Energetic Requirements for Processive Elongation of Actin Filaments by FH1FH2-formins*

Received for publication, November 11, 2008, and in revised form, February 5, 2009. Published, JBC Papers in Press, February 26, 2009, DOI 10.1074/jbc.M808587200

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Formin-homology (FH) 2 domains from formin proteins associate processively with the barbed ends of actin filaments through many rounds of actin subunit addition before dissociating completely. Interaction of the actin monomer-binding protein profilin with the FH1 domain speeds processive barbed end elongation by FH2 domains. In this study, we examined the energetic requirements for fast processive elongation. In contrast to previous proposals, direct microscopic observations of single molecules of the formin Bni1p from Saccharomyces cerevisiae labeled with quantum dots showed that profilin is not required for formin-mediated processive elongation of growing barbed ends. ATP-actin subunits polymerized by Bni1p and profilin release the γ-phosphate of ATP on average >2.5 min after becoming incorporated into filaments. Therefore, the release of γ-phosphate from actin does not drive processive elongation. We compared experimentally observed rates of processive elongation by a number of different FH2 domains to kinetic computer simulations and found that actin subunit addition alone likely provides the energy for fast processive elongation of filaments mediated by FH1FH2-formin and profilin. We also studied the role of FH2 structure in processive elongation. We found that the flexible linker joining the two halves of the FH2 dimer has a strong influence on dissociation of formins from barbed ends but only a weak effect on elongation rates. Because formins are most vulnerable to dissociation during translocation along the growing barbed end, we propose that the flexible linker influences the lifetime of this translocative state.

Formins are multidomain proteins that assemble unbranched actin filament structures for diverse processes in eukaryotic cells (reviewed in Ref. 1). Formins stimulate nucleation of actin filaments and, in the presence of the actin monomer-binding protein profilin, speed elongation of the barbed ends of filaments (2–6). The ability of formins to influence elongation depends on the ability of single formin molecules to remain bound to a growing barbed end through multiple rounds of actin subunit addition (7, 8). To stay associated during subunit addition, a formin molecule must translocate processively on the barbed end as each actin subunit is added (1, 9–12). This processive elongation of a barbed end by a formin is terminated when the formin dissociates stochastically from the growing end during translocation (4, 10).

The formin-homology (FH)2 1 and 2 domains are the best conserved domains of formin proteins (2, 13, 14). The FH2 domain is the signature domain of formins, and in many cases, is sufficient for both nucleation and processive elongation of barbed ends (2–4, 7, 15). Head-to-tail homodimers of FH2 domains (12, 16) encircle the barbed ends of actin filaments (9). In vitro, association of barbed ends with FH2 domains slows elongation by limiting addition of free actin monomers. This “gating” behavior is usually explained by a rapid equilibrium of the FH2-associated end between an open state competent for actin monomer association and a closed state that blocks monomer binding (4, 9, 17).

Proline-rich FH1 domains located N-terminal to FH2 domains are required for profilin to stimulate formin-mediated elongation. Individual tracks of polyproline in FH1 domains bind 1:1 complexes of profilin-actin and transfer the actin directly to the FH2-associated barbed end to increase processive elongation rates (4–6, 8, 10, 17).

Rates of elongation and dissociation from growing barbed ends differ widely for FH1FH2 fragments from different formin homologs (4). We understand few aspects of FH1FH2 domains that influence gating, elongation or dissociation. In this study, we examined the source of energy for formin-mediated processive elongation, and the influence of FH2 structure on elongation and dissociation from growing ends. In contrast to previous proposals (6, 18), we found that fast processive elongation mediated by FH1FH2-formins is not driven by energy from the release of the γ-phosphate from ATP-actin filaments. Instead, the data show that the binding of an actin subunit to the barbed end provides the energy for processive elongation. We found that in similar polymerizing conditions, different natural FH2 domains dissociate from growing barbed ends at substantially different rates. We further observed that the length of the flexible linker between the subunits of a FH2 dimer influences dissociation much more than elongation.

