Formin Differentially Utilizes Profilin Isoforms to Rapidly Assemble Actin Filaments

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Cells contain multiple formin isoforms that drive the assembly of profilin-actin for diverse processes. Given that many organisms also contain several profilin isoforms, specific formin/profilin pairs might be matched to optimally stimulate actin polymerization. We utilized a combination of bulk actin polymerization and single filament total internal reflection fluorescence microscopy assays to measure the effect of different profilin isoforms on the actin assembly properties of the cytokinesis formins from fission yeast (Cdc12p) and the nematode worm (CYK-1). We discovered that Cdc12p only effectively utilizes the single fission yeast profilin isoform SpPRF. Conversely, CYK-1 prefers the essential worm cytokinesis profilin CePFN-1 to the two non-essential worm profilin isoforms (SpPRF = CePFN-1 > CePFN-2 > CePFN-3). Chimeras containing the profilin-binding formin homology 1 (FH1) domain from one formin and the barbed-end associated FH2 domain from the other formin, revealed that both the FH1 and FH2 domains help confer profilin isoform specialization. Although the Cdc12p and CYK-1 FH1 domains cannot differentiate between profilin isoforms in the absence of actin, formin FH1 domains appear to preferentially select specific isoforms of profilin-actin. Surprisingly, analysis of profilin point mutants revealed that differences in highly conserved residues in both the poly-L-proline and actin binding regions of profilin do not explain their differential utilization by formin. Therefore, rapid formin-mediated elongation of profilin-actin depends upon favorable interactions of profilin-actin with the FH1 domain as well as the barbed-end associated FH2 domain. Specific formin FH1FH2 domains are tailored to optimally utilize actin bound to particular profilin isoforms.

Formins proteins assemble actin filaments for various cellular processes such as division, motility, establishing polarity and adhesion (1–3). The formin family is diverse with at least 20 formin genes in plants, 18 in mammals, six each in Drosophila melanogaster and Caenorhabditis elegans, three in fission yeast and two in budding yeast (4). A prevalent model is that multiple formin isoforms are necessary because they are required for disparate cellular processes. Although we do not yet know the cellular role(s) of many formins, the nematode worm C. elegans formin CYK-1 and the fission yeast Schizosaccharomyces pombe formin Cdc12p are required for cytokinesis (5–7).

The molecular basis for the functional specificity of formins is not entirely clear, but likely involves a combination of regulatory and actin assembly properties. Formins are large multidomain proteins that contain a highly conserved actin assembly formin homology 2 (FH2) domain and associated proline-rich profilin-binding FH1 domain, which are flanked on either side by regulatory domains. Some formins are regulated by auto-inhibition through association of their N- and C-terminal regulatory domains (1, 2, 8). Activated RhoGTPase binds to the N-terminal regulatory region and dissociates the C-terminal regulatory region, exposing the internal actin assembly FH1 and FH2 domains. The precise regulatory mechanism of many formins is not known, but activation at the right time and place is certainly important for functional specialization.

Upon activation, formin FH1FH2 domains stimulate actin filament nucleation and then remain processively associated with the elongating barbed-end and direct the addition of profilin-actin at significantly higher rates than a filament without formin (3). However, the mechanism of formin-mediated elongation of profilin-actin is poorly understood.

The specific actin assembly rates may be physiologically relevant, since the barbed-end elongation rate of profilin-actin differs between formin isoforms by as much as 10-fold (9–14). Some formin FH2 domains also bind to and sever and/or bundle actin filaments (15–18). Furthermore, formins associate with multiple ligands that influence their function. For example, the budding yeast cell polarity factor BUD6 binds to and enhances the actin assembly properties of the formin Bni1p, but not Bnr1p (18, 19).

Finally, because most organisms including mammals and plants contain multiple profilin isoforms, it is possible that specific formin-profilin pairs are optimally tailored for particular cellular roles. Although fission yeast contains a single profilin isoform SpPRF, the nematode worm has three differentially expressed profilins CePFN-1, CePFN-2, and CePFN-3 (20). However, the profilin isoform specificity of formin had not previously been explored.

Utilizing a combination of bulk actin polymerization assays and time-lapse evanescent wave fluorescence microscopy of single actin filaments we discovered for the first time that form-

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Ins differentially utilize specific profilin isoforms. The fission yeast cytokinesis formin Cdc12p exclusively utilizes fission yeast profilin SpPRF. On the other hand, the nematode worm cytokinesis formin CYK-1 differentially utilizes all three worm profilin isoforms (CePFN-1 > CePFN-2 > CePFN-3). CYK-1 elongates actin in the presence of the essential cytokinesis profilin CePFN-1 at ~60 subunits/s, significantly faster than in the presence of the non-essential profilin isoforms CePFN-2 (~40 subunits/s) and CePFN-3 (~15 subunits/s). Furthermore, exploration of the molecular basis of profilin isoform specificity revealed important mechanistic details of formin-mediated elongation of profilin-actin. Surprisingly, both the profilin-binding FH1 and the barbed-end associated FH2 domains of formin are important for profilin isoform specialization. We also found that although formin FH1 domains bind with similar low μM affinity to profilin, the key molecular property of the formin FH1 domain is to select specific profilin-actin complexes. Additionally, the formin FH2 domain plays a previously undetected role in the assembly of profilin-actin. The formin FH2 may make a critically important association with profilin (or the profilin-actin complex) that is necessary for either the favorable addition of profilin-actin monomers to formin-associated barbed-ends or for the subsequent dissociation of profilin from the barbed-end. Interestingly, profilin residues required for binding to either poly-l-proline or actin do not seem to dictate differential utilization by specific formin isoforms. Therefore, both the FH1 and FH2 domains contribute to rapid formin-mediated elongation of profilin-actin, and assembly of actin bound to different profilin isoforms may help tune each formin for specific cellular tasks.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Bacterial expression constructs for formin (FH1FH2) and formin (FH2) domains have been described: 1) FH1FH2<sup>Sp</sup>; pET21a-cdc12(882–1390)-HIS (11), 2) FH2<sup>Sp</sup>; pET21a-cdc12(973–1390)-HIS (12), 3) FH1FH2<sup>Ce</sup> and FH2<sup>Ce</sup>; pET21a-MBP(TEV)-cyk-1(700–1210)-HIS and pET21a-MBP(TEV)-cyk-1(802–1210)-HIS (12). Formin (FH1) bacterial expression constructs, pET21a-MBP(TEV)-cdc-12(882–973)-HIS and pET21a-MBP(TEV)-cyk-1(700–802)-HIS, were prepared by amplifying the appropriate FH1 domain (iProof, Bio-Rad) and standard cloning procedures. Formin (FH1FH2) chimera bacterial expression constructs, pGEXKT-cdc12(882–973)-cyk-1(802–1210)-HIS and pGEXKT-cyk-1(700–802):cdc12(973–1390)-HIS, were prepared by amplifying the appropriate FH1 and FH2 domains and cloning in-frame. Bacterial expression constructs for fission yeast profilin pMW172-SpPRF and nematode worm profilin isoform one pMW172-CePFN-1 have been described (12, 21). Bacterial expression constructs for nematode worm profilin isoforms two pMW172-CePFN-2 and three pMW172-CePFN-3 were prepared by amplifying from C. elegans cDNA (a generous gift from Michael Glotzer, The University of Chicago). Bacterial expression constructs for nematode worm profilin isoform three mutants (see Table 3) were prepared by overlap PCR with primers carrying the appropriate mutations. Inserts of the recombinant plasmids were sequenced to confirm fidelity of the PCR amplification and/or presence of desired mutations.

