 25 mM potassium phosphate, pH 7.0 (15.37 mM H₂KPO₄ and 9.63 mM HK₂PO₄), with 19.9 mM KCl. Mg-ATP actin and Mg-ADP actin were assembled in the presence of 25 mM potassium sulfate with 3.5 mM KCl to keep the conductivity consistent (Mahaffy and Pollard, 2006).

We incubated 4.0 μ M SpTm (2.0 μ M SpTm dimers) with a range of concentrations of preassembled actin filaments for 20 min at 25°C and then spun the filaments for 20 min at 100,000 $\times g$ at 25°C. Equal volumes of total (before centrifugation) supernatants and pellets were separated by 12.5% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The intensity of protein bands was then determined by an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE). Plots of the dependence of the concentration of bound SpTm (determined from the loss of SpTm from the supernatants) on the concentration of actin were fit with a quadratic function. Because Mg-ADP actin assembles 10-fold less well in the presence of Cdc12(FH1FH2)p and profilin (Kovar *et al.*, 2006), for ADP actin sedimentation experiments with Cdc12(FH1FH2)p and profilin, the amount of filamentous actin was determined by subtracting the amount of actin in the postspin supernatant from the total actin before centrifugation.

Fluorescence Spectroscopy

Actin assembly and disassembly was measured from the fluorescence of pyrene-actin with Spectramax Gemini XPS (Molecular Devices, Sunnyvale, CA) and Safire² (Tecan, Durham, NC) fluorescent plate readers. Spontaneous assembly, seeded assembly, depolymerization, and critical concentration assays have been described in detail previously (Neidt *et al.*, 2008). Briefly, assembly of a mixture of unlabeled and pyrene-labeled Mg-ATP-actin monomers was initiated by the addition of KMEI (50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM imidazole, pH 7.0) and other proteins to be assayed [SpTm, Cdc12(FH1FH2)p, profilin, etc.]. Final protein concentrations and percentage of pyrene-labeled actin are indicated in the figure legends. Calculation of spontaneous polymerization and depolymerization rates, and the affinity of Cdc12(FH1FH2)p for actin filament barbed ends, have been described previously (Neidt *et al.*, 2008).

Actin Filament Annealing

Annealing reactions were initiated by two methods: 1) mixing parallel spontaneous assembly reactions or 2) mixing and shearing preassembled filaments. For spontaneous assembly, parallel 3.0 μ M Mg-ATP actin spontaneous assembly reactions containing either 33% tetramethylrhodamine (TMR)-labeled or 33% Oregon Green-labeled actin, Cdc12(FH1FH2)p, and SpTm were mixed 30 min after initiation (reactions had reached steady state). For shearing, 4.0 μ M Mg-ATP actin was preassembled with either 33% TMR-labeled or 33% Oregon Green-labeled actin for 1 h. Equal volumes of red and green filaments (0.25 μ M, final concentration of each), Cdc12(FH1FH2)p, and SpTm were mixed and sheared by pushing 20 times through a 3/8-in. 26-gauge needle on a 1.0-ml tuberculin syringe. Final protein concentrations are indicated in figure legends. Both types of annealing reactions were terminated at various times by an 83-fold dilution in fluorescence buffer (50 mM KCl, 1 mM MgCl₂, 100 mM DTT, 20 μ g/ml catalase, 100 μ g/ml glucose oxidase, 3 mg/ml glucose, 0.5% methylcellulose, and 10 mM imidazole, pH 7.0) with 0.4 μ M unlabeled phalloidin (Sigma-Aldrich), absorbed to coverslips coated with poly-L-lysine, and images were collected with a cooled charge-coupled device camera (Orca-ER) on an IX-81 microscope (Olympus).

Figure 1. Simultaneous overexpression of formin Cdc12p and SpTm drives lethal ectopic actin filament assembly. (A) Wild-type fission yeast cells with either control vectors, a vector expressing an active Cdc12p fragment *pREP81-cdc12(FH1FH2)* at low levels, and/or vectors expressing SpTm at low (*pREP82X-cdc8*), medium (*pREP42X-cdc8*), and high (*pREP4X-cdc8*) levels, were grown on minimal media (EMM) plates to induce expression for 72 h at 25°C. Cells simultaneously overexpressing Cdc12(FH1FH2)p at low levels and SpTm at any level were not able to form colonies. (B–D) Wild-type fission yeast cells with control, low level *pREP81-cdc12(FH1FH2)*, and/or medium level *pREP42X-cdc8* vectors were grown exponentially in liquid EMM minimal media at 25°C to induce expression after thiamine removal at time zero. (B and C) Quantification of the time course of morphological features of cells individually or simultaneously overexpressing Cdc12(FH1FH2)p and SpTm. (B) Percentage of cells with two or more nuclei. (C) Percentage of septa that are abnormal (partial, misplaced, misaligned, and/or broad). (D) DIC and fluorescence micrographs of cells grown in the absence of thiamine for 22 h and stained with Hoechst (bisbenzimidazole) to visualize nuclei and septa or rhodamine-phalloidin to visualize filamentous actin. Bars, 5 μ m. (E) Fluorescence micrographs of GFP in wild-type fission yeast cells with either a low level expression *p573-81X-GFP-cdc8* vector alone (GFP-SpTm only), or *p573-81X-GFP-cdc8* with a low level expression *pREP81-cdc12(FH1FH2)* vector (GFP-SpTm and cdc12). Cells were grown in the absence of thiamine for 22 h. Bar, 5 μ m.

Total Internal Reflection Fluorescence (TIRF) Microscopy

TIRF microscopy images of a mixture of 1.0 μ M unlabeled Mg-ATP actin and 0.5 μ M Oregon Green-labeled Mg-ATP actin (33% labeled), excited by evanescent wave fluorescence (IX-71 microscope [Olympus] fit with through-the-objective TIRF illumination), were collected at 15-s intervals with an iXon EMCCD camera (Andor Technology, South Windsor, CT) as described in detail previously (Neidt *et al.*, 2008). For seeded assembly experiments (Figure 5), initial reactions contained 1.5 μ M unlabeled Mg-ATP actin with 0.25 μ M Oregon Green-labeled Mg-ATP actin (15% labeled), Cdc12(FH1FH2)p alone, or Cdc12(FH1FH2)p and SpTm. Initial reactions were diluted 200-fold into fresh reactions containing 1.5 μ M unlabeled Mg-ATP actin with 1.0 μ M Oregon Green-labeled Mg-ATP actin (40% labeled) with or without SpTm. Final protein concentrations are indicated in figure legends.

