The Cytokinesis Formins from the Nematode Worm and Fission Yeast Differentially Mediate Actin Filament Assembly*

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Formins drive actin filament assembly for diverse cellular processes including motility, establishing polarity, and cell division. To investigate the mechanism of contractile ring assembly in animal cells, we directly compared the actin assembly properties of formins required for cytokinesis in the nematode worm early embryo (CYK-1) and fission yeast (Cdc12p). Like Cdc12p and most other formins, CYK-1 nucleates actin filament assembly and remains processively associated with the elongating barbed end while facilitating the addition of profilin-actin above the theoretical diffusion-limited rate. However, specific properties differ significantly between Cdc12p and CYK-1. Cdc12p efficiently nucleates filaments that in the presence of profilin elongate at approximately the same rate as control filaments without formin (~10.0 subunits/s). CYK-1 is an inefficient nucleator but allows filaments to elongate profilin-actin 6-fold faster than Cdc12p (~60 subunits/s). Both Cdc12p and CYK-1 bind to pre-assembled actin filaments with low nanomolar affinity, but CYK-1 dissociates 2 orders of magnitude more quickly. However, CYK-1 rapidly re-associates with free barbed ends. Cdc12p allows barbed ends to elongate in the presence of excess capping protein, whereas capping protein inhibits CYK-1-mediated actin assembly. Therefore, these evolutionarily diverse formins can drive contractile ring assembly by a generally similar mechanism, but cells with unique dimensions and physical parameters might require proteins with carefully tuned actin assembly properties.

The final step of cell division is cytokinesis, the physical separation of a mother cell into two daughter cells (1, 2). Animal cells spatially and temporally coordinate cleavage site positioning through both astral and spindle microtubules (3, 4). Upon choosing a division site, far less is known about how actin and myosin II motor filaments assemble into the contractile ring. The mechanism is clearer in the unicellular fission yeast Schizosaccharomyces pombe (see Fig. 7A) (5). The fission yeast contractile ring is constructed from ~60 “pre-ring” nodes through the coordinated activities of numerous proteins including the anillin-like protein Mid1p (6–8), the type II myosin motor Myo2p (9–11), the actin monomer binding protein profilin Cdc3p/SpPRF (12), and the actin filament nucleator formin Cdc12p (13).

A major question is whether animal cells assemble the contractile ring by a similar mechanism as fission yeast. Many of the proteins required for cytokinesis are conserved between fission yeast and animal cells including anillin, type II myosin, profilin, and formin (1, 2). Formins are large multidomain proteins that, in addition to cytokinesis, assemble actin filaments for multiple cellular processes including motility and establishing polarity (14–16). Formins contain a highly conserved actin assembly FH2 domain, whereas capping protein inhibits CYK-1 rapidly re-associates with free barbed ends. Cdc12p allows barbed ends to elongate in the presence of excess capping protein, whereas capping protein inhibits CYK-1-mediated actin assembly. Therefore, these evolutionarily diverse formins can drive contractile ring assembly by a generally similar mechanism, but cells with unique dimensions and physical parameters might require proteins with carefully tuned actin assembly properties.

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2 The abbreviations used are: FH, formin homology; OG, Oregon green; a.u., arbitrary units; DTT, dithiothreitol.
cellular roles. An active CYK-1 construct containing the actin assembly domains CYK-1(FH1FH2) nucleates actin filament assembly, remains processively associated with the elongating actin filament barbed end, and drives the addition of actin bound to profilin CePFN-1 above the theoretical diffusion-limited rate. However, the specific rates of CYK-1 are significantly different from Cdc12p. Cdc12p efficiently nucleates actin filaments that elongate slowly. CYK-1 is an inefficient nucleator but has a high affinity (low nanomolar) for preassembled actin filament barbed ends and drives exceptionally fast actin monomer addition. Therefore, the general mechanism of formin-mediated contractile ring assembly may be similar between fission yeast and the nematode worm embryo. However, differences in their specific actin assembly properties may tune these formins for contractile ring assembly in diverse cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Bacterial expression constructs for formin(FH1FH2) and formin(FH2) domains were prepared by standard cloning procedures as follows: 1) Cdc12(FH1FH2)p, pET21a-cdc12(882–1390)-HIS has been described (24); 2) Cdc12(FH2)p, pET21a-cdc12(973–1390)-HIS was prepared by PCR amplification (iProof, Bio-Rad); 3) CYK-1(FH1FH2) and CYK-1(FH2), pET21a-MBP(TEV)-cyk-1(700–1210)-HIS and pET21a-MBP(TEV)-cyk-1(802–1210)-HIS were prepared by amplifying the appropriate regions from C. elegans cDNA (Michael Glotzer, The University of Chicago). A bacterial expression construct for fission yeast profilin pMW172-SpPRF has been described (31). A bacterial expression construct for nematode worm profilin isoform 1 pMW172-CePFN-1 was prepared by amplifying from C. elegans cDNA. Inserts of the recombinant plasmids were sequenced to confirm fidelity of the PCR amplification.

Protein Purification—Cdc12(FH1FH2)p, Cdc12(FH2)p, mouse capping protein, SpPRF, and CePFN-1 were purified by capping, SpPRF, and CePFN-1 were purified from bacteria as described (24, 28, 31–33).