**Protein Purification**—FH1FH2<sup>Sp</sup>, FH2<sup>Sp</sup>, FH1FH2<sup>Ce</sup>, FH2<sup>Ce</sup>, mouse-capping protein, SpPRF, CePFN-1, CePFN-2, CePFN-3, and CePFN-3 mutants were purified from bacteria as described (11, 12, 20–23). Recombinant FH1<sup>Ce</sup> FH2<sup>Sp</sup> and FH1<sup>Sp</sup> FH2<sup>Ce</sup> were purified by expressing in *E. coli* strain BL21-Codon Plus (DE3)-RP (Stratagene) with 0.5 mM isopropyl β-D-thiogalactopyranoside for 6 h at 25 °C. Harvested frozen cells were resuspended in extraction buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 10 mM BME) supplemented with protease inhibitors, and homogenized in an EmulsiFlex-C3 (Avestin). The homogenate was clarified at 30,000 and 50,000 × g for 20 min each, and incubated with Talon Metal Affinity Resin (Clontech) for 1 h at 4 °C and then loaded onto a disposable column. After a 50-ml extraction buffer wash, formin was eluted with Talon elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM BME, 250 mM imidazole) and dialyzed overnight versus either Source S Buffer A (FH1<sup>Sp</sup> FH2<sup>Ce</sup>; 10 mM Heps, pH 7.2, 50 mM NaCl, 5% glycerol, 0.01% NaN<sub>3</sub>, 1 mM DTT) or Source Q Buffer A (FH1<sup>Ce</sup> FH2<sup>Sp</sup>; 20 mM Tris, pH 8.5, 50 mM NaCl, 1 mM DTT, 5% glycerol, and 0.01% NaN<sub>3</sub>) with thrombin protease to remove GST. Dialyzed protein was loaded on either a 1.0-ml Source S or Source Q column (GE Healthcare Life Sciences) and eluted with a linear gradient from 50 to 500 mM NaCl. Pure formin was dialyzed into formin buffer (20 mM Heps, pH 7.4, 1 mM EDTA, 200 mM KCl, 0.01% NaN<sub>3</sub>, and 1 mM DTT), flash-frozen in liquid nitrogen and stored at −80 °C.

Recombinant FH1<sup>Ce</sup> and FH1<sup>Sp</sup> 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 frozen cells were resuspended in extraction buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 10 mM BME) supplemented with protease inhibitors, and homogenized in an EmulsiFlex-C3 (Avestin). The homogenate was clarified at 30,000 and 50,000 × g for 20 min each, and 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 extraction buffer wash, formin (FH1) was eluted with Talon elution buffer and dialyzed overnight versus extraction buffer with 3 μM TEV protease to remove MBP. Dialyzed protein was re-incubated with and eluted from Talon Resin, and then dialyzed versus Source Q Buffer A. Dialyzed protein was loaded on a 1.0-ml Source Q column and eluted with a linear gradient from 50 to 500 mM NaCl. Pure protein was dialyzed into formin buffer (20 mM Heps, pH 7.4, 1 mM EDTA, 200 mM KCl, 0.01% NaN<sub>3</sub>, and 1 mM DTT), flash-frozen in liquid nitrogen and stored at −80 °C.

Unlabeled, Oregon Green 488-labeled, and pyrenyl iodoacetamide-labeled Ca-ATP actin were prepared from chicken muscle as described (12, 24). Immediately prior to each experiment, Ca-ATP actin was converted to Mg-ATP actin by adding 0.2 volumes of 5 mM EGTA and 0.5 mM MgCl<sub>2</sub> for 2 min at 25 °C.

Protein concentrations were determined with extinction coefficients (10, 12, 21, 23, 25, 26). Extinction coefficients for
Flow Cell for Imaging.

For spontaneous assembly, seeded assembly, and critical concentration assays, actin assembly was measured from the fluorescence of 10–20% pyrene-actin (excitation at 364 nm and emission at 407 nm) with SpectraMax Gemini XPS (Molecular Devices) and Safire2 (Tecan) fluorescent plate readers as described in detail previously (12). Final protein concentrations are indicated in the figure legends. Calculation of spontaneous polymerization rates and barbed-end affinity has been described (12).

Profilin affinity for poly-L-proline (PLP; Sigma) or formin (FH1) was determined by measuring profilin’s intrinsic tryptophan fluorescence by excitation at 295 nm and emission at 325 nm (27, 28). 5.0 or 1.0 μM profilin was incubated with either a range of PLP or formin (FH1) concentrations for 30 min, read in a Safire2 fluorescence plate reader, and plotted versus profilin concentration. The fluorescence of profilin (FH1) in the absence of profilin was measured and subtracted from the fluorescence in the presence of profilin. Plots of the dependence of the concentration of bound profilin on the concentration of PLP or formin (FH1) were fit with a quadratic function.

Microscopy of Fluorescently Labeled Filaments—Products of spontaneous assembly reactions were examined by fluorescence microscopy as described previously (10, 29). 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 (9–11, 30), a mixture of unlabeled Mg-ATP actin and 33% Mg-ATP Oregon Green actin was mixed with 2× TIRF buffer (1×: 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 50 mM DTT, 0.2 mM ATP, 50 μM CaCl2, 15 mM glucose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, and 0.5% [50 centipoise] meth-ylcellulose) and formin and/or profilin and transferred to a flow cell for imaging.

RESULTS

Formin Variously Stimulates Actin Assembly in the Presence of Different Profilin Isoforms—Because polymerization-competent actin is bound to profilin in cells, an intriguing possibility is that particular formin isoforms prefer actin bound to specific profilin isoforms. We investigated the effect of various profilin isoforms on actin assembly in the presence of Cdc12p (7) and CYK-1 (5), formin proteins required for cytokinesis in fission yeast and the nematode worm embryo. Fission yeast has a single formin SpPRF, whereas the nematode worm contains three differentially expressed profilins CePFN-1, CePFN-2, and CePFN-3 (20). CePFN-1 is required for cytokinesis of the nematode worm early embryo, while CePFN-2 and CePFN-3 are nonessential (5, 20).

In the absence of formin, all four profilin isoforms inhibit spontaneous actin filament nucleation (supplemental Fig. S1, A and B). Plots of the dependence of the actin assembly rate on the concentration of profilin reveal equilibrium dissociation constants of profilin for Mg-ATP muscle actin monomers of 0.8, 0.08, 0.08, and 0.05 μM for SpPRF, CePFN-1, CePFN-2, and CePFN-3 (supplemental Fig. S1B).