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* This work was supported, in whole or in part, by National Institutes of Health Research Grant GM026338 (to T. D. P.). This work was also supported by a National Science Foundation predoctoral fellowship (to A. S. P.).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Discussion, Movies S1–S3, Tables S1–S6, Figs. S1–S6, and Equations S1–S5.

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2 The abbreviations used are: FH, formin-homology; GFP, green fluorescent protein; QD, quantum dot; GST, glutathione S-transferase; DTT, dithiothreitol; MESG, 2-amino-6-mercaptop-7-methylpurine riboside; PNP, purine nucleoside phosphorylase.
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EXPERIMENTAL PROCEDURES

Molecular Cloning of Recombinant Formins—All formin constructs were generated by standard cloning methods. For all FH1FH2 constructs containing either the Bni1p FH1 domain fused to a heterologous FH2 domain, or the Bni1p-FH2 domain with a heterologous FH2 linker, we generated the open-reading frames by overlap-extension PCR. All constructs were ultimately cloned into either the plasmid pGv67 (gift from B. J. Nolen, University of Oregon, Eugene, OR) for expression with an N-terminal GST tag or pQE70 (Stratagene, La Jolla, CA) for expression with a C-terminal His6 tag. For detailed description of cloning, see supplemental Experimental Procedures.

Protein Purification—All recombinant formins were expressed in Escherichia coli BL21-CodonPlus (DE3)-RP (Stratagene) (5). His6-tagged formin proteins and GST-tagged expressed in E. coli of cloning, see supplemental Experimental Procedures. Expression with a C-terminal His6 tag or pQE70 (Stratagene, La Jolla, CA) for approximately cloned into either the plasmid pGV67 (gift from B. J. JOURNAL OF BIOLOGICAL CHEMISTRY

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TIRF Microscopy—We performed TIRF microscopy with formin proteins, profilin, and Oregon Green-actin (10). The widely varying concentrations of each formin required to observe both free and formin-associated filaments in the same TIRF microscopy viewing field (Table 2) were likely due to differences in the abilities of these constructs to nucleate new ends. For experiments with quantum dots (Fig. 1), biotinylated molecules of Bni1(FH1FH2)pbio attached to streptavidin-conjugated QDot 625 nanocrystals (Invitrogen Cat. No. A10196, Carlsbad, CA) were imaged separately from actin filaments in a rhodamine-detecting channel as detailed in supplemental Experimental Procedures.

Measurement of γ-Phosphate Release from ATP-actin Filaments—We measured γ-phosphate release from polymerizing ATP-actin filaments (22) over time by monitoring the enzymatic conversion of 2-amino-6-mercapto-7-methylpyrimidine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpyrimidine by purine nucleoside phosphorylase (PNP) (EnzChek Phosphate Assay kit, Invitrogen, Cat. No. E-6646) as detailed in supplemental Experimental Procedures.

Data Analysis—Time-lapse movies of filament growth observed by TIRF microscopy were processed and analyzed with ImageJ software (10). The reported rates are an average of typically at least 6 individual filaments in a viewing field. Average rates of dissociation of formin from growing ends were determined by measuring either the duration of slow barbed end growth (without profilin), or duration of dim fluorescence of a filament (with profilin) (10) for at least 48 growing barbed ends. For detailed descriptions, see supplemental Experimental Procedures.