Recombinant CYK-1(FH1FH2) and CYK-1(FH2) were purified by expressing in Escherichia coli strain BL21-Codon Plus (DE3)-RP (Stratagene) with 0.5 mm isopropyl β-D-thiogalactopyranoside for 6 h at 25 °C. Harvested cells were resuspended in extraction buffer (50 mM NaH2PO4, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 10 mM M-mercaptoethanol) supplemented with protease inhibitors and homogenized in an Emulsiflex-C3 (Avestin). The homogenate was clarified at 30,000 rpm for 20 min each, and the extract was incubated with Talon Metal Affinity Resin (Clontech) for 1 h at 4 °C and then loaded onto a disposable column. After a 50-ml wash with extraction buffer, CYK-1 was eluted with Talon elution buffer (50 mM NaH2PO4, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 250 mM imidazole) and dialyzed overnight versus Source S buffer A (10 mM Hepes, pH 7.2, 50 mM NaCl, 5% glycerol, 0.01% NaN3, 1 mM DTT) with 3 mM tobacco etch virus protease to remove maltose-binding protein. Dialyzed protein was loaded on a 1.0-ml Source S column (GE Healthcare) and eluted with a linear gradient from 50 to 500 mM NaCl. Pure CYK-1 was dialedyzed into formin buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 200 mM KCl, 0.01% NaN3, and 1 mM DTT) and flash-frozen in liquid nitrogen and stored at −80 °C. Unfrozen and flash-frozen CYK-1 slowly lost activity over time, so CYK-1(FH1FH2) and CYK-1(FH2) were utilized within 1 month of purification.

Ca-ATP actin was purified from chicken skeletal muscle (Trader Joe’s) as described for rabbit skeletal muscle (34). Gel-filtered actin was labeled with Cys-374 with pyrenyl iodoacetamide or Oregon green 488 iodoacetamide (Invitrogen) (23, 35). Tetramethylrhodamine-labeled actin was a gift from Ron Rock (The University of Chicago). Immediately before each experiment, 5–15 μM 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.

Protein concentrations were determined with extinction coefficients as follows: unlabeled actin, A290 = 26,600 M−1 cm−1 (36); pyrene-actin, (A290 – [A444 × 0.127]) × 38.5 μM (37); Oregon green actin, [total Ca-actin] = (A290 – [A491 × 0.16991])/26,600 M−1 cm−1; [Ca-OG-actin] = A291/77,800 M−1 cm−1 (23); SpPRF, A280 = 1.63 absorbance mg−1 ml−1 (31); CePFN-1, A280 = 18,450 M−1 cm−1; mouse capping protein, A280 = 76.3 M−1 cm−1 (33); Cdc12(FH1FH2)p and Cdc12(FH2)p, A280 = 49,860 M−1 cm−1 (23). Extinction coefficients for CYK-1(FH1FH2) and CYK-1(FH2) were estimated with ProtParam from the amino acid composition, A280 = 34,170 M−1 cm−1.

Fluorescence Spectroscopy—Actin assembly was measured from the fluorescence of a trace of pyrene-actin (excitation at 364 nm and emission at 407 nm) with Spectramax Gemini XPS (Molecular Devices) and Safire2 (Tecan) fluorescent plate readers. Final protein concentrations are indicated in the figure legends.

For spontaneous assembly assays, a 15 μM mixture of pyrenelabeled and unlabeled Mg-ATP-actin with 100X anti-foam 204 (0.005%; Sigma) was placed in a upper row of a 96-well non-binding black plate (Corning). Other proteins to be assayed (formin, profilin, etc.), 10× KMEI (500 mM KCl, 10 mM MgCl2, 10 mM EGTA, and 100 mM imidazole, pH 7.0) and Mg-buffer G (2 mM Tris, pH 8.0, 0.2 mM ATP, 0.1 mM MgCl2, and 0.5 mM DTT) were placed in a lower row of the plate. Reactions were started by mixing contents of the lower wells and the actin monomers in the upper wells with a 12-channel Pipetman (Eppendorf).

For seeded assembly assays, 5.0 μM unlabeled Mg-ATP-actin was preassembled in the upper row of the plate followed by addition of anti-foam, other proteins to be assayed (formin, profilin, etc.), and Mg-buffer G. A 5.0 μM mixture of pyrenelabeled and unlabeled Mg-ATP-actin with Mg-buffer G was placed in the lower plate row. Mixing actin monomers in lower wells with preassembled actin filaments in upper wells started reactions.

For depolymerization assays, a 5.0 μM mixture of unlabeled and pyrenelabeled Mg-ATP-actin monomers was preassembled in the upper row of the plate for 2 h followed by the addition of anti-foam. Forinm, 10× KMEI, and Mg-buffer G were placed in the lower plate row. Reactions were started by mixing lower wells with upper wells, diluting the pre-assembled filaments to 0.1 μM.

The critical concentration for actin assembly was determined by assembling a 1.0 μM mixture of unlabeled and pyrene-
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labeled Mg-ATP-actin monomers in the presence of a range of concentrations of formin in a 96-well black plate. The final amount of filamentous actin was determined after a 16-h incubation in the dark at 25 °C.

**Calculation of Initial Polymerization Rates, Depolymerization Rates, Barbed End Affinity, and Nucleation Efficiency**—The actin assembly rates from spontaneous assembly reactions were determined by measuring the slopes from the points where 10–50% of the actin had assembled. Polymerization rates from pre-assembled actin filament seeds were measured from the slope of a linear fit of the first 300 s. The rate of depolymerization was calculated by fitting the data from 300 to 1000 s with a single exponential curve. Depolymerization rates were expressed as a percent normalized to the rate of actin alone. The affinity of formin for actin filament barbed ends was determined by fitting plots of the dependence of either the initial assembly rate or the initial depolymerization rate on the concentration of formin, with the equation $V_i = V_{if} + (V_b - V_{if})((K_d + [ends] + [formin]) - ((K_d + [ends] + [formin])^2 - 4[ends][formin])/2[ends])^{1/2}$, where $V_i$ is the observed elongation or depolymerization rate, $V_{if}$ is the elongation or depolymerization rate when barbed ends are free, $V_b$ is the elongation or depolymerization rate when barbed ends are bound, [ends] is barbed-end concentration, and [formin] is formin concentration (32). The nucleation efficiency was calculated by dividing the spontaneous assembly rate (slope) by $k^+$ [where $k^+ = 0.3$ and $10.6 \text{ nM}^{-1}\text{s}^{-1}$ for Cdc12(FH1FH2)p in the absence and presence of profilin and 6.2 and 63.2 $\text{nM}^{-1}\text{s}^{-1}$ for CYK-1(FH1FH2) in the absence and presence of profilin] and then dividing by the formin concentration.