RESULTS

Simultaneous Overexpression of Formin Cdc12p and SpTm Is Lethal in Fission Yeast

To investigate how tropomyosin Cdc8p (SpTm) influences formin Cdc12p-mediated actin assembly in fission yeast, we began by examining the consequences of overexpressing SpTm and Cdc12p in wild-type cells. Overexpression of either SpTm alone, or low levels of an active fragment of Cdc12(FH1FH2)p containing the formin homology 1 and 2 domains, has little effect on cell survival and morphology

(Figure 1, A–D) (Skoumpla *et al.*, 2007). However, when SpTm and Cdc12(FH1FH2)p are simultaneously overexpressed, cell growth is arrested as the percentage of cells with at least two nuclei, abnormal septa, and abnormal morphology increases (Figure 1, A–D).

Cells overexpressing only SpTm, or Cdc12(FH1FH2)p at low levels, contain normal actin cytoskeletal arrays, including endocytic patches, polar cables, and contractile rings (Figure 1D). Fission yeast overexpressing both SpTm and Cdc12(FH1FH2)p lack normal actin cytoskeletal architecture, but they contain an extraordinary accumulation of actin filaments in aberrant thick bundles (Figure 1D). On its own, GFP-SpTm localizes predominantly to contractile rings, whereas GFP-SpTm accumulates in thick bundles when overexpressed with Cdc12(FH1FH2)p (Figure 1E).

SpTm Binds Differentially to ATP-, ADP-Pi-, and ADP-loaded Actin Filaments

SpTm was purified directly from fission yeast extracts by successive steps of boiling, a 30–70% ammonium sulfate precipitation cut, and anion exchange chromatography (Figure 2A). Purified SpTm was >99% pure as judged by a Coomassie Blue-stained gel. More than 90% of purified

Figure 2. Formin Cdc12p and profilin increase the affinity of SpTm to Mg-ADP-actin filaments. The conditions were as follows: 10 mM imidazole, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, and 90 μM $CaCl_2$. (A) Coomassie Blue-stained gel showing purification of SpTm. Lane 1, fission yeast extract; lane 2, extract after being boiled for 10 min; lane 3, 30–70% ammonium sulfate cut; lane 4, Source 15Q column. Molecular weights are indicated on the left. (B–F) High-speed sedimentation of 2.0 μM SpTm dimer with a range of concentrations of Mg-actin filaments. (B) Coomassie Blue-stained gel showing the supernatants (s) and pellets (p) after SpTm was incubated with the indicated concentrations of preassembled Mg-ATP actin filaments for 20 min at 25°C and then spun at $100,000 \times g$ for 20 min at 25°C. Actin and tropomyosin are marked with arrows to the left. (C) Plot of the dependence of the concentration of SpTm bound on the concentration of preassembled Mg-ATP (○), Mg-ADP-Pi (◆), or Mg-ADP (□) actin. (D) Representative regions of Coomassie Blue-stained gels showing the total (t, before centrifugation) and supernatants (s) after SpTm was incubated for 20 min with 7.0 μM Mg-ADP actin preassembled alone or in the presence of Cdc12(FH1FH2)p and profilin, and spun at $100,000 \times g$ for 20 min. Formin, hexokinase, actin, tropomyosin, and profilin are marked with arrows to the right. (E) Plot of the dependence of the concentration of SpTm bound on the concentration of Mg-ADP actin preassembled alone (●) or in the presence of Cdc12(FH1FH2)p and profilin (■). Because Mg-ADP actin assembles 10-fold less well in the presence of Cdc12(FH1FH2)p and profilin (Kovar *et al.*, 2006), the amount of filamentous actin was determined by subtracting the supernatant from the total actin. (F) Average dissociation constants from at least two independent experiments.

SpTm runs as a lower band of a doublet (Supplemental Figure 2D), consistent with the finding that the majority of SpTm is acetylated at the amino terminus (Skoumpla *et al.*, 2007).

The affinity of SpTm for chicken skeletal muscle actin filaments was determined by high-speed centrifugation at $100,000 \times g$ (Figure 2, B–F). Curve fits of the dependence of the amount of SpTm bound on the concentration of actin revealed that SpTm's affinity for actin filaments depends upon the nucleotide (Figure 2C). SpTm binds to ATP-, ADP-Pi-, and ADP-loaded actin filaments with affinities of ~ 35 , ~ 220 , and ~ 900 nM (Figure 2F). Therefore, SpTm binds an order of magnitude more strongly to newly assembled ATP-actin filaments than older ADP-Pi- and ADP-actin filaments. Because ATP is rapidly hydrolyzed as Mg-ATP-actin is assembled (Blanchoin and Pollard, 2002), the ATP-actin sample contains a mixture of ATP- and ADP-Pi-loaded actin filaments. SpTm may therefore have a higher affinity for purely ATP-actin filament. The affinity of SpTm for preassembled actin filaments was reported previously to be 600 nM, suggesting that the actin filaments were primarily ADP-loaded in those studies (Skoumpla *et al.*, 2007).

Formin Cdc12p Increases the Affinity of SpTm for ADP-loaded Actin Filaments

Understanding how diverse actin regulators localize to specific actin-dependent cellular structures is of utmost importance. Given the fast rate of ATP hydrolysis, it is unlikely that SpTm can accumulate on ATP-loaded actin filaments in cells. SpTm localizes to and is necessary for formin-dependent actin structures in fission yeast (contractile ring, actin cables, mating projection) but not the Arp2/3 complex-dependent endocytic actin patches (Balasubramanian *et al.*, 1992; Arai *et al.*, 1998; Pelham and Chang, 2001; Kurahashi *et al.*, 2002; Skoumpla *et al.*, 2007). However, we found that SpTm binds with similar affinity to actin filaments assembled from Mg-ATP actin monomers in the absence and presence of Cdc12p and profilin (Figure 2F), suggesting that formin does not build filamentous structures that preferentially bind SpTm.