We began investigating formin/profilin isoform specialization by comparing the effect of SpPRF, CePFN-1, CePFN-2, and CePFN-3 on the rate of spontaneous actin monomer assembly in the presence of wild-type Cdc12p and CYK-1 constructs containing either both the formin homology 1 and 2 domains (Cdc12p, FH1FH2Ce; CYK-1, FH1FH2Sp) or just the FH2 domain (Cdc12p, FH2Ce; CYK-1, FH2Ce) (Fig. 1A). A range of profilin concentrations has two possible effects on formin-mediated actin monomer assembly, depending upon both the formin construct and type of profilin (Fig. 1 and supplemental Fig. S2). In the case of favorable formin/profilin pairs, lower profilin concentrations up to ~2.5 μM maximally increase the spontaneous actin assembly rate, whereas higher concentrations progressively reduce the assembly rate (Fig. 1, B and D; FH1FH2Ce with SpPRF). The biphasic effect is due to profilin enhancing the barbed-end elongation rate while inhibiting nucleation, and because excess profilin competes with profilin-actin for binding to formin (9, 10, 31). The effect with unfavorable formin/profilin pairs is monophasic, because all profilin concentrations from low to high increasingly reduce the spontaneous actin assembly rate (Fig. 1, B and D; FH1FH2Ce with CePFN-1). These different outcomes show that formins are molecularly suited for particular profilin isoforms.

First, FH2Ce, constructs lacking the profilin-binding FH1 domain, do not effectively assemble actin in the presence of any profilin isoform (supplemental Fig. S2, A and B). Second, FH1FH2Sp effectively assembles actin in the presence of fission yeast profilin but not in the presence the three worm profilins (Fig. 1, B and D). Third, FH1FH2Ce effectively assembles actin in the presence of fission yeast profilin SpPRF and worm profilins CePFN-1 and CePFN-2, but not actin in the presence of worm profilin CePFN-3 (Fig. 1, C and F).

Profilin Isoforms Differentially Enhance Fornin-mediated Barbed-end Elongation—Profilin’s contribution to formin-mediated actin monomer assembly is complicated because profilin reduces the nucleation efficiency while increasing the barbed-end elongation rate (3). Therefore, we sought to determine whether differences between favorable and unfavorable formin/profilin pairs are due to their effects on the barbed-end elongation rate and/or nucleation efficiency. We began our investigation of the elongation rate by determining the effect of profilin on the addition of Mg-ATP actin monomers to the barbed-end of pre-assembled actin filaments in the presence of wild-type formin (FH1FH2) and formin (FH2) constructs (Fig. 2 and supplemental Fig. S2).

Without profilin, FH1FH2Sp and FH2Ce reduce the seeded assembly rate by ~99%, whereas FH1FH2Ce and FH2Ce reduce the seeded assembly rate by ~50% (Fig. 2, A–D) (12). All profilin isoforms further reduce the seeded assembly rate in the
Formin and Profilin Isoform Specificity

A

Fission yeast Cdc12p

Wild-Type Cdc12p

FH1FH2<sup>Sp</sup> [882-1390]
FH2<sup>Sp</sup> [973-1390]
FH1<sup>Sp</sup> [882-972]

Nematode worm CYK-1

Wild-Type CYK-1

FH1FH2<sup>Ce</sup> [700-1210]
FH2<sup>Ce</sup> [802-1210]
FH1<sup>Ce</sup> [700-801]

Chimeras

FH1<sup>Ce</sup>FH2<sup>Sp</sup>
FH1<sup>Ce</sup>FH2<sup>Ce</sup>
FH1<sup>Ce</sup>SpFH2<sup>Ce</sup>

B

C

D

E

F

G

Visualization of Formin-mediated Actin Assembly by Time-lapse Evanescent Wave Fluorescence Microscopy—To quantify the specific barbed-end elongation rate in the presence of formin and different profilin isoforms we directly visualized actin assembly in real time by total internal reflection fluorescence (TIRF) microscopy (Fig. 3, supplemental Fig. S2). We followed individual filaments as they assemble from a mixture of unlabeled 1.0 μM Mg-ATP-actin monomers and 0.5 μM Mg-ATP-actin monomers labeled with Oregon Green (9, 11, 30). In the absence of formin, all filaments elongate their barbed-ends at the same constant rate of ~10 subunits/s both with and without profilin (Table 1) (9, 11). Two distinct populations of filaments assemble in the presence of all formin constructs, both with and without profilin (Fig. 3). The first population consists of control filaments that elongate their barbed-ends at a similar rate to filaments in the presence of formin FH2<sup>Ce</sup> with 2.5 μM worm profilin CePFN-3 (solution D). In this case, the two worm forms of profilin increase the assembly rate significantly above the rate of FH1FH2<sup>Ce</sup> alone (Fig. 2, B and D).

On the other hand, all four profilin isoforms differentially increase the seeded assembly rate of both FH1FH2<sup>Sp</sup> and FH1FH2<sup>Ce</sup> constructs (Fig. 2, A–D). Fission yeast profilin SpPRF increases the rate of FH1FH2<sup>Sp</sup>-mediated seeded assembly to near the assembly rate in the absence of formin (Fig. 2, A and C). However, the three worm profilin isoforms increase the assembly rate significantly less than SpPRF. In the presence of FH1FH2<sup>Ce</sup>, both fission yeast profilin SpPRF and worm profilin CePFN-1 increase the assembly rate significantly above the rate in the absence of formin, whereas worm profilin CePFN-2 immediately increases the assembly rate and worm profilin CePFN-3 minimally increases the rate over that of FH1FH2<sup>Ce</sup> alone (Fig. 2, B and D).

FIGURE 1. CeCYK-1 and SpCdc12p differentially stimulate spontaneous actin assembly in the presence of various profilin isoforms. The conditions were as follows: 10 mM imidazole, pH 7.0, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 90 μM CaCl<sub>2</sub>. A, wild-type and chimera FH 1 and 2 domain constructs used in this study. FH<sup>Sp</sup> domains from fission yeast Cdc12p. FH<sup>Ce</sup> domains from nematode worm CYK-1. Specific residues are indicated in brackets. Each P signifies a putative profilin binding site of at least 6 prolines within 8 residues. B–G, spontaneous actin assembly; time course of the polymerization of 2.5 μM Mg-ATP actin monomer (20% pyrene-labeled). B, plot of pyrene-fluorescence over time in the absence (○) or presence of 20 nM FH1FH2<sup>Sp</sup> (●), FH1FH2<sup>Ce</sup> with 2.5 μM fission yeast profilin SpPRF (○), and FH1FH2<sup>Ce</sup> with 2.5 μM worm profilin CePFN-1 (□), C, plot of pyrene-fluorescence over time in the absence (○) or presence of 20 nM FH1FH2<sup>Sp</sup> (●), FH1FH2<sup>Ce</sup> with 2.5 μM worm profilin CePFN-1 (□), and FH1FH2<sup>Ce</sup> with 2.5 μM worm profilin CePFN-3 (△). D–G, plots of the dependence of the actin assembly rate (slope) on the concentration of SpPRF (○), CePFN-1 (□), CePFN-2 (○), or CePFN-3 (△). E, 20 nM FH1FH2<sup>Sp</sup>, F, 20 nM FH1FH2<sup>Ce</sup>, G, 200 nM FH1FH2<sup>Sp</sup> and FH1FH2<sup>Ce</sup>, respectively.
Formin and Profilin Isoform Specificity

absence of formin. The second population consists of formin-nucleated filaments, which grow at a different rate than control filaments because formin remains processively associated with the elongating barbed-end and modifies the elongation rate (9, 11). The specific elongation rate of formin-associated filaments depends upon both the type of formin and profilin (Fig. 3 and Table 1).