**Microscopy of Fluorescently Labeled Filaments**—Products of spontaneous assembly reactions were examined by fluorescence microscopy as described previously (23, 38). Actin filament annealing (39) was examined by assembling a blend of unlabeled Mg-ATP-actin with either rhodamine-labeled Mg-ATP-actin or Oregon green-labeled Mg-ATP-actin. Mixtures of equal amounts of red and green filaments were sheared by pushing 16 times through a 3/8-inch 26-gauge needle on a 1.0-ml tuberculin syringe and allowed to anneal for 60 min at room temperature. Reactions were terminated by a 250-fold dilution in fluorescence buffer (50 mM KCl, 1 mM MgCl$_2$, 100 mM DTT, 20 $\mu$g/ml catalase, 100 $\mu$g/ml glucose oxidase, 3 mg/ml glucose, 0.5% methylene cellulose, 10 mM imidazole, pH 7.0) and absorbed to coverslips coated with 0.05 g/ml poly-L-lysine. Fluorescence images were collected with a cooled CCD camera (Orcal-ER) on an Olympus IX-81 microscope.

**Total Internal Reflection Fluorescence (TIRF) Microscopy**—Images of Oregon green-labeled actin filaments excited by total internal reflection (Olympus IX-71 microscope; fit with through-the-objective TIRF illumination) were collected at 15-s intervals with an iXon EMCCD camera (Andor). As described in detail previously (22–24, 35), a mixture of unlabeled Mg-ATP actin and 33% Mg-ATP Oregon green actin was mixed with 2 $\times$ TIRF buffer (1 $\times$, 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 $\mu$M CaCl$_2$, 15 mM glucose, 20 $\mu$g/ml catalase, 100 $\mu$g/ml glucose oxidase, and 0.5% (500 centipoise) methylene cellulose) and formin and/or profilin and transferred to a flow cell for imaging.

**RESULTS**

The Worm Cytokinesis Formin CYK-1 Reduces the Rate of Monomer Association and Dissociation from the Actin Filament Barbed End—Most formins bind processively to actin filament barbed ends with low nanomolar affinity and in the absence of profilin lower the rate of both assembly and disassembly (16). We began our investigation of the worm cytokinesis formin CYK-1 by determining whether CYK-1 binds actin filament-barbed ends and influences monomer association and dissociation and whether the specific rates are similar in the presence of CYK-1 and the fission yeast cytokinesis formin Cdc12p.

A fission yeast Cdc12p construct containing both the formin homology 1 and 2 domains Cdc12(FH1FH2)p as well as a construct lacking the profilin binding FH1 domain Cdc12(FH2)p shifts the critical concentration for assembly from that of the barred end near 0.1 $\mu$m to that of the pointed end near 0.9 $\mu$m (Fig. 1A) (23, 32). A range of concentrations of CYK-1(FH1FH2) and CYK-1(FH2)p constructs had no effect on the critical concentration (Fig. 1A), suggesting that CYK-1 does not strongly inhibit barred end assembly.

Next we investigated how CYK-1 influences the addition or loss of Mg-ATP actin monomers to preassembled actin filaments (Figs. 1, B–E). Both CYK-1 and Cdc12p reduce the seeded assembly rate, revealing similar low nanomolar equilibrium dissociation constants for the actin filament barred end of 1.5, 1.7, 1.7, and 10.6 nM for Cdc12(FH1FH2)p, Cdc12(FH2)p, CYK-1(FH1FH2), and CYK-1(FH2) (Figs. 1, B and C). However, whereas Cdc12p reduces the seeded assembly rate by ~99%, CYK-1 reduces the seeded assembly rate by ~50%. Conversely, both CYK-1 and Cdc12p inhibit barred end disassembly by ~80%, revealing similar low nanomolar equilibrium dissociation constants for the actin filament barred end of 0.63, 0.60, 0.76, and 2.6 nM for Cdc12(FH1FH2)p, Cdc12(FH2)p, CYK-1(FH1FH2), and CYK-1(FH2) (Figs. 1, D and E).

**CYK-1 Inefficiently Nucleates Actin Filament Assembly**—Although formins reduce the rate of barred end addition (Figs. 1, B and C) (16), formins enhance the overall rate that actin monomers assemble into filaments by stimulating nucleation. CYK-1(FH1FH2) increases the spontaneous assembly of Mg-actin monomers by reducing the lag at the outset of the reaction and increasing the maximum rate (slope) of assembly (Fig. 2A) (23). The general ability of CYK-1(FH1FH2) and Cdc12(FH1FH2)p to stimulate spontaneous actin monomer assembly is similar over a range of formin concentrations (Fig. 2B). Constructs containing only the formin homology 2 domain CYK-1(FH2) and Cdc12(FH2)p also stimulate the spontaneous assembly of actin monomers (Fig. 2B). Because CYK-1 does not shift the critical concentration for assembly near that of the pointed end (Fig. 1A), CYK-1(FH1FH2) enhances spontaneous actin assembly at lower actin concentrations than Cdc12(FH1FH2)p (Fig. 1E).

The rate of spontaneous actin assembly is dependent upon both the number of filaments (nucleation) as well as the rate that the filaments assemble (elongation). CYK-1 allows actin filaments to elongate significantly faster than Cdc12p (Fig. 1C), suggesting that Cdc12(FH1FH2)p is a more efficient nucleator than CYK-1(FH1FH2) since the spontaneous actin assembly
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activity of these formins is similar (Fig. 2B). To compare the relative nucleation efficiency of CYK-1 and Cdc12p, we looked at the length of filaments after the spontaneous assembly reactions shown in Fig. 2A reached a plateau. Fluorescence images of the products of the spontaneous actin assembly reactions labeled with rhodamine-phalloidin showed that filaments formed in the presence of CYK-1 constructs are ≈9× longer than filaments formed in the presence of Cdc12p constructs, indicating that CYK-1 is a less efficient nucleator (Fig. 2C). Actin filaments in the absence of formin averaged 16.0 μm, whereas actin filaments in the presence of either 100 nM Cdc12(FH1FH2)p or Cdc12(FH2)p averaged 0.7 and 1.0 μm. Actin filaments in the presence of either 100 nM CYK-1(FH1FH2) or CYK-1(FH2) averaged 8.8 and 9.2 μm.