Alternatively, it has been proposed that formins promote rapid ATP hydrolysis and phosphate release as they ride processively on the elongating actin filament barbed end (Romero *et al.*, 2004; Romero *et al.*, 2007). It is therefore possible that Cdc12p and profilin simultaneously increase

Figure 3. Effects of SpTm on the spontaneous polymerization of Mg-ATP actin. The conditions were as follows: 10 mM imidazole, pH 7.0, 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, and 90 μ M CaCl₂. (A) The time course of the polymerization of 3.0 μ M Mg-ATP actin (20% pyrene labeled) was monitored by fluorescence in the absence (thick curve) or presence of 25 nM Cdc12(FH1FH2)p (○), 3 μ M profilin (◇), 4 μ M SpTm (□), profilin and SpTm (△), Cdc12(FH1FH2)p and profilin (▲), Cdc12(FH1FH2)p and SpTm (●), and Cdc12(FH1FH2)p, profilin, and SpTm (■). (B) Fluorescence micrographs of actin filaments after the indicated reactions reached steady state (10 h). Samples were labeled with rhodamine-phalloidin and absorbed to glass coverslips coated with poly-L-lysine. Bar, 10 μ m. (C–E) The time course of the polymerization of 3.0 μ M Mg-ATP actin in the presence of a range of indicated SpTm concentrations. (C) Plot of actin polymer over time. (D) After reaching steady state (6 h), the reactions in C were spun at 100,000 $\times$ g for 20 min at 25°C, and the supernatants and pellets were separated on a 12.5% gel and stained with Coomassie Blue. SpTm concentrations are indicated above each lane. Actin and tropomyosin are marked with arrows to the left. (E) Plots of the dependence of the actin assembly rate (slope) on the concentration of SpTm (left y-axis, ●), and the normalized amount of SpTm bound as determined by densitometry of the pellets in (D) (right y-axis, ■).

the rates of ATP hydrolysis, phosphate release, and the affinity of SpTm for ADP-loaded actin filaments. In agreement, we found that preassembling ADP-actin monomers in the presence of Cdc12(FH1FH2)p and profilin increased SpTm's affinity for actin filaments by approximately fivefold from 1100 to 210 nM (Figure 2, D and E). Therefore, by driving ATP hydrolysis and increasing filament flexibility (Romero *et al.*, 2004, 2007; Bugyi *et al.*, 2006; Papp *et al.*, 2006), processive formin-mediated actin assembly is capable of promoting the association of tropomyosin to particular cellular structures while inhibiting the association/activity of other regulators such as the Arp2/3 complex, which is inhibited by tropomyosin and dissociates from ADP-loaded actin filaments (Blanchoin *et al.*, 2001; Mahaffy and Pollard, 2006).

Formin Cdc12p Overcomes the Inhibition of Actin Assembly by SpTm

We next examined the combined effect of Cdc12(FH1FH2)p and SpTm on actin dynamics. Actin filament assembly is a complex process governed by the rates of both nucleation and elongation. On its own, SpTm inhibits spontaneous actin monomer assembly (Figure 3A). The extent of inhibition depends on the concentration of SpTm, but saturating

amounts of SpTm do not fully block assembly (Figure 3, C and E). High-speed sedimentation (100,000 $\times$ g) after the spontaneous assembly reactions reached plateau revealed that SpTm maximally inhibits the actin assembly rate when filaments are saturated with SpTm ($\sim$ 3 μ M; Figure 3, D and E). Therefore, SpTm most likely inhibits actin assembly by binding filaments rather than monomers. Supporting this, SpTm does not seem to inhibit nucleation because the average length of actin filaments in reactions with or without tropomyosin is $\sim$ 20 μ m after reaching plateau (Figure 3B). We verified that SpTm reduces the barbed end elongation rate by following actin filament assembly with TIRF microscopy (Figure 4, A, B, F, and G and Table 1). Actin filament barbed ends elongate at $\sim$ 10.0 and $\sim$ 6.0 subunits s⁻¹ μ m⁻¹ in the absence and presence of SpTm.

Profilin prevents spontaneous actin assembly by binding to and inhibiting the nucleation of actin monomers (Figure 3A) (Lu and Pollard, 2001). By preventing both nucleation and elongation, the combination of profilin and SpTm strongly inhibits actin polymerization (Figure 3A). Conversely, Cdc12(FH1FH2)p dramatically increases actin polymerization by efficiently nucleating new filaments (Figure 3A) (Kovar *et al.*, 2003). Cdc12(FH1FH2)p completely overcomes the inhibition of actin assembly by SpTm, profilin,

Figure 4. Time-lapse evanescent wave fluorescent microscopy of the effect of SpTm on Mg-ATP-actin polymerization. The spontaneous assembly of 1.0 μM ATP-actin with 0.5 μM ATP-actin labeled with Oregon Green (ATP-OG-actin) on slides coated with *N*-ethylmaleimide (NEM)-myosin II. Conditions: 10 mM imidazole pH 7.0, 50 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 50 μM CaCl_2 , 15 mM glucose, 20 $\mu\text{g}/\text{ml}$ catalase, 100 $\mu\text{g}/\text{ml}$ glucose oxidase, and 0.5% methylcellulose (500 cP) at 25°C. Bar, 5 μm . (C, H, M, and R) Time-lapse micrographs with time in seconds indicated at top. Blue arrows and yellow arrowheads mark barbed and pointed ends. Lowercase letters indicate filaments shown in kymographs to the right (a, control filaments; b and c, Cdc12(FH1FH2)p-nucleated filaments). (A, D, F, I, K, N, P, and S) Kymographs of the length (y-axis) of the filaments marked to the left versus time (x-axis, 900 s; N and S, 300 s). (B, E, G, J, L, O, Q, and T) Plots of the growth of eight individual filament barbed ends (and pointed ends for [B, G, L, and D]) versus time for control and formin-nucleated filaments. (A and B) 1.0 μM ATP-actin only control. (C–E) 1.0 μM ATP-actin with 10 nM Cdc12(FH1FH2)p. (F and G) 1.0 μM ATP-actin with 2.0 μM SpTm. (H–J) 1.0 μM ATP-actin with 10 nM Cdc12(FH1FH2)p and 2.0 μM SpTm. (K and L) 1.0 μM ATP-actin with 2.5 μM profilin. (M–O) 1.0 μM ATP-actin with 1 nM Cdc12(FH1FH2)p and 2.5 μM profilin. (P and Q) 1.0 μM ATP-actin with 2.5 μM profilin and 2.0 μM SpTm. (R–T) 1.0 μM ATP-actin with 1 nM Cdc12(FH1FH2)p, 2.5 μM profilin and 2.0 μM SpTm.

and SpTm with profilin (Figure 3A) (Kovar *et al.*, 2003). Therefore, Cdc12p is able to promote actin filament assembly for contractile ring formation even though fission yeast cells contain high micromolar concentrations of profilin and tropomyosin (Lu and Pollard, 2001; Wu and Pollard, 2005).