In the absence of profilin, FH1FH2Sp and FH2Sp-associated filaments grow at only ~0.3 subunits/s (Fig. 3G and supplemental Fig. S2E and Table 1) (9, 11), whereas FH1FH2Ce and FH2Ce-associated filaments grow at 6.2 and 4.8 subunits/s (Fig. 3, A and I, and supplemental Fig. S2F, Table 1) (12). With either FH2Sp or FH2Ce, constructs lacking the profilin-binding FH1 domain, all four profilin isoforms further reduce the barbed-end elongation rate (supplemental Fig. S2, E and F and Table 1).

With both FH1FH2Sp and FH1FH2Ce all four profilin isoforms differentially increase the barbed-end elongation rate (Fig. 3, C–G and I and Table 1). With FH1FH2Sp, 4.0 μM fission yeast profilin SpPRF increases the barbed-end elongation rate ~45-fold, whereas the three worm profilins CePFN-1, CePFN-2, and CePFN-3 increase the elongation rate by only ~4-, ~6-, and ~8-fold (Fig. 3G and Table 1). With FH1FH2Ce, 4.0 μM fission yeast profilin SpPRF increases the barbed-end elongation rate ~10-fold, whereas the three worm profilins CePFN-1, CePFN-2, and CePFN-3 differentially increase the elongation rate by ~10-, ~6.5-, and ~2.0-fold (Fig. 3, C–F and I and Table 1).

Differences in the FH1FH2Ce-mediated elongation rate occur over a large range of worm profilin concentrations (Fig. 4A). A plot of the fraction of filaments bound to formin over time show that FH1FH2Ce allows the addition of thousands of subunits before dissociating in the presence of all three worm profilins (Fig. 4C). Therefore, the rate of FH1FH2Ce dissociation is not affected by the elongation rate, which argues against the model that the dissociation rate increases proportionally with the elongation rate due to formin rapidly transitioning between closed and open states as has been proposed (9, 13).

CePFN-3 Does Not Poison CYK-1-mediated Assembly of Actin Bound to CePFN-1—The pathway for formin-mediated assembly of actin bound to profilin is complex (Fig. 6B). Proli-
lin-actin binds to the formin FH1 domain, is transferred to the FH2-associated barbed-end, and then profilin must dissociate from the barbed-end for subsequent rounds of actin monomer addition. To begin to investigate the molecular basis for profilin isoform specificity, as well as the general mechanism of formin-mediated assembly of profilin-actin, we tested the effects of equal mixtures of a favorable (CePFN-1) and unfavorable (CePFN-3) profilin on actin filament assembly in the presence of FH1FH2Ce. We found that equal mixtures of CePFN-1 and CePFN-3 intermediately increase both the spontaneous assembly of actin monomers, as well as the specific barbed-end elongation rate in the presence of FH1FH2Ce (Fig. 4, A and B). Therefore, CePFN-3 does not inhibit FH1FH2Ce-mediated assembly by irreversibly blocking one of the steps in the pathway, which would lead to a dominant negative inhibition of CePFN-1. CePFN-1 and CePFN-3 likely have different rate constants for all or some of the reactions along the pathway.

The Specificity of Worm Profilin Isoforms Is Not Determined by Their Affinity for the CYK-1 FH1 Domain—To ascertain important mechanistic insights into formin-mediated assembly of profilin-actin we continued to explore the basis for profilin isoform specialization. Given that the FH1 domain binds directly to profilin (7, 32), we measured the affinity of the fission yeast and worm profilin isoforms for the Cdc12p and CYK-1 FH1 domains (FH1Sp and FH1Ce; supplemental Fig. S3). We predicted that profilin/FH1 domain pairs that favorably stimulate actin assembly (such as CYK-1 with CePFN-1) bind more tightly than unfavorable pairs (such as CYK-1 with CePFN-3).

We initially determined the affinity of each profilin for poly-L-proline (PLP), a non-physiological ligand that is widely utilized to purify profilin and to compare the association of various profilin isoforms with proline-rich ligands (27, 28). The affinity for PLP was determined by measuring the increase in profilin intrinsic tryptophan fluorescence over a range of PLP concentrations, revealing equilibrium dissociation constants of 46, 653, 330, and 333 μM proline residues for SpPRF, CePFN-1,
TABLE 1
Comparison of Mg-ATP actin assembly rates in the presence of formin

| Conditions* | Barbed-end subunits/s | Conditions* | Barbed-end subunits/s |
|-------------|------------------------|-------------|------------------------|
| 1 μM Actin only | 9.7 ± 0.2              | FH1FH2<sup>c</sup> | 6.2 ± 0.1 (9.5 ± 0.2)<sup>c</sup> |
| 1 μM Actin + 4 μM SpPRF | 9.4 ± 0.3              | FH1FH2<sup>c</sup> + 4 μM SpPRF | 59.3 ± 0.7 (8.0 ± 0.1) |
| 1 μM Actin + 4 μM CePFN-1 | 9.6 ± 0.2              | FH1FH2<sup>c</sup> + 4 μM CePFN-1 | 61.8 ± 0.6 (8.1 ± 0.2) |
| 1 μM Actin + 4 μM CePFN-2 | 9.3 ± 0.1              | FH1FH2<sup>c</sup> + 4 μM CePFN-2 | 41.3 ± 0.6 (9.2 ± 0.2) |
| 1 μM Actin + 4 μM CePFN-3 | 9.2 ± 0.2              | FH1FH2<sup>c</sup> + 4 μM CePFN-3 | 14.2 ± 0.3 (8.8 ± 0.2) |

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

** Rates for Cdc12p are the sum of rates from both the barbed and pointed ends.

The rates of internal control filaments are reported in parentheses.

CePFN-2, and CePFN-3 (supplemental Fig. S3) were used in these experiments. Assuming profilin binds to ~10 contiguous proline residues (28), our experiments thus support the equilibrium dissociation constants of low to medium micromolar.

FH1<sup>c</sup> and FH1<sup>c</sup> constructs (supplemental Fig. S3A) also increase profilin’s intrinsic tryptophan fluorescence, revealing equilibrium dissociation constants in the low micromolar range (supplemental Fig. S3, C and D and Table 2). For FH1<sup>c</sup>, equilibrium dissociation constants are 3.4, 14.7, 17.8, and 16.4 μM with SpPRF, CePFN-1, CePFN-2, and CePFN-3. For FH1<sup>c</sup>, equilibrium dissociation constants are 1.7, 4.4, 5.8, and 4.8 μM with SpPRF, CePFN-1, CePFN-2, and CePFN-3. These binding constants are similar to the affinity of mouse profilin 2A and a mouse formin mDia1 FH1 peptide (32). FH1<sup>c</sup> contains six profilin-binding proline rich stretches whereas FH1<sup>c</sup> only contains two (supplemental Fig. S3A), possibly explaining why each profilin has an ~3-fold high affinity for FH1<sup>c</sup>. The severalfold higher affinity of SpPRF for both formin FH1 domains might explain why SpPRF works well with both formin isoforms. However, since all three worm profilin isoforms bind similarly to FH1<sup>c</sup>, the affinity of profilin for the FH1 domain cannot independently explain formin/profilin specialization.