Knowing that CYK-1-nucleated filaments elongate their barbed ends at ~6.0 subunits s⁻¹μm⁻¹ (see Fig. 5; Table 1) and Cdc12p nucleated filaments elongate their barbed ends at ~0.2 subunits s⁻¹μm⁻¹ (see Fig. 5; Table 1) (22–24), we calculated the nucleation efficiency of CYK-1 and Cdc12p (Fig. 2D). At low concentrations CYK-1(FH1FH2) and CYK-1(FH2) maximally produced 1 new filament per ~50 and ~80 molecules (Fig. 2D). This is very inefficient compared with Cdc12(FH1FH2)p and Cdc12(FH2)p, which maximally produced 1 new filament per ~2.5 and ~3.0 molecules at low concentrations (Fig. 2D) (23).

The Rate of CYK-1-mediated Spontaneous Actin Assembly Is Increased by Profilin—Profilin reduces the spontaneous assembly of Mg-ATP actin monomers by inhibiting nucleation (Fig. 3A), whereas formin enhances spontaneous assembly by promoting nucleation (Figs. 2 and 3A) (16). In the presence of CYK-1(FH2) or Cdc12(FH2)p, constructs that lack the proline-rich profilin binding FH1 domain, profilin inhibits formin-mediated spontaneous actin assembly (Figs. 3, A–C). In the presence of CYK-1(FH1FH2), worm profilin CepFN-1 increases the rate of spontaneous actin assembly (Fig. 3A).

The effect on spontaneous actin assembly of a range of concentrations of either CepFN-1 with CYK-1(FH1FH2) or fission yeast profilin SpPRF with Cdc12(FH1FH2)p is biphasic (Figs. 3, B and C) (23). Low profilin concentrations increasingly enhance the spontaneous assembly rate until an optimal con-
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FIGURE 2. Worm formin CYK-1 inefficiently nucleates actin filament assembly. Conditions were the same as in Fig. 1. A–D, spontaneous actin assembly; time course of the polymerization of 3 μM Mg-ATP actin (20% pyrene-labeled). A, plot of pyrene-fluorescence over time in the (thick curve) absence or presence of 10 ( ), 25 ( ), and 100 nm ( ) Cdc12(FH1FH2)p or 10 ( ), 25 ( ), and 100 ( ) nm CYK-1(FH1FH2). B, plot of the dependence of the actin assembly rate (slope) on the concentration of Cdc12(FH1FH2)p ( ), Cdc12(FH2)p ( ), CYK-1(FH1FH2) ( ), or CYK-1(FH2) ( ). C, fluorescence micrographs of actin filaments after the indicated reactions with 100 nm formin reached plateau (2 h). Samples were labeled with rhodamine-phalloidin and absorbed to glass cover slips coated with poly-l-lysine. Bar, 10 μm. D, nucleation efficiency; yield of new filaments (calculated from B) per formin molecule as a function of the concentration of Cdc12(FH1FH2)p ( ), Cdc12(FH2)p ( ), CYK-1(FH1FH2) ( ), or CYK-1(FH2) ( ). The inset shows the plot of CYK-1(FH1FH2) ( ) and CYK-1(FH2) ( ) with a focused y axis. E, spontaneous actin assembly; time course of polymerization of the indicated concentrations of Mg-ATP actin monomers alone (thick curve) or with 100 nm Cdc12(FH1FH2)p ( ) or 100 nm CYK-1(FH1FH2) ( ).

centration of ~2.5 μM is reached. Higher profilin concentrations decrease the assembly rate because profilin inhibits nucleation, and profilin unassociated with actin effectively excludes profilin-actin from formin (22, 40).

Knowing that Cdc12(FH1FH2)p- and CYK-1(FH1FH2)p-nucleated filaments elongate their barbed ends at ~12 and ~60 subunits s⁻¹ μm⁻¹ in the presence of 2.5 μM profilin (see Fig. 6G) allowed us to calculate formin nucleation efficiency in the presence of profilin (Fig. 3D). Profilin significantly decreases the nucleation efficiency of both Cdc12(FH1FH2)p and CYK-1(FH1FH2) to 1 new filament per ~50 and ~550 molecules (Fig. 3D).

The Rate of CYK-1-mediated Seeded Assembly Is Increased by Profilin—Although profilin reduces the nucleation efficiency of formin (Fig. 3D), profilin increases the “bulk” spontaneous actin assembly rate in the presence of both Cdc12(FH1FH2)p and CYK-1(FH1FH2) (Figs. 3, A–C). For Cdc12(FH1FH2)p and other forms, this paradox is because profilin significantly increases the barbed end elongation rate of formin nucleated actin filaments (16). We, therefore, investigated how a range of profilin concentrations affects the addition of Mg-ATP actin monomers to the barbed ends of preassembled actin filaments in the presence of formin(FH1FH2) and formin(FH2) constructs (Figs. 4, A and B).

In the absence of profilin, Cdc12(FH1FH2)p and Cdc12(FH2)p reduce the assembly rate by ~99%, whereas CYK-1(FH1FH2) and CYK-1(FH2) reduce the assembly rate by ~50% (Figs. 1C and 4A). Profilin further inhibits the barbed end elongation of preassembled filaments in the presence of both CYK-1(FH2) and Cdc12(FH2)p, constructs lacking the profilin-binding FH1 domain (Fig. 4B). However, profilin significantly increases the assembly rate of preassembled filaments associated with either CYK-1(FH1FH2) or Cdc12(FH1FH2)p (Figs. 4, A and B). In the presence of Cdc12(FH1FH2)p, SpPRF increases the assembly rate near that of control filaments in the absence of profilin (Figs. 4, A and B) (32). In the presence of CYK-1(FH1FH2), CePFN-1 increases the assembly rate well above the control rate without formin (Figs. 4, A and B).