SpTm Doubles the Elongation Rate of Formin Cdc12p-associated Actin Filaments

Alterations in the critical concentration (C_c) for assembly reflect the effect of regulatory proteins on the rate at which actin monomers add and dissociate from the actin filament barbed end. Cdc12(FH1FH2)p shifts the C_c from that of the barbed end (0.1 μM) to that of the pointed end (0.6 μM) by inhibiting barbed end elongation (Kovar *et al.*, 2003). To gain mechanistic insight into SpTm's effect on Cdc12p-mediated actin filament elongation, we began by investigating SpTm's effect on the C_c (Supplemental Figure 1). We found that although SpTm slightly increases the C_c on its own, SpTm lowers the C_c of Cdc12p-mediated actin assembly (Figure 3D and Supplemental Figure 1). Therefore, we hypothesized that SpTm increases the elongation rate of Cdc12p-associated barbed ends. By comparing the slope of "bulk" spontaneous actin assembly reactions with the filaments lengths

after the reactions reached steady-state (Figure 3, A and B), we predicted that SpTm increases the elongation rate by ~ 4.5 -fold.

We determined the elongation rate of Cdc12p-associated actin filaments directly by visualizing actin assembly with TIRF microscopy (Figure 4 and Table 1). Individual filaments were observed as they assembled from a mixture of 1.0 μM Mg-ATP-actin monomers and 0.5 μM Mg-ATP-actin monomers labeled with Oregon Green (Kovar and Pollard, 2004; Kuhn and Pollard, 2005; Kovar *et al.*, 2006). In the absence of Cdc12p and SpTm, all filaments elongate their barbed ends at the same rate of ~ 10.0 subunits/s (Figure 4, A and B). In the presence of SpTm, all filaments elongate significantly slower at rates of 6.0 and 5.5 subunits/s with and without profilin (Figure 4, F and G and P and Q).

In the presence of Cdc12(FH1FH2)p, two filament populations are present. The first population consists of control filaments not associated with formin that elongate at 6.8 and 10.7 subunits/s with and without SpTm (Figure 4, C–E and H–J, filament a). The second population elongates at a significantly different rate because Cdc12(FH1FH2)p remains processively associated with the barbed end and modifies the addition and loss of actin subunits (Figure 4, C–E, fila-

Table 1. Comparison of Mg-ATP actin assembly rates in the presence of Cdc12p, Profilin, and SpTm

Condition ^a	Barbed end assembly rate subunits/s
1.0 μ M Actin only ^b	9.9 $\pm$ 0.5
1.0 μ M Actin + 10 nM Cdc12(FH1FH2)p	0.3 $\pm$ 0.03 (10.7 $\pm$ 0.4) ^c
1.0 μ M Actin + 2.5 μ M profilin	8.5 $\pm$ 0.2
1.0 μ M Actin + 2.0 μ M SpTm	6.0 $\pm$ 0.3
1.0 μ M Actin + Profilin with SpTm	5.5 $\pm$ 0.2
1.0 μ M Actin + Cdc12p with profilin	11.7 $\pm$ 0.4 (9.1 $\pm$ 0.3)
1.0 μ M Actin + Cdc12p with SpTm	0.6 $\pm$ 0.02 (6.8 $\pm$ 0.2)
1.0 μ M Actin + Cdc12p with profilin and SpTm	9.0 $\pm$ 0.1 (6.1 $\pm$ 0.3)
1.5 μ M Actin only ^d	17.2 $\pm$ 0.8
1.5 μ M Actin + 15 nM Cdc12(FH1FH2)p	0.56 $\pm$ 0.06 (18.6 $\pm$ 0.7)
1.5 μ M Actin + Cdc12p with 2.0 μ M SpTm	1.21 $\pm$ 0.1 (12.8 $\pm$ 0.4)

^a At least 10 individual filaments were measured for each population. Rates are represented as mean $\pm$ SD.

^b Data from the experiment shown in Figure 4.

^c The rates of control filaments not associated with formin are shown in parentheses.

^d Data from the experiment shown in Figure 5.

ments b and c) (Kovar *et al.*, 2003, 2006; Kovar and Pollard, 2004; Neidt *et al.*, 2008, 2009). In the absence of SpTm, Cdc12(FH1FH2)p-associated filaments elongate at $\sim$ 0.3 subunits/s (Figure 4, C–E). In the presence of SpTm, Cdc12(FH1FH2)p-associated filaments elongate twofold faster at $\sim$ 0.6 subunits/s (Figure 4, H–J). In the presence of profilin, Cdc12(FH1FH2)p-associated filaments elongate at 11.7 subunits/s, a 1.3-fold increase over the rate of control filaments (9.1 subunits/s) (Figure 4, M–O). In the presence of profilin and SpTm, Cdc12(FH1FH2)p-associated filaments elongate at 9.0 subunits/s, a 1.5-fold increase over the rate of control filaments (6.1 subunits/s) (Figure 4, R–T). The presence of two filament populations, control and Cdc12(FH1FH2)p-associated, demonstrates that Cdc12p remains processively associated with the barbed end of actin filaments saturated with SpTm.

Without profilin, Cdc12(FH1FH2)p-associated filaments elongate too slowly for accurate measurement of the elongation rate at both filament ends. Because Cdc12(FH1FH2)p shifts the critical concentration of assembly to that of the pointed end (Supplemental Figure 1), we have proposed that in the absence of other actin-binding proteins, Cdc12(FH1FH2)p-associated filaments elongate exclusively from their pointed ends (Kovar *et al.*, 2003). SpTm might double the elongation rate of Cdc12(FH1FH2)p-associated filaments by allowing them to slowly elongate their barbed ends.