Computer Simulations of FH1FH2-formin-mediated Barbed End Elongation—We simulated processive elongation by FH1FH2-formins in profilin and actin with Virtual Cell software (National Resource for Cell Analysis and Modeling and the National Center for Research Resources) and the model proposed by Vavylonis et al. (17). For detailed descriptions of fibrinogen molecules track the growing barbed ends of actin filaments. TIRF microscopy conditions: 1.5 μM actin monomers (25% Oregon Green-labeled) in 10 mM imidazole (pH 7.0), 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 100 mM DTT, 0.2 mM ATP, 17 μM CaCl2, 15 mM glucose, 40 μg/ml catalase, 200 μg/ml glucose oxidase, and 0.5% methylcellulose (4000 cP at 2%, w/v). These reactions were prepared with microscopy buffer II as described under supplemental Experimental Procedures. A, time-series of TIRF microscopy images of actin filaments growing in 18 nM Bni1(FH1FH2)pbio attached to streptavidin-conjugated QD. Actin filaments were imaged in a fluorescein-detecting channel and molecules of QD-Bni1(FH1FH2)pbio separately in a rhodamine-detecting channel. Each composite image shows actin filaments in green pseudo-color and QDs in red pseudo-color. Yellow wedges indicate two examples of QDs tracking with growing barbed ends. QD-Bni1(FH1FH2)pbio-associated ends grew at an average of 6.0 subunits/s and free barbed ends grew at 12.3 subunits/s, as in A, but with 12 nM QD-Bni1(FH1FH2)pbio and 5 μM profilin. A purple wedge marks a free barbed end not associated with a QD from the 50-s time point to highlight the differences in fluorescent intensities between filaments with free ends and QD-Bni1(FH1FH2)pbio-associated filaments. Ends associated with QD-Bni1(FH1FH2)pbio grew at average of 20.2 subunits/s and free ends grew at 16.1 subunits/s.

RESULTS

Direct Observation of Processive Association of Bni1(FH1FH2)p with Growing Barbed Ends—To confirm processive association of formin molecules with growing ends, we attached S. cerevisiae Bni1(FH1FH2)p with an N-terminal biotin group (Bni1(FH1FH2)pbio) to streptavidin-conjugated fluorescent Quantum Dots (QD) and used two-color TIRF microscopy to observe both growing actin filaments and QDs attached to formins (Fig. 1, supplemental Movies S1 and S2). In these reactions, 25% of the actin monomers were labeled with the fluorescent dye Oregon Green. Without profilin, QDs tracked closely with ends elongating at 6.0 subunits/s, approximately half the rate of apparently free barbed ends growing in the same field at 12.3 subunits/s (Fig. 1A). In the presence of S. cerevisiae profilin, ends associated with individual molecules of QD-Bni1(FH1FH2)pbio were dimmer and grew faster than free filaments in the same field (Fig. 1B). In the presence of profilin and actin labeled with a fluorescent dye on Cys-374 filaments growing in association with FH1FH2 are dimmer than free filaments (4, 17). The lower fluorescence arises in a mixture of unlabeled and labeled actin, because profilin binds more strongly to the unlabeled actin and FH1FH2-associated ends preferentially incorporate profilin-actin over free actin monomers (10, 17). While other interpretations are possible, our
Formin and profilin do not stimulate the release of γ-phosphate from ATP-actin filaments—To examine the energetic requirements of processive elongation of actin filaments with FH1FH2 domains and profilin, we tested the hypothesis that FH1FH2-formins with profilin promote the release of γ-phosphate from ATP-actin filaments (18). We measured the time course of γ-phosphate release into solution during polymerization of 18 μM ATP-actin in the presence or absence of 50 nM Bni1(FH1FH2)p and profilin (Fig. 2). In these experiments, actin with formin polymerized faster than actin alone, showing that formin nucleates filaments in these conditions (Fig. 2).

Under the conditions of these experiments, the actin was nearly polymerized by the beginning of data acquisition (dead-time ~30–45 s), while the half time for release of γ-phosphate into solution was >2.5 min with either Bni1(FH1FH2)p alone, or Bni1(FH1FH2)p with 10 or 50 μM profilin (Fig. 2 and Table 1). For all of these samples, the rate constants for γ-phosphate release of 0.0032–0.0047 s⁻¹ were similar to those rate constants observed previously for the polymerization of ATP-actin alone or ATP-actin with profilin (22, 23) (Table 1). These data show directly that addition of profilin-actin onto barbed ends by FH1FH2-formin is not directly coupled to the release of γ-phosphate from ATP-actin subunits incorporated into the filaments.

