How ATP Hydrolysis Controls Filament Assembly from Profilin-Actin

IMPLICATION FOR FORMIN PROCESSIVITY

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Formins catalyze rapid filament growth from profilin-actin, by remaining processively bound to the elongating barbed end. The sequence of elementary reactions that describe filament assembly from profilin-actin at either free or formin-bound barbed ends is not fully understood. Specifically, the identity of the transitory complexes between profilin and actin terminal subunits is not known; and whether ATP hydrolysis is directly or indirectly coupled to profilin-actin assembly is not clear. We have analyzed the effect of profilin on actin assembly at free and FH1–FH2-bound barbed ends in the presence of ADP and non-hydrolyzable CrATP. Profilin blocked filament growth by capping the barbed ends in ADP and CrATP/ADP-P, states, with a higher affinity when formin is bound. We confirm that, in contrast, profilin accelerates depolymerization of ADP-F-actin, more efficiently when FH1–FH2 is bound to barbed ends. To reconcile these data with effective barbed end assembly from profilin-MgATP-actin, the nature of nucleotide bound to both terminal and subterminal subunits must be considered. All data are accounted for quantitatively by a model in which a barbed end whose two terminal subunits consist of profilin-ATP-actin cannot grow until ATP has been hydrolyzed and Pi released from the penultimate subunit, thus promoting the release of profilin and allowing further elongation. Formin does not change the activity of profilin but simply uses it for its processive walk at barbed ends. Finally, if profilin release from actin is prevented by a chemical cross-link, formin processivity is abolished.

Formins are involved in various motile processes such as cytokinesis, adhesion, or filopodia formation, where they nucleate and catalyze processive assembly of actin filaments.

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3 The abbreviations used are: FH, formin homology; Ap5A, 5-di(adenosine 5’)-pentaphosphate; AMP-PNP, adenosine 5’-β,γ-iminotriphosphate; PAcov, covalent profilin-actin complex.
recent review). There is general agreement that the profilin-actin complex does not assemble at pointed ends of actin filaments, but can productively associate at barbed ends (28–30). Profilin thus enhances the efficiency of actin filaments treadmilling, in favoring steady-state barbed end growth events. This property is at the origin of the positive effect of profilin in actin-based motile processes, which are powered by treadmilling (31). Filament assembly from profilin-actin requires dissociation of profilin from the barbed end following association of profilin-actin, to allow further elongation. Several mechanisms have been proposed.

In a first model, the dissociation of profilin from the barbed end is caused by ATP hydrolysis because profilin has a 20-fold lower affinity for ADP-actin than for ATP-actin (29, 32). In this view, ATP hydrolysis is mechanistically coupled to filament assembly from profilin-actin, which is not the case for assembly from pure actin (33). Three independent facts are consistent with this view. First, the rate of barbed end growth increases linearly with the concentration of G-actin, but reaches a limit at high concentration of profilin-actin, which was attributed to the rate of ATP hydrolysis at the terminal subunit; second, profilin causes a decrease in the partial critical concentration of G-actin (33); third, filaments do not assemble from profilin–ADP-actin nor from profilin–CaATP-actin, which hydrolyzes ATP very slowly (29, 32).

In a second model, profilin dissociation is due to its lower affinity for filament barbed ends than for G-actin and is not dependent on ATP hydrolysis (34). In agreement with this view, ATP hydrolysis was found to lag slightly behind profilin-actin assembly, indicating that the release of profilin from barbed ends was not coupled to ATP hydrolysis (35).

To resolve the above discrepancies on profilin and formin mechanisms, we have analyzed the assembly of ADP-actin and CrATP-actin in the absence and presence of profilin, formin, or both profilin and formin. We demonstrate that the dissociation of profilin from barbed ends is directly coupled to ATP hydrolysis and phosphate release on actin. We confirm that profilin binds to ADP-actin at barbed ends and increases the rate of depolymerization (34, 36). Profilin inhibits barbed end assembly from ADP-actin as well as from CrATP-actin by blocking the barbed ends.

These fundamental properties of profilin are conserved and enhanced when FH1–FH2 is bound to profilin-actin at barbed ends, and are used to promote the processive assembly by formins. The present results provide new insight into the correlation between mechanical and chemical steps in the processive cycle of formins.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Actin was purified from rabbit muscle and was pyrenyl- or rhodamine-labeled (33, 57). Mouse profilin 1 and His-tagged FH2 or FH1–FH2 constructs were expressed in *Escherichia coli* BL21 and purified (24, 58). Covalently cross-linked profilin-actin complex (PACov) was prepared (32).