Formin (FH1) Constructs Differentially Inhibit Formin (FH1FH2)-mediated Actin Assembly—Although formin FH1 domains bind with similar affinity to different profilin isoforms in the absence of actin, it is possible that formin FH1 domains select actin that is bound to particular profilin isoforms. To indirectly test this possibility we examined the effect of adding exogenous formin (FH1) to actin filament assembly assays in

** FIGURE 4. Effect of profilin concentration on formin-mediated actin assembly.** A, time-lapse evanescent wave microscopy of 1.0 μM Mg-ATP actin with 0.5 μM Mg-ATP OG-actin. Dependence of the barbed-end elongation rate of FH1FH2<sup>c</sup>-associated filaments on the concentration of worm profilin isoforms CePFN-1 (○), CePFN-2 (□), CePFN-3 (■), or an equal mixture between CePFN-1 and CePFN-3 (●). B, dependence of the percent of FH1FH2<sup>c</sup>-associated filaments on time, in the presence of 20 μM Mg-ATP CePFN-1 (○), CePFN-2 (□), or CePFN-3 (■), as determined by time-lapse evanescent wave microscopy. Exponential fits indicate dissociation rates of FH1FH2<sup>c</sup> from the elongating barbed-end: 8.3 × 10<sup>−5</sup> s<sup>−1</sup> for CePFN-1, 1.5 × 10<sup>−5</sup> s<sup>−1</sup> for CePFN-2, and 1.1 × 10<sup>−5</sup> s<sup>−1</sup> for CePFN-3. C, CePFN-2, and CePFN-3 (supplemental Fig. S3A and Table 2). Assuming profilin binds to ~10 contiguous proline residues (28), these results correspond to equilibrium dissociation constants of low to medium micromolar.

FH1<sup>v</sup> and FH1<sup>c</sup> constructs (supplemental Fig. S3A) also increase profilin’s intrinsic tryptophan fluorescence, revealing equilibrium dissociation constants in the low micromolar range (supplemental Fig. S3, C and D and Table 2). For FH1<sup>v</sup>, equilibrium dissociation constants are 3.4, 14.7, 17.8, and 16.4 μM with SpPRF, CePFN-1, CePFN-2, and CePFN-3. For FH1<sup>c</sup>, equilibrium dissociation constants are 1.7, 4.4, 5.8, and 4.8 μM with SpPRF, CePFN-1, CePFN-2, and CePFN-3. These binding constants are similar to the affinity of mouse profilin 2A and a mouse formin mDia1 FH1 peptide (32). FH1<sup>c</sup> contains six profilin-binding proline rich stretches whereas FH1<sup>v</sup> only contains two (supplemental Fig. S3A), possibly explaining why each profilin has an ~3-fold high affinity for FH1<sup>c</sup>. The severalfold higher affinity of SpPRF for both formin FH1 domains might explain why SpPRF works well with both formin isoforms. However, since all three worm profilin isoforms bind similarly to FH1<sup>c</sup>, the affinity of profilin for the FH1 domain cannot independently explain formin/profilin specialization.

Formin (FH1) Constructs Differentially Inhibit Formin (FH1FH2)-mediated Actin Assembly—Although formin FH1 domains bind with similar affinity to different profilin isoforms in the absence of actin, it is possible that formin FH1 domains select actin that is bound to particular profilin isoforms. To indirectly test this possibility we examined the effect of adding exogenous formin (FH1) to actin filament assembly assays in

** FIGURE 3. Visualization of formin-mediated actin assembly by time-lapse evanescent wave fluorescence microscopy.** 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 MgCl<sub>2</sub>, 1 mm EGTA, 50 mm DTT, 0.2 mm ATP, 50 μM CaCl<sub>2</sub>, 15 mm glucose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 0.5% (500 cP) methylcellulose at 25°C. Scale bar, 5 μm. A, C, E, time-lapse micrographs with time in seconds indicated at the bottom. Blue arrows and yellow arrowheads indicate barbed and pointed ends. Internal control filaments (yellow c) and formin-associated filaments (yellow f) are indicated. B, D, F, plots of the growth of eight individual filament barbed-ends versus time for control (black) and formin-associated (red) filaments. The average barbed-end elongation rates are indicated. A–B, 50 nM FH1FH2<sup>c</sup> (C–D, FH1FH2<sup>c</sup> with 4 μM worm profilin CePFN-3 (E–F, FH1FH2<sup>c</sup> with 4 μM CePFN-1. G–J, bar graphs of average barbed-end elongation rates for control (black), formin only (red), formin with SpPRF (blue), formin with CePFN-1 (green), formin with CePFN-2 (brown), formin with CePFN-3 (pink). G, 10 nM FH1FH2<sup>c</sup> with 4 μM profilin. H, 150 nM FH1FH2<sup>c</sup> with 4 μM profilin. I, 50 nM FH1FH2<sup>c</sup> with 4 μM profilin. J, 250 nM FH1FH2<sup>c</sup> with 4 μM profilin.
the presence of formin (FH1FH2) and formin (FH2) constructs (Fig. 5).

First, formin FH1 and FH2 domains cannot work in trans, because FH1<sup>Sp</sup> and FH1<sup>Ce</sup> do not increase the rate of spontaneous actin assembly in the presence of either FH2<sup>Sp</sup> with SpPRF or FH2<sup>Ce</sup> with CePFN-1 (Fig. 5C). This is an important substantiation of the direct transfer model for formin-mediated assembly of profilin-actin (Fig. 6B) (31), which requires that the FH1 and FH2 domains must be covalently attached. As expected, FH1<sup>Sp</sup> and FH1<sup>Ce</sup> have little effect on the spontaneous assembly of actin alone, actin with profilin, and actin with either FH2<sup>Sp</sup> or FH2<sup>Ce</sup> alone (data not shown).

Neither FH1<sup>Sp</sup> nor FH1<sup>Ce</sup> alter FH1FH2<sup>Sp</sup>- or FH1FH2<sup>Ce</sup>-mediated spontaneous actin assembly in the absence of profilin (Fig. 5, A and B). However, FH1<sup>Sp</sup> inhibits both FH1FH2<sup>Sp</sup>-mediated spontaneous actin monomer assembly and barbed-end elongation in the presence of SpPRF (Fig. 5, A, C, and D). Similarly, FH1<sup>Ce</sup> inhibits both FH1FH2<sup>Ce</sup>-mediated spontaneous actin monomer assembly and barbed-end elongation in the presence of CePFN-1 (Fig. 5, B–D). Exogenous formin FH1 domain most likely inhibits formin FH1FH2-mediated actin assembly through binding and sequestering profilin-actin complexes. Surprisingly, FH1FH2<sup>Sp</sup>-mediated spontaneous assembly of SpPRF-actin is more effectively inhibited by FH1<sup>Sp</sup> than by FH1<sup>Ce</sup> (Fig. 5E). Conversely, FH1FH2<sup>Ce</sup>-mediated spontaneous assembly of CePFN-1-actin is more effectively inhibited by FH1<sup>Ce</sup> than by FH1<sup>Sp</sup> (Fig. 5F). Although other interpretations are possible, these results are consistent with the hypothesis that formin FH1 domains are tailored to preferentially bind specific profilin-actin complexes, FH1<sup>Ce</sup> to CePFN-1-actin and FH1<sup>Sp</sup> to SpPRF-actin.

In the Absence of Profilin the FH2 Domain Determines Formin Actin Assembly Properties—To test whether the FH2 domain also contributes to profilin isoform specificity, we constructed chimeras between Cdc12p and CYK-1 (Fig. 1A). Chimeric formin (FH1FH2) constructs, containing the FH1 domain from one formin and the FH2 domain from the second formin, are not physiologically relevant but allowed us to explore the molecular contribution of the FH1 and FH2 domains to formin-mediated assembly of actin-actin.