Influence of the FH2 Domain on FH1FH2-formin-mediated Elongation in Profilin-actin—We studied the effect of different FH2 domains on the rate of elongation of barbed ends in mixtures of S. cerevisiae profilin and muscle actin. We used a set of chimeric FH1FH2 constructs containing identical FH1 domains from S. cerevisiae Bni1p covalently attached to FH2 domains from other formins. We selected FH2 domains from Schizosaccharomyces pombe Cdc12p, mouse mDia1 and mouse mDia2, because their FH2 gating factors span a broad range (4, 5, 19) (Table 2). The gating factor is the fraction of the time the formin spends in the open state (17). Each of these FH2 domains has been shown to mediate elongation of filaments of muscle actin (2–5). While these FH2 domains might be optimized for interaction with the species of actin found in their respective cell types, the use of only one combination of FH1 domain, profilin, and actin throughout our experiments ensured that the observed differences between FH1FH2 constructs arose only from differences in the activities of the FH2 domains.

For each of these constructs, we measured by TIRF microscopy the rates of formin-mediated elongation of barbed ends growing in fluorescent actin monomers (typically 33% Oregon Green-labeled) and profilin (Fig. 3 and Table 2). Fig. 3 also shows rates measured in association with Bni1(FH1FH2)p (10). From the rates of elongation measured in the absence of profilin, we calculated the FH2 gating factor for each FH1FH2 construct. Without profilin each chimeric FH1FH2 had a gating factor similar to the FH2 domain of the parent formin (Table 2). Bni1(FH1)-Cdc12(FH2) allowed barbed ends to grow very slowly, at less than 1% the rate of free barbed ends, as shown by the incorporation of bright fluorescent actin at both ends of dim, photobleached filaments (supplemental Fig. S1 and Movie S3). Previous studies (4, 5, 8) did not detect this very slow elongation of barbed ends associated with Cdc12(FH1FH2)p. The gating factor of Bni1(FH1)-mDia2(FH2) was 0.05, lower than 0.2 reported for mDia2(FH1FH2) (4). Bni1(FH1)-mDia1(FH2) did not slow elongation, similar to mDia1(FH1FH2) with a gating factor of 0.9 (4).

As observed previously for FH1FH2-formins (4, 10), a concentration of profilin similar to the concentration of actin monomers stimulated formin-mediated elongation to its maximum rate (Fig. 3A). At this profilin concentration, most of the actin was bound to profilin but minimal free profilin competed with profilin-actin for binding to FH1 polyproline tracks. Without profilin the rate of elongation was (by definition) directly proportional to the FH2 gating factor. By contrast, with profilin the rate of elongation did not scale linearly with the gating factor (Fig. 3B). For example, without profilin barbed ends associated with Bni1(FH1)-mDia1(FH2) grew 100-fold faster than ends associated with Bni1(FH1)-Cdc12(FH2), but with 5 μM profilin these formin-associated rates converged such that ends associated with the construct containing the mDia1-FH2

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**TABLE 1**

| Conditions: 18 μM ATP-actin with: | Observed rate constant of γ-phosphate release, s⁻¹ |
|----------------------------------|-----------------------------------------------|
| Actin alone                      | 0.0035 ± 7 × 10⁻⁵ |
| 50 nM Bni1(FH1FH2)p              | 0.0047 ± 0.0002 |
| 50 nM Bni1(FH1FH2)p, 10 μM profilin | 0.0042 ± 0.0001 |
| 50 nM Bni1(FH1FH2)p, 50 μM profilin | 0.0032 ± 0.0001 |

observations indicate that individual formin molecules bound to QDs processively associate with growing barbed ends.
domain grew only ~6-fold faster than ends associated with Bni1(FH1)-Cdc12(FH2) (Fig. 3, A and B, Table 2).