Capping Protein Inhibits Actin Assembly in the Presence of CYK-1—Most formins inhibit capping protein, allowing the assembly of long actin filaments (16). We initially compared the ability of capping protein to inhibit the addition of profilin-Mg-ATP actin monomers to pre-assembled actin filaments in the
TABLE 1

Comparison of Mg-ATP actin assembly rates in the presence of formin

| Conditions | Control filaments |
|------------|-------------------|
| 1 µM Actin only | Barbed-end subunits/s 9.2 ± 0.3 |
| 1 µM Actin + 4 µM SpPrF | 8.8 ± 0.3 |
| 1 µM Actin + 4 µM CePFN-1 | 9.0 ± 0.2 |
| Cdc12(FH2)p | 0.26 ± 0.04 (10.2 ± 0.4) |
| Cdc12(FH2)p + 4 µM SpPrF | 0.02 ± 0.01 (9.1 ± 0.3) |
| Cdc12(FH1FH2)p | 0.3 ± 0.02 (8.5 ± 0.2) |
| Cdc12(FH1FH2)p + 4 µM SpPrF | 10.5 ± 0.5 (9.6 ± 0.2) |
| CYK-1(FH2) | 5.4 ± 0.1 (10.4 ± 0.2) |
| CYK-1(FH2) + 4 µM CePFN-1 | 1.6 ± 0.2 (9.1 ± 0.3) |
| CYK-1(FH1FH2) | 6.1 ± 0.2 (9.9 ± 0.2) |
| CYK-1(FH1FH2) + 4 µM CePFN-1 | 63.2 ± 0.5 (9.1 ± 0.2) |

*a At least 10 individual filaments were measured for each population. Rates are represented as the mean ± S.D.

*b The rates of internal control filaments are reported in parentheses.

*Rates for Cdc12 are the sum of rates from both the barbed and pointed ends.

FIGURE 3. Effect of profilin on spontaneous actin assembly in the presence of worm CYK-1 and fission yeast Cdc12p. Conditions were the same as in Fig. 1. A–D, spontaneous actin assembly; time course of polymerization of 2.5 µM Mg-ATP actin (20% pyrene-labeled). A, plot of pyrene fluorescence over time in the absence (thick curve) or presence of 2.5 µM worm profilin CePFN-1 (●), 20 nM CYK-1(FH1FH2) (○), CYK-1(FH1FH2) with CePFN-1 (□), 100 nM CYK-1(FH2) (■), and CYK-1(FH2) with CePFN-1 (□). B, plot of the dependence of the actin assembly rate (slope) on the concentration of profilin for either CYK-1(FH1FH2) (○) or CYK-1(FH2) (□). C, plot of the dependence of the actin assembly rate (slope) on the concentration of profilin for either Cdc12(FH1FH2)p (●) or Cdc12(FH2)p (□). D, nucleation efficiency; yield of new filaments per formin molecule with 2.5 µM profilin.

presence of Cdc12(FH1FH2)p and CYK-1(FH1FH2) (Figs. 4, C and D). In the absence of formin, capping protein concentrations as low as 5 nM completely inhibit the barbed end elongation of preassembled filaments with an equilibrium dissociation constant of 0.1 nM (Figs. 4, C and D). Both Cdc12(FH1FH2)p and CYK-1(FH1FH2) reduce the affinity of capping protein for actin filament barbed ends by ~50-fold. Concentrations of capping protein up to 250 nM slightly inhibit the barbed end elongation of filaments associated with Cdc12(FH1FH2)p (Figs. 4, C and D). However, capping protein completely inhibits barbed end elongation in the presence of CYK-1(FH1FH2) (Figs. 4, C and D).

We also compared the ability of capping protein to inhibit the stimulation of spontaneous actin monomer assembly by
Cdc12(FH1FH2)p with SpPRF and CYK-1(FH1FH2) with CePFN-1 (Figs. 4, E and F). With Cdc12(FH1FH2)p and SpPRF, capping protein concentrations up to 500 nM had little effect on both the spontaneous assembly rate as well as the average filament length 5 min into the reaction. However, with CYK-1(FH1FH2) and CePFN-1, capping protein inhibited the spontaneous assembly rate and reduced the average filament length near that of Cdc12(FH1FH2)p and SpPRF.

**Real-time Visualization of Actin Assembly in the Presence of CYK-1 by Total Internal Reflection Fluorescence Microscopy**—We utilized time-lapse evanescent wave fluorescent microscopy to directly observe formin-mediated actin assembly in real-time to determine 1) whether CYK-1(FH2) and CYK-1(FH1FH2) remain processively associated with the elongating barbed end in both the absence and presence of profilin, 2) the specific elongation rates of CYK-1(FH2)- and CYK-1(FH1FH2)-associated actin filament barbed ends with and without profilin, and 3) the rate that CYK-1(FH1FH2) dissociates from the actin filament barbed end (Figs. 5 and 6; Table 1). We followed the assembly of individual filaments elongating from a pool of 1.0 μM Mg-ATP-actin monomers supplemented with a trace of 0.5 μM Mg-ATP-actin monomers labeled with Oregon green for visualization (22, 24, 35).

In the absence of formin all filaments elongate their barbed ends at the same constant rate of ~9.0 subunits/s (Figs. 5, A and D; Table 1) (22, 24). As seen previously with Cdc12p (22–24), two distinct filament populations are detected in both the absence and presence of profilin (Figs. 5, E and F). The first population consists of internal control filaments that elongate at ~9.0 subunits/s, the same rate as filaments in the absence of formin. The second population consists of Cdc12p-associated filaments that elongate at a significantly different rate, which depends upon profilin SpPRF (Figs. 5, E and F). In the absence of SpPRF, Cdc12(FH1FH2)p-nucleated filaments elongate at only 0.3 subunits/s (Fig. 5E). Four micromolar SpPRF increases the elongation rate of Cdc12(FH1FH2)p-nucleated filaments to 10.6 subunits/s (Fig. 5F). Filaments nucleated by Cdc12(FH2)p, which lacks the profilin binding FH1 domain, elongate at 0.26 and 0.02 subunits/s in the absence and presence of 4.0 μM SpPRF (Fig. 5, G and H). The presence of two filament populations verifies processive association of Cdc12p with the elongating barbed end. If formin rapidly came on and off, only one filament population would be detected that elongates at an intermediate rate (22, 24).