We examined the contribution of elongation at each filament end by using TIRF microscopy to directly visualize the addition of new “bright” actin monomers to “dim” preassembled Cdc12(FH1FH2)p-associated actin filament seeds (Figure 5). Initially, 1.5 μ M unlabeled Mg-ATP-actin with 0.25 μ M Mg-ATP-Oregon Green actin ($\sim$ 15% Oregon Green labeled) was polymerized in the presence of either 15 nM

Cdc12(FH1FH2)p alone or Cdc12(FH1FH2)p with 2.0 μ M SpTm. After 45 min, the initial reactions containing dim Cdc12(FH1FH2)p-associated seeds were diluted 200-fold into fresh reactions containing 1.5 μ M unlabeled Mg-ATP-actin with 1.0 μ M Mg-ATP-Oregon Green actin ($\sim$ 40% Oregon Green labeled) in the absence or presence of 2.0 μ M SpTm. When visualized by TIRF microscopy, two filament populations are present that differ by brightness and elongation rate (Figure 5, A and G). Faster elongating bright control filaments surround slowly elongating dim Cdc12(FH1FH2)p-associated filaments. In the absence of SpTm, Cdc12(FH1FH2)p-associated filaments show up as dumbbells with new bright actin subunits adding slowly at each end (Figure 5, A–F). Therefore, Cdc12(FH1FH2)p-associated filaments elongate at both the barbed and pointed ends at the same “pointed end rate” of $\sim$ 0.2 subunits s^{-1} μ M⁻¹ (Figure 5F and Table 1). In the presence of SpTm, Cdc12(FH1FH2)p-nucleated filaments show up as lopsided dumbbells with new bright actin accumulating significantly faster at one end (Figure 5, G–L). Therefore, SpTm has no effect on the pointed end but allows the presumed Cdc12(FH1FH2)p-associated barbed end to elongate significantly faster at $\sim$ 0.5 subunits s^{-1} μ M⁻¹ (Figure 5L and Table 1).

SpTm Inhibits Actin Filament Disassembly

It is possible that SpTm increases the elongation rate of Cdc12p-associated barbed ends by either increasing the rate of actin monomer addition, decreasing the rate of actin monomer dissociation, or both. We explored the possibility that SpTm inhibits the disassembly of Cdc12p-associated filaments by following barbed end depolymerization upon dilution below the critical concentration for assembly (Figure 6).

In the absence of Cdc12(FH1FH2)p, SpTm lowers the rate of barbed end disassembly in a concentration-dependent manner (Figure 6, A and C). At saturating concentrations, SpTm inhibits the disassembly rate by $\sim$ 60% (Figure 6C). Cdc12(FH1FH2)p reduces the disassembly rate by $\sim$ 80% (Figure 6B) (Kovar *et al.*, 2003). Although profilin has little effect on the disassembly rate on its own, profilin increases the disassembly rate of Cdc12(FH1FH2)p-associated actin filaments (Figure 6B) (Kovar *et al.*, 2003; Yonetani *et al.*, 2008). In contrast, SpTm reduces the disassembly rate of Cdc12(FH1FH2)p-associated actin filaments in the presence of profilin in a concentration-dependent manner (Figure 6, B and C). Therefore, SpTm could increase the elongation rate of Cdc12(FH1FH2)p-associated filaments by inhibiting dissociation of subunits. Fits of the dependence of the depolymerization rate on the concentration of SpTm revealed equilibrium dissociation constants of SpTm for filaments assembled from Mg-ATP actin between 9.5 and 21.6 nM (Figure 6C), which is in good agreement with the dissociation constant determined from high-speed sedimentation assays (Figure 2).

SpTm Allows Formin Cdc12p-nucleated Filaments to Anneal End to End

Although spontaneous assembly reactions suggested that SpTm increases the elongation rate of Cdc12p-associated barbed ends by $\sim$ 4.5-fold (Figure 3, A and B), we found that SpTm only doubles the elongation rate from $\sim$ 0.3 to $\sim$ 0.6 subunits s^{-1} μ M⁻¹ (Figures 4 and 5). Therefore, we investigated whether SpTm also increases the length of Cdc12p-nucleated filaments by another mechanism.

We began by determining the length of actin filaments as they spontaneously assemble from 3.0 μ M Mg-ATP-actin

monomers over a period of 10 h (Figure 7, A and B). Reactions with Cdc12(FH1FH2)p plateau ~30 min after initiation, suggesting that filament lengths are no longer increasing via actin monomer addition (Figure 7A). As expected from a twofold increase in the elongation rate, Cdc12(FH1FH2)p-nucleated filaments in the absence and

presence of SpTm are ~0.6 and 1.1 μ m upon reaching the plateau (Figure 7B). Over the next 9 h, the length of Cdc12(FH1FH2)p-nucleated filaments increases significantly in the presence of SpTm but not in the absence of SpTm (Figure 7B). After 9 h, the filament length with and without SpTm increases from 1.1 to 6.8 μ m and from 0.6 to 0.8 μ m.