Natural FH2 Domains Differ in their Processivities—We measured the rates that Bni1(FH1FH2)p and each chimeric FH1FH2 dissociated randomly from growing barbed ends in the presence and the absence of profilin (Fig. 4, Table 2). In the absence of profilin, dissociation of most formins was signaled by an abrupt increase in the rate of barbed end elongation to the rate characteristic of free filaments (Fig. 4A). Bni1(FH1)-mDia2(FH2) dissociated from ends slower (0.0001 s⁻¹) than Bni1(FH1FH2)p (0.00032 s⁻¹). No molecules of Bni1(FH1)-Cdc12(FH2) dissociated from barbed ends growing over 1300 s of observation, so it dissociated on a time scale much longer than that of the experiment (Fig. 4C, Table 2). We could not measure dissociation of Bni1(FH1)-mDia1(FH2) from ends without profilin, because these formin-associated filaments are as bright as free filaments and elongate at approximately the same rate as free ends (Table 2).

Profilin increased the rates of dissociation of all the formins (Fig. 4C, Table 2). The ability of profilin to stimulate dissociation can be explained by considering that profilin increases formin-mediated elongation rates and actin subunit addition stimulates dissociation of formin from growing ends (10). In the presence of profilin, the fluorescence intensity of a growing filament increased suddenly when an FH1FH2-formin dissociated from a barbed end (Fig. 4B). Bni1(FH1)-mDia1(FH2) with profilin dissociated faster (0.020 s⁻¹) than the other constructs (Fig. 4C) and had by far the shortest processive runs (Table 2). Profilin increased the dissociation rates of Bni1(FH1FH2)p and Bni1(FH1)-mDia2(FH2) by ~6–7-fold. Cdc12p-FH2 formins with profilin incorporated on average ~29 times more actin subunits per processive run (75,000) than mDia1-FH2 formins (2600) (Table 2).

Influence of the FH2 Flexible Linker on Processive Elongation—The flexible linker connecting the two halves of the FH2 dimer is the only element of FH2 that undergoes a major conformational change upon binding an actin filament (9). To study the role of the FH2 linker in processive elongation by formins, we created chimeric FH2 domains containing the lasso, knob, coiled-coil, and post regions from Bni1p-FH2, with either the 14-amino acid linker from Cdc12p (Bni1pCdc12p), the 19-amino acid linker from mDia2 (Bni1pmDia2), or the 23-amino acid linker from mDia1 (Bni1pmDia1). The native linker in Bni1p-FH2 contains 17 amino acids. Each of these natural linker sequences is predicted to contain short α-helical elements (Fig. S2). To test the possible role of an α-helix in the linker, we replaced the native linker in Bni1p FH2 domain with a 17-amino acid poly-Gly-Ser linker (G₄S(GS)₄G₄) that is not expected to have secondary structure (Bni1pGS) (data not shown). Each
of these variant Bni1p FH2 domains was attached to a Bni1p FH1 domain.

Based on a correlation between linker lengths and rates of elongation mediated by several natural FH2 domains, we previously hypothesized that the availability of the open state of the formin-associated barbed end, and therefore the elongation rate, is proportional to the length of this linker (4, 10). The rates of elongation measured in association with the Bni1p linker chimeras provided strong evidence against this hypothesis (Table 2). We instead observed that both with and without profilin, barbed end elongation was fastest in association with the constructs carrying the shortest linkers.