Constructions containing either the FH1 domain from Cdc12p and the FH2 domain from CYK-1 ( FH1<sup>Sp</sup> and FH2<sup>Ce</sup>), or the FH1 domain from CYK-1 and the FH2 domain from Cdc12p ( FH1<sup>Ce</sup> and FH2<sup>Sp</sup>) are functional. FH1<sup>Sp</sup> and FH1<sup>Ce</sup> increase the spontaneous assembly of Mg-ATP actin monomers similarly to wild-type FH1FH2<sup>Sp</sup> and FH1FH2<sup>Ce</sup> (supplemental Fig. S4, A and B). As expected, in the absence of profilin the actin assembly properties of the chimera constructs follow the FH2 domain; FH1<sup>Ce</sup> FH2<sup>Sp</sup> behaves like wild-type FH1FH2<sup>Sp</sup> and FH1<sup>Sp</sup> FH2<sup>Ce</sup> behaves like wild-type FH1FH2<sup>Ce</sup> (supplemental Fig. S4). Conversely, FH1FH2<sup>Sp</sup> and FH1FH2<sup>Ce</sup> is an efficient nucleation factor, increases the critical concentration for actin assembly, and binds to pre-assembled actin filament barbed-ends with high affinity and inhibits the elongation rate by ~99%. Like wild-type FH1FH2<sup>Ce</sup>, FH1<sup>Sp</sup>FH2<sup>Ce</sup> is an inefficient nucleation factor, does not increase the critical concentration for actin assembly, and binds to pre-assembled actin filament barbed-ends with high affinity and inhibits the elongation rate by ~50%.

Both the FH1 and FH2 Domains Contribute to Profilin Isoform Specificity—Because formin (FH2) constructs inhibit the assembly of profilin-actin (supplemental Fig. S2), and the FH1 domain binds directly to profilin (supplemental Fig. S3 and Table 2), the simplest hypothesis is that preference of formin (FH1FH2) constructs for particular profilin isoforms depends exclusively on the FH1 domain. However, it is important to explore the possibility that the formin FH2 domain contributes to profilin isoform specificity, and therefore to formin-mediated assembly of profilin-actin in general. We tested the effect of the various profilin isoforms on the ability of the formin chimeras to: 1) stimulate spontaneous assembly of Mg-ATP actin monomers (Fig. 1), 2) increase the addition rate of Mg-ATP actin monomers to pre-assembled filament seeds (Fig. 2), and 3) increase the specific barbed-end elongation rate by directly observing actin filament assembly with time-lapse evanescent wave fluorescence microscopy (Fig. 3 and Table 1). For these experiments, fission yeast profilin SpPRF provided a convenient positive control because both wild-type FH1FH2<sup>Sp</sup> and FH1FH2<sup>Ce</sup> effectively assemble actin in the presence of SpPRF.

Wild-type fission yeast FH1FH2<sup>Sp</sup> only works well with fission yeast SpPRF, whereas wild-type nematode worm FH1FH2<sup>Ce</sup> differentially utilizes all three worm profilin isoforms (qualitatively summarized in Fig. 6A). We hypothesized that if the FH2 domain is not important for formin-mediated assembly of profilin-actin, the ability of each formin chimera construct to use various profilin isoforms should follow the FH1 domain. To our surprise, we discovered that profilin utilization depends upon both the FH1 and FH2 domains because the use of the various profilin isoforms for each chimera construct is intermediate between the parent wild-type constructs (qualitatively summarized in Fig. 6A).

**TABLE 2**

| Profilin | Proline residues (PLP) | FH1<sup>Sp</sup> | FH1<sup>Ce</sup> |
|----------|------------------------|-----------------|-----------------|
| SpPRF    | 46 ± 4.6<sup>a</sup>   | 3.4 ± 0.8       | 1.7 ± 0.3       |
| CePFN-1  | 653 ± 12.8             | 14.7 ± 1.3      | 4.4 ± 0.8       |
| CePFN-2  | 330 ± 9.5              | 17.8 ± 1.8      | 5.8 ± 1.1       |
| CePFN-3  | 353 ± 10.7             | 16.4 ± 1.1      | 4.8 ± 0.6       |

<sup>a</sup> Determined by the increase in the profilin intrinsic tryptophan fluorescence upon binding to proline-rich ligands (27, 28), as shown in supplemental Fig. S3.

<sup>b</sup> Equilibrium dissociation constants (K<sub>d</sub>) are the average of two independent experiments.
Formin and Profilin Isoform Specificity

FIGURE 5. Inhibition of formin (FH1FH2)-mediated assembly by exogenous formin (FH1). A–C, spontaneous assembly of 2.0 μM Mg-ATP actin (20% pyrene-labeled). A, plot of pyrene-fluorescence over time in the absence (thick curve) or presence of either 10 nM FH1FH2Sp (○), FH1FH2Sp with 30 μM FH1Sp (●), FH1FH2Sp with 2.0 μM SpPF (□), or FH1FH2Sp + SpPF and FH1Sp (■). B, plot of pyrene-fluorescence over time in the absence (thick curve) or presence of either 25 nM FH1FH2CeSp (○), FH1FH2CeSp with 30 μM FH1Ce (●), FH1FH2CeSp with 2.0 μM CePFN-1 (■), or FH1FH2CeSp + CePFN-1 and FH1Ce (□). C, plot of the dependence of the actin assembly rate (slope) on the concentration of exogenous formin (FH1), for either FH1FH2Sp and SpPF with FH1Sp (○), FH2Sp and SpPF with FH1Sp (●), FH1FH2CeSp and CePFN-1 with FH1Ce (●), or FH2CeSp with CePFN-1 and FH1Ce (□). D, dependence of the barbed-end elongation rate of formin (FH1FH2)-associated filaments on the concentration of formin (FH1), as determined by time-lapse evanescent wave fluorescent microscopy of 1.0 μM Mg-ATP actin with 0.5 μM Mg-ATP G-actin: 50 nM FH1FH2Sp and 2.0 μM CePFN-1 with FH1Ce (●), 10 nM FH1FH2Sp and 2.0 μM SpPF with FH1Sp (○). The inset shows the plot of FH1FH2Sp and SpPF (□) with a focused Y-axis. E–F, spontaneous assembly of 2.0 μM Mg-ATP actin (20% pyrene-labeled). E, dependence of the actin assembly rate (slope) of 25 nM FH1FH2Sp with 2.0 μM SpPF on the concentration of either FH1Sp (●) or FH1Ce (○). F, dependence of the actin assembly rate (slope) of 25 nM FH1FH2CeSp with 2.0 μM CePFN-1 on the concentration of either FH1Ce (●) or FH1Sp (○).