Two filament populations are also present with the CYK-1 constructs, confirming processive association with the elongating barbed end in both the absence (Figs. 5, B and I) and presence of profilin CePFN-1 (Figs. 5, C and J). CYK-1(FH1FH2)-associated filaments elongate at 6.1 and 63.2 subunits/s with and without 4.0 μM CePFN-1 (Figs. 5, I and J). Filaments nucleated by CYK-1(FH2) elongate at 5.4 and 1.6 subunits/s in the absence and presence of 4.0 μM CePFN-1 (Figs. 5, K and L).

The barbed-end elongation rate in the presence of both CYK-1(FH1FH2) and Cdc12(FH1FH2)p has a biphasic dependence on the concentration of profilin (Fig. 6G) (22). Lower profilin concentrations increase the elongation rate, with maximal effect in the range of 2.5–5.0 μM profilin. However, elongation is increasingly inhibited at higher profilin concentrations. CYK-1(FH1FH2)-associated actin filament barbed ends elongate significantly faster than Cdc12(FH1FH2)p-associated barbed ends at all profilin concentrations.

**CYK-1 Dissociates More Rapidly Than Cdc12p from the Elongating Barbed End**—In the absence of formin, all filaments visualized by total internal reflection fluorescence microscopy that assemble from a pool of 33% Oregon green-labeled Mg-ATP actin monomers elongate at a constant rate and are uniformly bright (Fig. 5A). In the presence of formin, two filament populations are detected that differ by elongation rate because formin remains processively associated with the elongating barbed end and influences actin monomer addition (Figs. 5, B and C). In the presence of formin and profilin, rapidly growing formin-associated filaments are also less bright than control filaments because profilin selects against the labeled actin (Figs. 6, A–E) (5, 22). Switches from a dim fast filament to a bright slow filament provide a convenient method to identify CYK-1 dissociation events (Fig. 6A). Kymographs of the length of individual filaments over time allow easy visualization of switches between periods of formin-dependent fast dim elongation and formin-independent bright slow elongation, apparent formin dissociation events (Figs. 6, B–D). A plot of the fraction of filaments bound to formin versus time shows that on average both Cdc12(FH1FH2)p and CYK-1(FH1FH2) allow the addition of thousands of subunits before dissociating (Fig. 6F). However, CYK-1(FH1FH2) dissociates 2 orders of magnitude more rapidly than Cdc12p: 7.1 × 10⁻⁵ s⁻¹ for Cdc12(FH1FH2)p and 3.9 × 10⁻³ s⁻¹ for CYK-1(FH1FH2).

Although CYK-1 dissociates more rapidly than Cdc12p, both formins have a low nanomolar affinity for the actin filament barbed end (Figs. 1, C and E), suggesting that the barbed end association rate constant for CYK-1 must be high. In agreement, CYK-1(FH1FH2) molecules rapidly re-associate with free actin filament barbed ends revealing filaments with multiple alternating stretches of dim fast-growing segments and bright slow-growing segments (Figs. 6, D and E).

As another measure of the dissociation rate, we also compared the ability of formins to inhibit end-to-end actin filament annealing (Figs. 6, H and I) (23, 39). Mixtures of sheared red (rhodamine-labeled) and green (Oregon green-labeled) filaments will anneal over time resulting in longer filaments that alternate between red and green segments (Fig. 6H) (23). Five min after shearing, actin filaments average 1.0 μm in length. After 60 min of annealing, control actin filaments without formin average 3.8 μm in length. Annealing is strongly inhibited by 250 nM Cdc12(FH1FH2)p in both the absence (0.96 μm) and presence of 5.0 μM profilin SpPRF (0.95 μm) (Fig. 6H). However, annealing is only partially inhibited by 250 nM CYK-1(FH1FH2) in both the absence (2.4 μm) and presence of 5.0 μM profilin CePFN-1 (2.3 μm) (Fig. 6H). A plot of the dependence of the actin filament length on the concentration of formin shows that high concentrations of CYK-1(FH1FH2) allow
FIGURE 5. Time-lapse total internal reflection fluorescence microscopy of the effect of worm CYK-1 and fission yeast Cdc12p on actin assembly. 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 NEM-myosin II. Conditions: 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM EGTA, 50 mM EDTA, 0.2 mM ATP, 50 μM CaCl2, 15 mM glucose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 0.5% (500 centipoise) methylcellulose at 25°C. Scale bar = 5 μm. A–C, time-lapse micrographs with time in seconds indicated in the bottom corner. Blue arrows and yellow arrowheads indicate barbed and pointed ends. Internal control filaments (yellow c) and formin-associated filaments (yellow f) are indicated. D–L, plots of the growth of eight individual filament barbed ends versus time for control (black) and formin-nucleated (red) filaments. The average barbed end elongation rates are indicated. D, 1.0 μM actin-only control. E, actin with 10 nM Cdc12(FH1FH2)p. F, actin with Cdc12(FH1FH2)p and 4.0 μM fission yeast profilin SpPRF. G, actin with 25 nM Cdc12(FH2)p. H, actin with Cdc12(FH2)p and SpPRF. I, actin with 50 nM CYK-1(FH1FH2). J, actin with CYK-1(FH1FH2) and 4.0 μM worm profilin CePFN-1. K, actin with 200 nM CYK-1(FH2). L, actin with CYK-1(FH2) and CePFN-1. sub/s, subunits/s.
**DISCUSSION**

By direct comparison, we discovered that the actin assembly properties are generally similar between the cytokinesis forms from the nematode worm *C. elegans* CYK-1 and fission yeast *S. pombe* Cdc12p. However, various rate constants differ significantly. Therefore, the general mechanism of contractile ring assembly in fission yeast and the nematode worm early embryo may be similar, but cells with different dimensions and physical characteristics require proteins with carefully tuned actin assembly properties.