Indeed, there was no obvious relationship between the rates of elongation mediated by the Bni1p linker variants and the native FH2 domains from which the linkers were derived (Table 2). In 1.5 μM actin, instead of strongly inhibiting elongation like Cdc12p-FH2, Bni1pCdC12p behaved more similarly to Bni1(FH1FH2)p and slowed elongation to approximately half the rate of free barbed ends. Most surprisingly, the long linkers in Bni1pCdDia1 and Bni1pCdDia2 slowed processive barbed end elongation more strongly than Bni1(FH1FH2)p. Ends associated with these “slow” formins grew at 17 to 20% of the rate of free barbed ends without profilin. Similarly, the unstructured linker in Bni1pGS slowed elongation to 25% the rate of free ends. Five micromolar profilin stimulated elongation in association with Bni1pCdC12p to rates similar to those observed with Bni1(FH1FH2)p in the same conditions, ~28–31 subunits/s. With profilin, ends associ-
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ated with Bni1p_mDia2, Bni1p_mDia1, or Bni1p_GS, elongated only at ~12–15 subunits/s.

While substitution with the foreign linkers had only a modest effect on elongation by Bni1p, linker substitution strongly influenced dissociation of formin from ends growing in profilin (Table 2). The average number of subunits incorporated per processive run by these formins spanned a broad range with a low of 360 with Bni1p_GS to a high of 14,000 with Bni1(FH1FH2)p (Table 2).

DISCUSSION

Tracking Formins on Growing Ends Confirms Processive Association—For the first time, direct observations of the positions of both individual formin molecules and growing actin filaments show that formins track growing barbed ends (Fig. 1). This finding corroborates other evidence that formins processively elongate barbed ends: (i) formins inhibit association of capping protein with growing barbed ends (16, 19, 24, 25); (ii) GFP-tagged formins undergo directed translocation in live cells (7, 26); (iii) the growing barbed ends of actin filaments associate with formins immobilized on glass slides (8); and (iv) formins modulate the barbed end elongation rates and fluorescence intensities of filaments growing in profilin (4, 10) (Figs. 3 and 4).

Contrary to the proposal that the interaction of profilin with the FH1 domain is required for processive association (6, 18), our direct observations (Fig. 1A) confirm that profilin is not required for processive elongation by formins (4, 8).

Dissociation of γ-Phosphate from Polymerizing Actin Does Not Drive Formin-mediated Processive Elongation—Romero et al. (18) proposed that γ-phosphate release from actin provides the energy for processive elongation. This proposal was based on two observations. First, mDia1-FH1FH2 with profilin stimulates assembly of Mg-ATP-actin more strongly than Cr-ATP-actin. Second, chromium chelates the γ-phosphate of actin-bound ATP more strongly than magnesium and inhibits its release into solution (27). However, our results show unambiguously that FH1FH2-formins and profilin do not stimulate the release of γ-phosphate from polymerized actin because γ-phosphate release lags far behind elongation. Therefore, γ-phosphate release is not coupled to processive elongation (Fig. 2, Table 1).

Romero et al. (6) also observed that mDia1(FH1FH2) and profilin eliminated the lag in the time courses between the polymerization of Ca-ATP-actin and hydrolysis of the bound ATP, so they proposed that FH1FH2-formins and profilin stimulate the intrinsically slow hydrolysis of Ca-ATP bound to polymerized actin. Interpretation of their experiments, however, is complicated by their use of actin labeled with pyrene on Cys-374 to monitor bulk actin polymerization. Polymerization enhances the fluorescence of pyrenyl-actin, so the fluorescence of samples containing pyrenyl-actin can be used to monitor bulk assembly (28). However, barbed ends associated with FH1FH2-formins growing in profilin incorporate much less actin modified at Cys-374 than unlabeled actin (4, 17). Therefore, in reactions containing FH1FH2-formin, profilin, pyrenyl- and unlabeled actin, the pyrene fluorescence at every time point underestimates the total polymerized actin (10). In the experiments of Romero et al. (6) with mDia1(FH1FH2) and profilin, the unlabeled fraction of actin (95%) must have polymerized faster than ATP hydrolysis even though the time course of pyrenyl-actin polymerization was similar to that of ATP hydrolysis. Without additional data, it is premature to conclude that ATP hydrolysis is coupled to subunit addition to barbed ends during formin-mediated elongation.