With wild-type FH1FH2Ce, fission yeast SpPF, and worm CePFN-1 increase the elongation rate ~15-fold, whereas CePFN-2 (~6-fold) and CePFN-3 (~8-fold) increases elongation significantly less (Fig. 3G and Table 1). In addition, the worm CYK-1 FH2 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1). Therefore, the CYK-1 FH1 domain to the Cdc12p FH2 domain in the chimera FH1CeFH2Sp has little affect on the impact of SpPF (~40-fold) over formin (FH1FH2CeSp (~4-fold), CePFN-2 (~10-fold), and CePFN-3 (~8-fold) increase elongation significantly less (Fig. 3G and Table 1).
Formin and Profilin Isoform Specificity

A, qualitative score card from the quantitative data in Figs. 1–3 of the ability of wild-type and chimera formin constructs to utilize actin bound to various profilin isoforms. B, schematic model for the addition of profilin-actin to a formin-associated actin filament barbed-end (31). 8(II), profilin-actin complexes bind to a proline-rich stretch in the FH1 domain (o). It is less likely that actin binds to profilin pre-associated with the FH1 domain (a’), because the formin FH1 domain binds similarly to all profilins but appears to select specific profilin-actin complexes. 8(II), FH1-bound profilin-actin associates with the actin filament barbed-end, which includes a previously undetected favorable interaction between profilin or profilin-actin and the FH2 domain (dashed green circle). 8(II), profilin must dissociate from the barbed-end for subsequent rounds of elongation. 8(II), profilin might dissociate from the barbed-end first and then from the FH1 domain. 8(II), profilin might detach from the FH1 domain first and then from the barbed-end. A favorable interaction between profilin and the FH2 domain could also be necessary to dissociate profilin from the elongating barbed-end (dashed brown circle). Finally, profilin residues outside the poly-L-proline (formin)- and actin-binding regions, and/or the profilin overall fold, play a key role in formin-mediated assembly of profilin actin and profilin isoform specificity.

because the Cdc12p FH1 domain has four fewer profilin-binding sites than the CYK-1 FH1 domain (supplemental Fig. S3A), all four profilin isoforms do not increase the elongation rate of barbed-ends associated with the chimera FH1<sup>Sp</sup>FH2<sup>Ce</sup> as much as with wild-type FH1FH2<sup>Ce</sup> (Fig. 3/ and Table 1). However, just as with wild-type FH1FH2<sup>Ce</sup>, the elongation rate of FH1<sup>Sp</sup>FH2<sup>Ce</sup>-associated barbed-ends is variably increased by SpPRF (9-4-fold), CePFN-1 (9-2.5-fold), CePFN-2 (1.5-fold), and CePFN-3 (no fold increase) (Fig. 3/ and Table 1). Therefore, the CYK-1 FH2 domain contributes significantly to worm profilin specificity (CePFN-1 > CePFN-2 > CePFN-3).

Profilin Poly-L-proline (PLP)- and Actin-binding Regions Do Not Intrinsically Confer Utilization by Specific Formin Isoforms—Formin/profilin isoform specialization likely requires that both the formin and profilin isoform be optimized. From the formin side we established that both the profilin-binding FH1 domain and the barbed-end associated FH2 domain are important for driving the assembly of profilin-actin in a profilin isoform-dependent manner. We next began to explore whether specific profilin residues are important for utilization by particular formin isoforms.

We aligned profilin sequences to look for residues that might explain why worm profilin CePFN-3 does not work well with the worm cytokinesis formin CYK-1 (supplemental Fig. S5). Worm profilin CePFN-1 and CePFN-2 are more identical to each other (58%) than to worm profilin CePFN-3 (27 and 31%) or fission yeast profilin SpPRF (33 and 32%). CePFN-3 is 34% identical to SpPRF. Despite the low sequence similarity with other profilins, homology modeling with the crystal structure of <i>Acanthamoeba castellanii</i> profilin IA as a template suggest that CePFN-1, CePFN-2, and CePFN-3 form similar folds as other profilins (20). Numerous residues important for ligand-specific interactions have been identified in the fission yeast profilin SpPRF (supplemental Fig. S5) (21). Interestingly, several residues in both the PLP-binding region (SpPRF Y6 and L122) and actin-binding region (SpPRF Y69, R73) that are absolutely conserved between CePFN-1, CePFN-2, and SpPRF, are different in the CePFN-3 sequence (supplemental Fig. S5). These differences do not hinder the ability of CePFN-3 to bind actin or PLP (supplemental Figs. S1 and S3 and Table 2), but could account for the inefficient utilization of CePFN-3 by worm formin CYK-1.

We tested this possibility by attempting to turn CePFN-3 into CePFN-1 by substituting several residues. We made an entire set of individual and combinatorial CePFN-3 correction substitutions in the N-terminal PLP-binding region (CePFN-3<sup>PLP-N</sup>; 16Y), C-terminal PLP-binding region (CePFN-3<sup>PLP-C</sup>; F121L), and actin-binding region (CePFN-3<sup>A</sup>; F68Y, N72R). As expected, the substitution mutations had little impact on the ability of CePFN-3 to bind actin or the CYK-1 FH1 domain (Table 3). However, as determined by both spontaneous actin monomer assembly and direct visualization of the barbed-end elongation rate by TIRF microscopy (supplemental Fig. S6 and Table 3), none of the CePFN-3 substitution mutants increased utilization by FH1FH2<sup>Ce</sup>. CePFN-3 proteins carrying substitutions in the PLP-binding regions were indistinguishable from wild-type CePFN-3. CePFN-3 proteins carrying substitutions in the actin-binding region were utilized slightly less efficiently than wild type. Therefore, the basis for differential utilization

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**Table 3** Comparison of CePFN-3 mutants

| Profilin<sup>a</sup> | FH1FH2<sup>Ce</sup> Elongation rate | Mg-ATP Actin, K<sub>MD</sub> | PLP K<sub>MD</sub> | FH1<sup>Ce</sup> K<sub>d</sub> |
|---------------------|-----------------------------------|----------------------|-------------------|-------------------|
| CePFN-1             | 57.4 ± 0.8                        | 0.08 ± 0.05          | 564 ± 378         | 2.1 ± 0.7         |
| CePFN-3             | 16.8 ± 0.3                        | 0.07 ± 0.06          | 415 ± 25          | 2.9 ± 0.7         |
| CePFN-3<sup>PLP-N</sup> | 16.0 ± 0.4                           | 0.05 ± 0.03          | 903 ± 74          | 0.9 ± 0.5         |
| CePFN-3<sup>PLP-C</sup> | 16.1 ± 0.4                           | 0.09 ± 0.07          | 454 ± 35          | 3.9 ± 1.9         |
| CePFN-3<sup>PLP-N,PLP-C</sup> | 15.2 ± 0.3                          | 0.10 ± 0.06          | 1,085 ± 85        | 4.0 ± 2.0         |
| CePFN-3<sup>A</sup> | 10.1 ± 0.2                        | 0.09 ± 0.04          | 355 ± 26          | 2.3 ± 0.7         |
| CePFN-3<sup>PLP-N,A</sup> | 11.4 ± 0.3                           | 0.07 ± 0.05          | 899 ± 56          | 1.7 ± 1.3         |
| CePFN-3<sup>PLP-C</sup> | 9.9 ± 0.9                           | 0.07 ± 0.07          | 310 ± 20          | 4.0 ± 3.0         |
| CePFN-3<sup>PLP-N,PLP-C</sup> | 12.6 ± 0.5                         | 0.13 ± 0.06          | 1,070 ± 93        | 3.1 ± 2.4         |

<sup>a</sup> Abbreviations indicate mutations in the CePFN-3 N-terminal PLP-binding region (16Y), C-terminal PLP-binding region (CePFN-3<sup>PLP-C</sup>; F121L), or actin-binding region (F68Y, N72R).
by CYK-1 is not determined by the most obvious ligand-binding residues on profilin (PLP and actin). It is possible that poorly conserved residues outside the PLP- and actin-binding regions preclude utilization of CePFN-3 by CYK-1 (see supplemental Fig. S5 and the discussion below). Differences in the profilin overall fold may also be crucial.