**FIGURE 6.** Worm CYK-1 dissociates from actin filament barbed ends faster than does fission yeast Cdc12p. A–G, time-lapse total internal reflection fluorescence microscopy of the spontaneous assembly of 1.0 μM ATP-actin with 0.5 μM ATP-OG-actin on slides coated with NEM-myosin II. Conditions were the same as in Fig. 5. Scale bar = 5 μm. A and E, time-lapse micrographs of 1.0 μM actin with 50 nM CYK-1(FH1FH2) and 2.0 μM worm profilin CePFN-1, with time in seconds indicated in the bottom left corner. Blue arrows and yellow arrow heads indicate barbed and pointed ends. Red and green dots indicate formin dissociation and association events. B–D, kymographs of the length (x axis) of filaments versus time (y axis). B, filament 1 from the time-lapse shown in A, C, filament 2 from the time-lapse shown in A, D. Filament from the time-lapse micrographs shown in E, F, dependence of the percent of Cdc12(FH1FH2)p- (●) or CYK-1(FH1FH2) (○)-bound filaments on time in the presence of 2.0 μM profilin. Exponential fits indicate dissociation rates of formin from the elongating barbed end: 7.1 × 10^-5 s^-1 for Cdc12(FH1FH2)p and 3.9 × 10^-5 s^-1 for CYK-1(FH1FH2), G, dependence of the barbed-end elongation rate of Cdc12(FH1FH2)p- (●) or CYK-1(FH1FH2) (○)-associated filaments on the concentration of profilin. H and I, effect of profilin on actin filament annealing. H, merged micrographs of red and green fluorescence. Equal concentrations (0.25 μM) of red (tetramethylrhodamine)- and green (Oregon green)-labeled actin filaments were sheared through a 26-gauge needle in the absence or presence of profilin alone or formin with 5 μM profilin as indicated and allowed to anneal for 60 min before dilution and absorption to poly-L-lysine-coated coverslips. I, dependence of actin filament length on the concentration of Cdc12(FH1FH2)p- (●) or CYK-1(FH1FH2) (○) after annealing for 60 min.
~10-fold better to fission yeast actin than to muscle actin (43), Cdc12p might be able to increase the assembly rate of fission yeast actin significantly more than muscle actin in the presence of profilin.

**Nucleation Efficiency**—Given that formin FH2 domains form homodimers (44), essentially each Cdc12p homodimer initiates assembly of a new filament. CYK-1 is a significantly less efficient nucleation factor, requiring ~25 CYK-1 homodimers for every new actin filament (Fig. 2D). Profilin reduces the nucleation efficiency of all formins. Cdc12p and CYK-1 require ~25 and ~225 homodimers to produce a new actin filament in the presence of profilin-actin (Fig. 3D).

**Association with Preassembled Actin Filaments**—Both Cdc12p and CYK-1 bind to the barbed end of preassembled filaments with low nanomolar affinity (Figs. 1, C and D). Formin isoforms like CYK-1 might circumvent inefficient nucleation by binding to filaments assembled by other actin nucleation factors such as the Arp2/3 complex or Spire. The “convergent elongation model,” originally proposed to explain how vasodilator-stimulated phosphoprotein (VASP) uses Arp2/3 complex-nucleated filaments at the leading edge of motile cells to initiate the assembly of filopodia (45), might also explain the formin-dependent assembly of particular actin-based cellular structures.

Although Cdc12p- and CYK-1-associated barbed ends elongate at significantly different rates, both depolymerize ~80% slower than control filaments without formin (Figs. 1, C and E). Dissimilar elongation rates may reflect differences in the equilibrium between “closed” and “open” states as formins “walk” the elongating barbed end (22, 40, 46). Therefore, the reverse closed and open state transitions of formins during monomer dissociation may occur at similar rates. It is important to determine whether formins remain processively associated with a depolymerizing barbed end.

**Barbed End Dissociation Rate**—In vitro formins are able to facilitate the addition of thousands of actin subunits to the elongating barbed end. These filaments can be 100s of microns in length, significantly longer than typical actin filaments in cells. Therefore, formins must ultimately be turned off in cells. Formin-dependent actin assembly might be stopped by re-activated autoinhibition (15, 21), the action of other factors such as capping protein, and/or the intrinsic dissociation rate of formin. CYK-1 dissociates from the barbed end 2 orders of magnitude more quickly than Cdc12p (Fig. 6F). The intrinsic dissociation rate may be enough to turn off CYK-1, whereas other mechanisms stall Cdc12p-mediated elongation.

**Inhibition of Capping Protein**—The ability of formin to allow barbed end seeded elongation in the presence of capping protein provided the first indirect evidence that formins remain processively associated with the elongating barbed end (32, 47–49). However, capping protein can inhibit barbed ends associated with the mouse formin mDia1 (26), suggesting that capping protein may have different effects depending upon the specific formin.

CYK-1 and Cdc12p both reduce the affinity of capping protein for actin filament barbed ends ~50-fold. However, capping protein completely blocks the stimulation of spontaneous actin monomer assembly and barbed end elongation in the presence of CYK-1 but not in the presence of Cdc12p (Figs. 4, D and F). The basis for this difference is not known, but it might simply reflect the 2 orders of magnitude faster barbed-end dissociation rate of CYK-1 compared with Cdc12p. Further work is required to determine the mechanism(s) by which Cdc12p is inhibited.

**Actin Filament Bundling and Severing**—In addition to nucleation and modification of the barbed end elongation rate, several formin isoforms are capable of binding to and bundling or severing actin filaments (41, 47, 50–52). A physiological relevance for these activities has not been demonstrated, although formin-dependent cellular structures such as the contractile ring and filopodia are typically composed of bundled actin filaments. CYK-1 and Cdc12p do not bind and bundle actin filaments at any concentration, and neither CYK-1 nor Cdc12p effectively sever actin filaments at the nanomolar concentrations that are sufficient to nucleate actin filament assembly and bind preassembled actin filament barbed ends (data not shown).