Actin Subunit Addition Provides the Energy for Processive Elongation—The γ-phosphate dissociates from polymerizing filaments long after addition of ATP-actin subunits, so this reaction cannot provide the energy for processive elongation (Fig. 2 and Table 1). An alternative explanation is that known pairwise interactions between profilin, ATP-actin monomers and a FH1FH2-formin on the barbed end of a filament explain the kinetics of processive elongation. To test this hypothesis, we compared experimentally observed rates of elongation with data from computer simulations of actin filament elongation with profilin, ATP-actin monomers and an FH1FH2-formin (Fig. 3). To simulate elongation, we used the model proposed by Vavylonis et al. (17) that considers all of the likely pairwise interactions among the molecules in the system and how gating by the FH2 domain influences the transitions between the individual chemical species in the reaction (Fig. 5 and a full model in supplemental Fig. S3). This model does not consider how profilin might inhibit FH1-independent actin subunit addition onto formin-associated ends (29).

We constrained the simulations with measured rate constants for some steps (10, 30, 31) and by assuming that the interactions between formin, profilin and actin, completely...
describe the flow of energy in the system. This latter condition requires that all reactions in supplemental Fig. S3 are reversible. Accordingly, we enforced detailed balance to limit the ratios of rate constants for the reactions in the model (17) (see supplemental Discussion). With these constraints, the energy for elongation is only derived from the condition that the total concentration of actin monomers and profilin-actin complexes exceeds the critical concentration for actin filament barbed end elongation.

The model in supplemental Fig. S3 with the set of rate constants described as “good fit to experimental” in supplemental Table S1 yielded simulated elongation rates similar to those observed experimentally over the broad range of tested FH2 gating factors (0.01 for Cdc12p to 1 for mDia1) and most of the profilin concentrations that we tested (Fig. 3). A “sensitivity analysis”, where we tested the uniqueness of the selected rate constants to describe the experimental data, showed that the “good fit” set of rate constants comes the closest to accounting for the observed formin-mediated rates of elongation (supplemental Fig. S4 and Tables S1 and S2). Moreover, in agreement with our previous experimental findings with Bni1p (10), this analysis showed that for FH2 domains with gating factors \( \approx 0.1 \), the binding of profilin-actin to an FH1 polyproline track limits the transfer of actin subunits by FH1 to the barbed end (see supplemental Discussion).

At profilin concentrations \( \leq 10 \, \mu M \), the experimental and simulated data agreed particularly well, though for Bni1p the simulations predicted elongation rates somewhat higher than those observed experimentally. We do not understand this discrepancy between the experimental measurements and the simulations. Though the experimental data were consistent over separate trials with different preparations of reagents, we needed to estimate some of the rate constants. Disagreements between experiment and simulation might have arisen from uncertainties in either these parameters, the model itself, or both.

At profilin concentrations \( > 10 \, \mu M \), the model predicts elongation rates slower than those observed experimentally. This discrepancy is probably independent of formin and more strongly related to an incomplete understanding of the direct interaction of profilin with barbed ends (17) (supplemental Fig. S5 and Discussion).

Because the model does not consider energy contributed by an extrinsic process such as ATP hydrolysis or \( \gamma \)-phosphate release, the agreement between the simulations and experiments shows that protein-protein interactions alone can account for the elongation rates observed by TIRF microscopy (Fig. 3). Thus neither actin-ATPase activity nor \( \gamma \)-phosphate release is required to explain processive elongation in ATP-actin (Figs. 2 and 3 and Table 1). This finding is consistent with the observation that formins processively elongate barbed ends growing in ADP-actin monomers (4).

Our findings indicate that in physiological conditions with ATP-actin and profilin, the process of actin subunit addition alone provides the energy for all aspects of formin-mediated processive elongation, including translocation of the FH2 domain on the growing barbed end. Our mechanical model for the formin-mediated subunit addition provides a plausible explanation for how association of an actin monomer with a formin-associated end might drive translocation toward the barbed end (10).