**DISCUSSION**

Formin proteins are exceptional molecules that remain processively associated with the elongating actin filament barbed-end while driving the rapid addition of profilin-actin. The mechanism by which formin transfers profilin-actin complexes to the elongating barbed-end is not well understood. Furthermore, although diverse formin isoforms are required for specific cellular tasks (1, 2), the mechanism for functional specialization is not clear. For the first time we discovered that formins drive the assembly of actin bound to diverse profilin isoforms by significantly different rates. Fission yeast formin Cdc12p works well only with fission yeast profilin. Nematode worm formin CYK-1 works well with either fission yeast profilin or the essential nematode worm profilin 1, but to a lesser extent with nematode worm profilin 2 and even less with nematode worm profilin 3. Utilization of specific profilin isoforms may therefore be an important mechanism for specializing formins for particular cellular processes.

Importantly, investigation of the molecular basis for profilin specificity revealed novel details of the basic mechanism(s) of formin-mediated assembly of profilin-actin. We found that despite their differential utilization by CYK-1, the three nematode worm profilin isoforms bind equally well to the CYK-1 proline-rich FH1 domain with low μM affinity. However, diverse formin FH1 domains differentially inhibit formin (FH1FH2)-mediated assembly of profilin-actin, suggesting that the FH1 domain is able to select specific profilin-actin isoforms. We also discovered that both the formin FH1 and FH2 domains are important for profilin specificity, which reveals a surprising and previously undetected role for the barbed-end associated FH2 domain in formin-mediated assembly of profilin-actin. Furthermore, differences in conserved residues within profilin PLP- and actin-binding regions do not account for differential utilization by formin. Therefore, either there are key residues outside the PLP- and actin-binding regions, or the overall fold of profilin is important for formin-mediated assembly of profilin-actin and profilin isoform specificity.

**Mechanism of Formin-mediated Assembly of Profilin-Actin**—Based on experiment and theory, the direct transfer mechanism has been proposed to explain formin-mediated elongation of profilin-actin (31) (Fig. 6B). Profilin-actin binds to proline-rich stretches in the formin FH1 domain, or actin binds to profilin pre-associated with the FH1 domain. FH1-bound profilin-actin then attaches to the FH2 domain-associated elongating barbed-end. Following addition of the actin monomer to the filament, profilin must dissociate prior to additional rounds of assembly. We validated the direct transfer hypothesis by showing that formin FH1 and FH2 domains cannot work in trans (Fig. 5). However, most of the pathway steps have not been experimentally tested.

Investigation of the basis for differential profilin utilization allowed us to explore individual reactions along the direct transfer pathway. Unfavorable profilins do not act as dominant negative inhibitors of favorable profilins (Fig. 4). Therefore, the general pathway for FH1 domain-dependent delivery of profilin-actin is similar between different profilins, but the specific rate constants vary.

First, our experiments support a scheme where new profilin-actin complexes are required for each round of addition (Fig. 6B, a). It is not likely that an individual profilin molecule remains continually associated with the formin FH1 domain and repeatedly transfers multiple actin molecules to the barbed-end (Fig. 6B, a'). The role of the FH1 domain is to differentially select specific profilin-actin complexes. This model is consistent with our discovery that the three worm profilin isoforms bind similarly to the CYK-1 FH1 domain (supplemental Fig. S3 and Table 2), whereas exogenously added FH1Ce and FH1Sp differentially inhibit formin-mediated actin assembly in the presence of fission yeast profilin and worm profilin CePFN-1 (Fig. 5).

Second, the prevailing model that the role of the FH2 domain is restricted to nucleation and processive barbed-end association is incorrect. Our investigation demonstrates for the first time that the FH2 domain also contributes significantly to the addition of profilin-actin to the elongating barbed-end. Observations of formin (FH1FH2)- and formin (FH2)-associated actin filaments in the presence and absence of profilin have indicated that the FH2 domain influences the elongation rate of profilin-actin (Table 1) (9). In the absence of formin, actin and profilin-actin add at nearly the same rate to free barbed-ends (Table 1; \( \sim 10 \) subunits s\(^{-1} \) μM\(^{-1} \)). However, profilin-actin adds at least 3-fold more slowly than actin to formin (FH2)-associated actin filament barbed-ends (Table 1) (9). Therefore, the FH2 domain must alter the barbed-end association and addition of profilin-actin. Conversely, profilin-actin adds significantly faster than actin to formin (FH1FH2)-associated actin filament barbed-ends (Table 1) (9). The FH1 domain binds profilin-actin and is thought to increase the local actin concentration and steer actin to the barbed-end (9, 13, 14, 31). Additionally, our findings suggest that the FH2 domain allows favorable barbed-end association and addition of profilin-actin, and that particular FH2 domains are favorably paired with specific profilin isoforms.

Determining the specific role of the FH2 domain in profilin-actin elongation will require further investigation. Our hypothesis is that delivery of profilin-actin to the FH1FH2-associated actin filament barbed-end involves an important interaction of profilin or profilin-actin with the FH2 domain (Fig. 6B, II). We hypothesize that this interaction enhances the accessibility of profilin-actin to the barbed-end. Alternatively, interaction of profilin with the FH2 domain may be important for detaching profilin after the associated actin has been delivered to the barbed-end (Fig. 6B, III'). Dissociation of profilin may also be fine-tuned by the associated FH1 domain. To address these possibilities, it will be important to determine exactly how profilin and profilin-actin associate with formin FH2 domains in both the presence and absence of the FH1 domain.
Formin and Profilin Isoform Specificity

Structural Basis for Profilin Isoform Specificity—Residue differences between CePFN-1 and CePFN-3 in highly conserved PLP- and actin-binding regions did not account for differential utilization by worm formins CYK-1 (Table 3). Other highly conserved profilin residues, that have thus far not been defined roles, might therefore be critical for formin profilin isoform specialization (supplemental Fig. S5). For example, groups of conserved residues cluster outside the actin- and PLP-binding regions, such as: 1) at the bottom of the profilin molecule away from the actin-binding surface (SpPRF 29S, 53D, 49A), and 2) near the top of the terminal helices at the transition between the PLP-binding and actin-binding regions (SpPRF 109G, 11T). Although diverse profilins have highly similar folds despite their low sequence conservation, important subtle differences might be critical for their interaction with specific formin proteins. Structures of different profilins in complex with formin FH1 domains and actin are essential to evaluate this interesting possibility.

Physiological Importance of Profilin Isoform Specialization—Although it has not yet been determined whether formin isoforms drive the assembly of actin bound to specific profilin isoforms in cells, we think that optimal formin/profilin pairing is functionally important. None of the three worm profilin isoforms, which do not work well with the essential fission yeast cytokinesis formin Cdc12p in vitro (Fig. 6A), are able to complement fission yeast profilin cdc3 temperature-sensitive mutants. The type of actin might also influence differential profilin utilization by formins. Although actin is one of the most well conserved proteins, both fission yeast and worm actin bind to some fission yeast and worm actin-binding proteins with different affinities than does rabbit muscle actin (33, 34). Higher eukaryotes with multiple actin isoforms might be critical for their interaction with specific formin proteins. Structures of different profilins in complex with formin FH1 domains and actin are essential to evaluate this interesting possibility.

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