Figure 7. SpTm allows formin Cdc12p-nucleated filaments to anneal. The conditions were as follows: 10 mM imidazole, pH 7.0, 50 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, and 90 μM CaCl_2 . (A) Time course of the spontaneous assembly of 3.0 μM Mg-ATP actin (20% pyrene labeled) in the absence (○) or presence of 25 nM Cdc12(FH1FH2)p (□), Cdc12(FH1FH2)p and 4.0 μM SpTm (■), Cdc12(FH1FH2)p and 3.0 μM profilin (△), or Cdc12(FH1FH2)p, profilin, and SpTm (▲). Arrows indicate points at which samples were removed for filament length determination by rhodamine-phalloidin staining. (B) Plot of the dependence of the filament length on reaction time in the presence of 25 nM Cdc12(FH1FH2)p (□), Cdc12(FH1FH2)p and 4.0 μM SpTm (■), Cdc12(FH1FH2)p and 3.0 μM profilin (△), or Cdc12(FH1FH2)p, profilin, and SpTm (▲). (C) Parallel 3.0 μM Mg-ATP actin spontaneous assembly reactions containing either 33% TMR-labeled actin or 33% Oregon Green-labeled actin were mixed 30 min after initiation. Representative two-color fluorescence micrographs of reactions initiated immediately and 7.0 h after mixing are shown. As indicated, reactions contained either 25 nM Cdc12(FH1FH2)p, Cdc12(FH1FH2)p with 4.0 μM SpTm, Cdc12(FH1FH2)p with 3.0 μM profilin, or Cdc12(FH1FH2)p with profilin and SpTm. Lengths are the average of 100 filaments. (D) Dependence of the length of annealed filaments on the concentration of SpTm in the presence of Cdc12(FH1FH2)p. Equal concentrations (0.25 μM) of TMR- and Oregon Green-labeled preassembled actin filaments were sheared through a 26-gauge needle in the presence of 100 nM Cdc12(FH1FH2)p and a range of SpTm concentrations. After 1.0 h reactions were diluted, absorbed to poly-L-lysine-coated coverslips, and the lengths of 100 filaments were measured. (E) Dependence of the length of annealed filaments on the concentration of capping protein in the presence of 5.0 μM SpTm (○), or SpTm and 100 nM Cdc12(FH1FH2)p (●). (F and G) Addition of profilin-actin to the barbed end of preassembled actin filament seeds. After 5 h, initial spontaneous assembly reactions containing 3.0 μM Mg-ATP actin and either 100 nM Cdc12(FH1FH2)p alone, or Cdc12(FH1FH2)p with 4.0 μM SpTm, were diluted by various amounts into new reactions containing 0.5 μM Mg-ATP actin (20% pyrene labeled) and 2.5 μM profilin. (F) Time course of the barbed end elongation of reactions containing no preassembled seeds (○) or a 250-fold dilution of seeds preassembled with Cdc12(FH1FH2)p alone (□), or Cdc12(FH1FH2)p with SpTm (■). (G) Plot of the dependence of the initial barbed end elongation rate (slope) on the concentration of actin filament seeds after dilution from reactions containing Cdc12(FH1FH2)p alone (□) or Cdc12(FH1FH2)p with SpTm (■).

SpTm also increases the length of Cdc12(FH1FH2)p-nucleated filaments in the presence of profilin (Figure 7B). After 9 h the filament length in the presence of profilin increases from 2.5 to 9.1 μm with SpTm and from 2.7 to 4.5 μm without SpTm.

To test the possibility that SpTm allows Cdc12p-nucleated filaments to anneal end to end (Andrianantoandro *et al.*, 2001), we visualized filaments after mixing two “parallel” spontaneous assembly reactions containing either red (TMR-labeled) or green (Oregon Green-labeled) actin (Figure 7C). In the absence of SpTm, Cdc12(FH1FH2)p-nucleated filaments do not anneal. Conversely, SpTm allows Cdc12(FH1FH2)p-nucleated filaments to anneal, as indicated by longer filaments containing alternating sections of red and green. The extent of annealing is dependent upon the concentration of SpTm (Figure 7D). SpTm also allows Cdc12(FH1FH2)p-nucleated filaments to anneal in the presence of profilin (Figure 7C), but the profilin-binding Cdc12 FH1 domain is not necessary for annealing because Cdc12(FH2)p-nucleated filaments also anneal in the presence of SpTm (data not shown). SpTm promotes the annealing of actin filaments in the absence of Cdc12p as well. After manual severing and annealing for 1 h, filaments increased from ~ 1.5 to 6.8 μm without SpTm and to 17.0 μm with 5.0 μM SpTm.

Does SpTm Induce Annealing by Dissociating Formin Cdc12p from the Barbed End?

We next investigated how SpTm might allow Cdc12p-nucleated filaments to anneal. In the absence of SpTm, Cdc12p blocks annealing by remaining continually associated with the barbed end (Kovar *et al.*, 2003). SpTm might promote annealing by increasing the dissociation rate of Cdc12p from the barbed end. Direction observation of elongating Cdc12(FH1FH2)p-associated filaments by TIRF microscopy suggests that Cdc12p rarely dissociates within 30 min in both the absence and presence of SpTm, although this is difficult to quantify (Figures 4 and 5).

We tested the possibility that SpTm increases the dissociation rate of Cdc12p by three assays (Figure 7E and Supplemental Figure 2). First, we determined the affinity of Cdc12(FH1FH2)p for actin filament barbed ends in both the absence and presence of SpTm. We measured the ability of Cdc12(FH1FH2)p to bind and inhibit the barbed end elongation of preassembled actin filament seeds (Supplemental Figure 2, A and B). Although SpTm approximately halves the barbed end elongation rate on its own, the dependence of the initial rate on the concentration of Cdc12(FH1FH2)p decreases with a similar slope in both the absence and presence SpTm (Supplemental Figure 2, A and B). Curve fits revealed that Cdc12(FH1FH2)p binds to actin filament barbed ends with similar low nanomolar affinities in the absence ($K_d = 0.9$ nM) and presence of SpTm ($K_d = 1.4$ nM). It is possible that the nearly doubled equilibrium dissociation constant explains annealing, although this difference is not significant by the two-tailed *t* test ($p = 0.35$). Second, we tested the ability of capping protein to prevent annealing (Figure 7E). In the absence of Cdc12p, capping protein binds to the free barbed ends of SpTm saturated filaments and prevents their annealing. However, capping protein does not prevent annealing in the presence of Cdc12(FH1FH2)p and SpTm. Results from these two experiments are consistent with a model whereby SpTm allows Cdc12p-associated actin filaments to anneal without removing Cdc12p from the barbed end, potentially “trapping” Cdc12p between annealed segments.

Third, we determined the amount of Cdc12(FH1FH2)p that sediments with annealed filaments (Supplemental Fig-

ure 2, C and D). In the absence of SpTm, Cdc12(FH1FH2)p-nucleated actin filaments remain short after 4 h (~ 0.7 μm ; Supplemental Figure 2C), and a small but detectable amount of Cdc12(FH1FH2)p pellets with the filaments at high speed (Supplemental Figure 2D). In the presence of SpTm, Cdc12(FH1FH2)p-nucleated actin filaments become significantly longer through annealing (~ 5.0 μm), and less Cdc12(FH1FH2)p pellets with the filaments (Supplemental Figure 2, C and D). This may indicate that SpTm dissociates Cdc12(FH1FH2)p from barbed ends to allow annealing. However, in addition to the barbed end, some formin proteins bind to the sides of actin filaments (Michelot *et al.*, 2005; Moseley and Goode, 2005; Harris *et al.*, 2006), and side-binding is inhibited by tropomyosin (Wawro *et al.*, 2007). We have not detected large quantities of Cdc12(FH1FH2)p bound to the sides of actin filaments (Kovar, unpublished observations). However, we cannot rule out the possibility that a small amount of Cdc12(FH1FH2)p binds to actin filament sides and is dissociated by SpTm.