**Influence of FH2 Flexible Linkers on Formin-mediated Elongation Rates and Processivity**—Actin filaments associated with the four well-characterized FH2 domains from Bni1p, Cdc12p, mDia1 and mDia2, differ by \( \geq 100 \)-fold in their elongation rates without profilin. By contrast, replacement of the native linker in the Bni1p FH2 domain with the linkers from any of the other three formins changes the formin-mediated elongation rate by only \( \sim 2\sim 3\)-fold (Table 2). While actin filament elongation rates are generally proportional to the linker length for native FH2 domains (4) (Fig. 3), no such trend is observed for the Bni1p chimeras with foreign FH2 linkers. Additionally, the ability of the Bni1p\(_{GS}\) to mediate processive elongation shows that no specific secondary structure is required in the linker for processive elongation.

It is not clear why the shorter FH2 linkers in Bni1p(FH1FH2)p and Bni1pCdc12p lead to somewhat faster elongation than observed for the other Bni1p linker variants. Because gating by FH2 domains is based on the differences in free energies between the open and closed states of the FH2-associated barbed end, short FH2 linkers might promote elongation by increasing the difference in free energies between these states. Indeed, the linker is expected to undergo various deformations during processive elongation (9, 10), so linker length might modulate the strain in the linker and thereby the energy of the formin bound to the end.

We considered what other aspects of FH2 structure besides the linker might influence rates of elongation. The distances between the two strong actin binding sites on FH2 domains (knob and post) differ by as much as 10 Å in crystal structures of mDia1, Bni1p, and DAAM1 (9, 12, 32–34) (supplemental Fig. S6A and Table S3). These FH2 domains must impose different structures on actin filaments to which they bind (33) (supplemental Fig. S6B) and perhaps influence elongation differently. These FH2 domains may be flexible enough to adopt the same conformation when bound to an actin filament, but the knob-post distances observed in the various FH2 crystal structures suggest that the conformations of a particular FH2 domain are limited in comparison to the range of conformations observed across diverse FH2 homologs (supplemental Table S3).

Natural FH2 domains differ more dramatically in their processivities than in their rates of elongation (Table 2). In 1.5 \( \mu M \) actin and 5 \( \mu M \) profilin, an mDia1-FH2 domain was almost 30-fold less processive and dissociated more than 200-fold faster from barbed ends than a Cdc12p-FH2 domain (Table 2). In the same conditions, however, these two formins differed by only \( \sim 6\)-fold in their rates of elongation.

Likewise, among the Bni1p FH2 domains differing only in their linker composition, rates of elongation in profilin differ by only \( \sim 2.5\)-fold, but the average number of actin subunits incorporated per processive run differs by almost 40-fold (Table 2). Our model of the mechanical cycle for formin-mediated subunit addition (10) provides a framework to consider the strong effect of linker composition on formin dissociation from growing barbed ends. This model proposes that the FH2 dimer is
most vulnerable to dissociation from a barbed end during translocation of formin along the growing barbed end.

The differing processivities of the Bni1p linker chimeras (Table 2) might be explained if linker length influences the lifetime of the translocative state. During translocation, one subunit of the FH2 dimer dissociates completely from the actin filament and searches for a new binding site on the terminal barbed end subunit (10). If this search is a diffusive process, the length of the linker might influence the volume explored by the dissociated FH2 subunit and therefore the length of time spent in translocation. The wild-type linker of Bni1(FH1FH2)p is apparently optimized for fast translocation by this formin, because it confers on Bni1p the greatest processivity (Table 2).

 FH2 and Formin-mediated Actin Assembly

Acknowledgments—We thank Dimitrios Vavylonis and Naomi Courtemanche for helpful discussions, Brad Nolen and Henry Higgs for providing reagents, and Chad McCormick for help with microscopy.

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