**Contractile Ring Assembly in Fission Yeast and the Nematode Worm Embryo**

The fission yeast contractile ring assemblies from ~60 pre-ring nodes composed of at least seven proteins including the actin filament motor protein myosin II Myo2p (9–11) and the formin Cdc12p (13). The coordinated effort of formin Cdc12p-mediated actin filament assembly coupled with type II myosin Myo2p-mediated actin filament pulling (53) connects pre-ring nodes and drives their coalescence into a mature contractile ring (Fig. 7A) (5, 54, 55). Pre-ring node-associated Cdc12p nucleates the assembly of a filament whose pointed end is pushed away by insertional barbed end elongation. Myo2p on an adjacent node captures the actin filament pointed end, and Myo2p “walking” toward the Cdc12p-associated barbed end pulls the pre-ring nodes together (Fig. 7A) (5). Before contractile ring assembly Cdc12p has also been visualized in a larger progenitor “spot” that might also contribute to contractile ring assembly (13, 56, 57).

The mechanism of contractile ring assembly in animal cells might be similar to fission yeast. In the early nematode worm embryo, the actin motor myosin II NMY-2 localizes to discrete “foci” that coalesce into the contractile ring (Fig. 7B) (58, 59). These foci may be analogous to the fission yeast pre-ring nodes as they are connected by filamentous actin (59). Although the formin CYK-1 localizes to the cleavage furrow (30) and CYK-1 and profilin CePFN-1 are required for early steps in contractile ring assembly (29), it is not known whether CYK-1 or other contractile ring proteins localize to the myosin II foci.

The general actin assembly properties of CYK-1 and Cdc12p are similar (Figs. 1–6), suggesting that the mechanism of contractile ring assembly in the nematode worm embryo may be similar to fission yeast. Differences in specific rates between evolutionarily diverse formins such as nucleation efficiency and elongation rate might be relevant for specializing actin assembly for cells with different physical parameters.

First, the fission yeast cortical actin cytoskeleton is sparse by comparison to animal cells, necessitating the need for an
efficiency nucleation factor such as Cdc12p to initiate the assembly of “new” filaments from pre-ring nodes. The Arp2/3 complex is not necessary for contractile ring assembly in fission yeast (55, 60, 61). Conversely, the worm embryo is filled with preassembled cortical actin filaments (59). CYK-1 might associate with preassembled cortical filaments and drive contractile ring assembly by a similar mechanism as Cdc12p. The source of preassembled filaments in the worm embryo is less clear. The actin related protein Arp2/3 complex is dispensable for actin filament assembly in the early embryo (29).

Second, the circumference of the worm embryo is ~6 times longer than fission yeast, which is similar to the difference in the barbed end elongation rate of profilin-actin in the presence of Cdc12p and CYK-1 (Fig. 5; Table 1). The efficient assembly of larger contractile rings might require a faster rate of actin elongation. However, cell size and the rate of formin-mediated elongation do not always correlate. Budding yeast and fission yeast cell sizes are similar, but the budding yeast formin Bni1 mediates the assembly of filaments that elongate twice as fast as Cdc12p (22, 24). It will be interesting to assess the consequence of the rate and efficiency of contractile ring assembly by modifying the formin-dependent actin filament elongation rate in an individual cell type.

Other factors, such as the number and length of individual filaments as well as stiffness of the cortex are also relevant parameters. Interestingly, because capping protein specifically inhibits CYK-1, in the presence of high capping protein concentrations the average length of filaments assembled with CYK-1 and Cdc12p are similar (Fig. 4D).

Third, a key parameter to contractile ring assembly in fission yeast is that pre-ring nodes are pulled together by transient connections rather than long-lived connections that favor clumping over coalescence (5). Given the slow intrinsic dissociation rate of Cdc12p, other factors are likely required to terminate connections between nodes, such as the actin-severing protein coflin. The faster dissociation rate of CYK-1 might be sufficient to provide temporary connections between myosin foci during contractile ring assembly in the worm embryo (Fig. 6F).

To understand how myosin II foci coalesce, we need to determine the spatial and temporal localization of CYK-1 and other contractile ring components as well as precisely image actin filaments in real-time during contractile ring assembly in the nematode worm embryo. Live cell imaging coupled with additional biochemical characterization of the participating set of proteins will be crucial for developing testable mechanistic models of contractile ring assembly in animal cells.

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REFERENCES

1. Balasubramanian, M. K., Bi, E., and Glotzer, M. (2004) Curr. Biol. 14, 806–818
2. Glotzer, M. (2005) Science 307, 1735–1739
3. Bringmann, H., and Hyman, A. A. (2005) Nature 436, 731–734
4. Dechant, R., and Glotzer, M. (2003) Dev. Cell 4, 333–344
5. Vavylonis, D., Wu, J. Q., Hao, S., O’Shea, B., and Pollard, T. D. (2008) Science 319, 97–100
6. Bahler, J., Steever, A. B., Wheatley, S., Wang, Y., Pringle, J. R., Gould, K. L., and McCollum, D. (1998) J. Cell Biol. 143, 1603–1616
7. Paoletti, A., and Chang, F. (2000) Mol. Biol. Cell 11, 2757–2773
8. Sohrmann, M., Fankhauser, C., Brodeck, C., and Simanis, V. (1996) Genes Dev. 10, 2707–2719
9. Balasubramanian, M. K., McCollum, D., Chang, L., Wong, K. C., Naqvi, N. I., He, X., Sazer, S., and Gould, K. L. (1998) Genetics 149, 1265–1275
10. Kitayama, C., Sugimoto, A., and Yamamoto, M. (1997) J. Cell Biol. 137, 1309–1319
11. May, K. M., Watts, F. Z., Jones, N., and Hyams, J. S. (1997) Cell Motil. Cytoskeleton 38, 385–396