SpTm-induced Annealing Limits Formin Cdc12p-mediated Actin Filament Elongation

Some formins are regulated by autoinhibition through association of their N- and C-terminal regulatory regions (Waller and Alberts, 2003; Higgs, 2005). There is no evidence that Cdc12p is autoinhibited (Yonetani *et al.*, 2008), and autoinhibition may not be the mechanism by which activated formins are subsequently inactivated. However, because actin filaments in the contractile ring are much shorter than expected from unregulated Cdc12p-mediated elongation in vitro (Kamasaki *et al.*, 2007; Vavylonis *et al.*, 2008), there must be mechanisms in place to limit Cdc12p-mediated elongation. Whether Cdc12p is trapped between annealed segments, or dissociated from the barbed end, SpTm induced annealing could ultimately inhibit the duration of Cdc12p-mediated actin filament elongation by reducing the number of available Cdc12p-associated barbed ends.

To test the possibility that annealed actin filaments contain fewer elongation competent Cdc12p-associated barbed ends, we diluted actin seeds assembled by Cdc12(FH1FH2)p alone or by Cdc12(FH1FH2)p and SpTm into new bulk pyrene actin assembly assays (Figure 7, F and G). Three micromolar Mg-ATP actin was assembled in the presence of 100 nM Cdc12(FH1FH2)p alone, or Cdc12(FH1FH2)p with 4.0 μM SpTm. After 5 h, the initial reactions were diluted into new reactions containing 0.5 μM Mg-ATP actin (20% pyrene labeled) and 2.5 μM profilin. Reactions without seeds did not elongate because 2.5 μM profilin strongly inhibits the nucleation of 0.5 μM actin monomers. Over a range of fold-dilutions, reactions doped with seeds preassembled in the presence of only Cdc12(FH1FH2)p elongated significantly faster than reactions doped with seeds preassembled in the presence of both Cdc12(FH1FH2)p and SpTm (Figure 7, F and G). Therefore, SpTm significantly reduces the number of Cdc12(FH1FH2)p-associated barbed ends available for elongation.

DISCUSSION

The single tropomyosin isoform Cdc8p (SpTm) is required for division in fission yeast (Balasubramanian *et al.*, 1992, 1994; Chang *et al.*, 1997). By antagonizing cofilin-mediated severing and regulating myosin-II binding to actin filaments (Nakano and Mabuchi, 2006; Skoumpla *et al.*, 2007), SpTm plays a role after actin filaments are assembled at the division site. However, it was not known how SpTm sorts to the contractile ring and whether SpTm affects Cdc12p-nucleated

Figure 8. Cartoon model for SpTm's influence on formin Cdc12p-mediated actin assembly. (1) Cdc12p nucleates actin filament assembly and then remains continually associated with the elongating barbed end (Kovar *et al.*, 2003, 2006; Kovar and Pollard, 2004; Yonetani *et al.*, 2008). (2) SpTm binds preferentially to newly assembled ATP-loaded actin filaments and Cdc12p-associated ADP-loaded actin filaments. (3) SpTm doubles the elongation rate of Cdc12p-associated barbed ends by inhibiting monomer dissociation. (4) In the presence of SpTm, profilin-actin adds ~15-fold faster than actin to Cdc12p-associated barbed ends. (5) SpTm allows Cdc12p-nucleated filaments to anneal end to end. (6) Cdc12p may be dissociated to allowing annealing, or Cdc12p may remain "sequestered/trapped" in annealed actin filaments. (7) Annealed actin filament barbed ends cannot incorporate new profilin-actin monomers. (8) SpTm protects filaments from cofilin-mediated severing (Nakano and Mabuchi, 2006). (9) SpTm regulates myosin-II binding to actin filaments (Skoumpla *et al.*, 2007).

actin filament assembly. We discovered that SpTm is recruited to filaments assembled by Cdc12p, and has diverse effects on Cdc12p-mediated actin filament assembly. We now have a detailed picture of the multifaceted mechanisms by which SpTm contributes to contractile ring assembly in fission yeast (Figure 8).

SpTm's Roles in Cdc12p-mediated Actin Assembly

Evidence has accumulated for two mechanisms for contractile ring assembly in fission yeast. In both models fission yeast uses Cdc12p-mediated assembly of profilin-actin to build a contractile ring composed of antiparallel actin filaments that constrict by type II myosin motor (Myo2p) activity. In the first model, Cdc12p accumulates in one or a few large spots that nucleates the assembly of a "leading cable" of actin filaments that wrap around the cell equator (Chang *et al.*, 1997; Chang, 1999, 2000; Arai and Mabuchi, 2002; Carnahan and Gould, 2003; Motegi *et al.*, 2004; Kamasaki *et al.*, 2007; Yonetani *et al.*, 2008). Alternatively, Cdc12p and Myo2p have been localized to ~60 smaller "pre-ring" nodes (Bahler *et al.*, 1998; Bezanilla *et al.*, 2000; Motegi *et al.*, 2000, 2004; Paoletti and Chang, 2000; Wu *et al.*, 2003, 2006; Vavylonis *et al.*, 2008). The coordinated activity of Cdc12p-mediated actin assembly coupled with Myo2p-mediated actin filament pulling (Lord *et al.*, 2005), connects nodes and drives their coalescence into a mature contractile ring (Wu *et al.*, 2006; Vavylonis *et al.*, 2008). Interestingly, both the Cdc12p-associated larger spot and smaller pre-ring nodes are not absolutely required for contractile ring assembly under all conditions (Wu *et al.*, 2006; Huang *et al.*, 2008; Yonetani *et al.*, 2008).

We now know that SpTm has diverse roles in actin filament assembly (Figure 8). Cdc12p overcomes the combined inhibition of actin polymerization by profilin and SpTm and remains processively associated with SpTm-saturated fila-

ments. SpTm selectively binds to newly assembled ATP-loaded actin filaments and/or Cdc12p-associated ADP-loaded filaments that have rapidly hydrolyzed ATP and released phosphate. Cdc12p-associated filaments saturated with SpTm elongate actin at $0.6 \text{ subs}^{-1} \mu\text{M}^{-1}$ and profilin-actin at $9.0 \text{ subs}^{-1} \mu\text{M}^{-1}$, which are 2- and 30-fold faster than actin adds to Cdc12p-associated filaments without SpTm ($0.3 \text{ subs}^{-1} \mu\text{M}^{-1}$). In part, SpTm's enhancement of the overall elongation rate can be explained by inhibiting the rate of actin subunit dissociation from the barbed end.

SpTm also allows Cdc12p-nucleated filaments to lengthen by end-to-end annealing, possibly without dissociating Cdc12p from the actin filament barbed end. Extensive attempts to directly visualize individual Cdc12p dimers by immunofluorescence at transitions between annealed segments have been inconclusive (Skau and Kovar, unpublished observations). Because Cdc12p in the contractile ring recovers within a few minutes after photobleaching (Yonetani *et al.*, 2008), annealing may depend upon Cdc12p dissociation. Although we do not yet know whether annealing is required for contractile ring assembly, annealing is theoretically possible for both the leading cable and pre-ring node models. Furthermore, because the fission yeast and budding yeast formins Cdc12p and Bnr1p localize throughout the contractile ring, and For3p and Bni1p localize as speckles throughout actin cables (Chang *et al.*, 1997; Imamura *et al.*, 1997; Martin and Chang, 2006; Buttery *et al.*, 2007), annealing may be important for multiple formin-dependent processes.

In contrast, *in vitro* annealing may simply reflect the ability of SpTm to dissociate Cdc12p from actin filament barbed ends and contribute to turning off Cdc12p-mediated barbed end elongation. Mechanisms for dissociating Cdc12p from the barbed end are particularly important. The intrinsic dissociation rate of Cdc12p is 2 orders of magnitude slower than other formins such as budding yeast Bni1p, mouse mDia1 and mDia2, and nematode worm CYK-1 (Kovar *et al.*, 2006; Neidt *et al.*, 2008). We found that annealed Cdc12p-nucleated filaments do not incorporate new profilin-actin monomers. "Sequestering" Cdc12p molecules between annealed segments, or dissociating Cdc12p from actin filament barbed ends, may provide an important mechanism for controlling actin filament lengths. Filaments in the fission yeast contractile ring average ~0.5 μm long (Kamasaki *et al.*, 2007), considerably shorter than expected from unregulated elongation (Kovar *et al.*, 2003, 2006; Kovar and Pollard, 2004).

Tropomyosin's Role in Diverse Actin-mediated Processes

SpTm is exclusively required for processes that depend upon the three fission yeast formins (Balasubramanian *et al.*, 1992; Arai *et al.*, 1998; Pelham and Chang, 2001; Kurahashi *et al.*, 2002; Skoumpla *et al.*, 2007). Formin and tropomyosin are also involved in cell division in animal cells (Clayton and Johnson, 1998; Higgs, 2005; Faix and Grosse, 2006; Goode and Eck, 2007), as well as other formin-dependent processes (Gunning *et al.*, 2005, 2008). Therefore, the influence of SpTm on Cdc12p-mediated actin assembly might be conserved, which is supported by the finding that tropomyosin isoforms differentially increase the seeded elongation rate of vertebrate formin isoforms including FRL1, mDia1 and mDia2 (Wawro *et al.*, 2007). It will be important to determine whether like Cdc12p, the other fission yeast and mammalian formins increase the affinity of tropomyosin for ADP actin filaments, remain processively associated with tropomyosin saturated filaments, anneal in the presence of tropomyosin, and are turned off by tropomyosin.

Mechanism of SpTm's Effect on Barbed End Dynamics in the Absence and Presence of Cdc12p

SpTm and Cdc12p have a complicated relationship. Cdc12p recruits SpTm to a specific actin filament structures, which increases the rate of Cdc12p-mediated elongation and allows annealing, but may ultimately turn off Cdc12p-mediated elongation. Although some mammalian formin and tropomyosin isoforms interact directly (Wawro *et al.*, 2007), we did not detect an interaction between SpTm and Cdc12p by the same method (data not shown). Therefore, we propose that Cdc12p and SpTm affect each other indirectly through altering actin filament conformation by influencing the twist and/or flexibility of the filament.

On its own, SpTm slows both the rate of polymerization and depolymerization. The ability of various tropomyosin isoforms to inhibit the rate of actin polymerization is well documented (Pragay and Grgely, 1968; Wegner, 1982; Lal and Korn, 1986; Hitchcock-DeGregori *et al.*, 1988; Wawro *et al.*, 2007). It has been proposed that tropomyosin inhibits polymerization by preventing filament fragmentation (Wegner, 1982; Hitchcock-DeGregori *et al.*, 1988) or by decreasing the elongation rate (Lal and Korn, 1986; Wawro *et al.*, 2007). Our direct observations by TIRF microscopy verify that SpTm inhibits polymerization by decreasing the barbed end elongation rate approximately twofold. It is possible that tropomyosin affects barbed end dynamics directly by steric blockade of the barbed end from subunit addition and dissociation, or indirectly through altering the twist and/or rigidity of the actin filament. Given that cofilin "locks" the filament in a specific twist and competes with some tropomyosin isoforms for binding (McGough *et al.*, 1997; Ono and Ono, 2002), tropomyosin isoforms may variously induce different twists and/or conformations of the actin filament that alter barbed end dynamics and access by cofilin (Nyakern-Meazza *et al.*, 2002).

Alterations in actin filament conformations may also provide the mechanism by which SpTm binds with higher affinity to Cdc12p-assembled ADP actin filaments, and they explain how SpTm increases the elongation rate of Cdc12p-associated barbed ends. We propose that Cdc12p alters long-range conformational properties of the filament by remaining processively associated with the elongating barbed end. This is consistent with the finding that mammalian formins increase the flexibility of actin filaments (Bugyi *et al.*, 2006; Papp *et al.*, 2006). A local increase of SpTm near the Cdc12p-associated barbed end could lead to cooperative saturation of the entire filament with SpTm. In turn, tropomyosin binding to "flexible" formin-associated filaments seems to stabilize the filament (Ujfalusi *et al.*, 2009). Filament stabilization might alter formin-associated barbed end properties such as the elongation rate and annealing. Ultimately, we anticipate that diverse effects on actin filament conformations by formin (promoting ATP hydrolysis and increased flexibility) and tropomyosin (decreased flexibility and twist), will allow a specific subset of actin binding proteins to bind particular actin filament structures and collectively influence actin filament dynamics, architecture, and function.

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