**ADP-Actin Polymerization Assays**—ADP-actin was prepared by polymerizing the ATP-actin (1:1) complex (2% pyrenyl-labeled) in the presence of 100 μM ADP and 15 units/ml hexokinase, and 5 mM glucose. F-ADP-actin was sedimented by centrifugation at 400,000 × g for 20 min. The pellet was resuspended in 5 mM Tris-Cl buffer, pH 7.8, containing 1 mM dithiothreitol, 100 μM CaCl2, 100 μM ADP, 10 μM Ap5A (Sigma), 15 units/ml hexokinase, and 5 mM glucose, incubated on ice for 1 h and gel filtered on G25 in the same buffer. Polymerization was induced by addition of 0.1 M KCl, 0.2 mM EGTA, and 1 mM MgCl2 to this solution. The increase in fluorescence of pyrenyl-labeled actin was monitored with a Safas spectrofluorimeter (λex 366 nm, λem 407 nm). Steady-state pyrene fluorescence measurements of F-actin assembly were carried out in a Spex spectrofluorimeter following a 3-h incubation at 20 °C to avoid denaturation of ADP-actin.

**Polymerization Assay of CrATP-Actin**—Polymerization of CrATP-actin was performed (45). Briefly, ADP-actin was prepared as described above, except that the F-ADP-actin pellet was resuspended in G0 buffer containing only 100 μM ADP. 5% pyrenyl-labeled ADP-actin was polymerized in I-buffer (5 mM imidazole, pH 6.6, 0.2 mM dithiothreitol) with the indicated concentrations of CrATP. CrATP was added at time 0 to minimize dissociation of CrATP into chromium ion and ATP.

**Fluorescence Microscopy Assay**—Filament elongation from a single FH1–FH2 molecule immobilized on a 2-μm diameter microsphere was performed as described (24) except that polymerization was performed in 5 mM imidazole, pH 6.8, 7 mM dithiothreitol, 0.1 M KCl, 1.5 mM diazobicyclooctane (DABCO, Sigma), 100 μM MgATP and CrATP as indicated.

**Electron Microscopy**—Filaments of actin, polymerized at 7 μM were negatively stained, using a 2% uranyl acetate solution, and observed at a magnification of ×50,000, under low electron dose conditions (less than 10 electrons per Å square) in a JEOL JEM 2100F electron microscope. Images were recorded on So163 Kodak films and treated for 12 min using pure D19 developer. Selected micrographs were digitized with a Nikon Coolscan 9000 microdensitometer, using a sampling distance and a scanning step of 2540 dots per inch, corresponding to a pixel size of 2 × 2 Å square.

**Modeling**—The capping of barbed ends by profilin in ADP and CrATP and the growth of filaments from profilin and actin in MgATP was analyzed using Scheme 1.

In Scheme 1, line 1 describes the interaction between profilin and G-actin. Line 2 describes filament barbed end growth from G-actin. Line 3 represents binding of profilin-actin to the barbed end of a filament that has n subunits (called Fₙ), making a Fₙ₊₁P end with profilin bound to the terminal subunit. Line 4...
represents binding of profilin to the terminal subunit of a filament that has $n$ subunits, resulting in a $F_nP$ filament end. Line 5 represents binding of profilin to a $F_n$ barbed end, resulting in a $F_nP$ end, corresponding to a filament that has $n$ subunits but with a subterminal profilin-actin subunit, hence is structurally different from the $F_nP$ filament. Line 6 represents binding of G-actin to a $F_nP$ filament, resulting in a $F_{n+1}P$ filament. Line 7 represents binding of PA to a $F_nP$ filament, resulting in a filament $F_{n+1}PP_0$ that has $n+1$ subunits and profilin bound to the two terminal subunits. The same $F_{n+1}PP_0$ filament can be obtained by binding of profilin either to $F_{n+1}P$ (line 8) or to $F_{n+1}PP_0$ (line 9). Note that lines 4 and 5 are repeated on a $F_{n+1}$ filament, giving $F_{n+1}P$ and $F_{n+1}PP_0$ as well as lines 8 and 9, giving $F_{n+1}PP_0$. Lines 10 and 11 describe irreversible hydrolysis of MgATP on the penultimate subunit of actin filaments assembling from profilin-actin, allowing cycles of assembly. The model is restricted to lines 1–9 in ADP and CrATP. In these cases, the profilin-bound filament ends are nonproductive, filament growth occurs only via line 2.

The rate of barbed end growth was modeled in ADP, CrATP, and MgATP, with and without formin, using the Madonna-Berkeley software, using the rate constants known for association dissociation of G-actin to free filament ends and using experimentally determined values of equilibrium dissociation constants for profilin binding to G-actin in ADP ($K_p = 4 \mu M$ in ADP, 0.1 $\mu M$ in ATP). Identical values were taken for $K_F$ and $K_s$ (1.5 $\mu M$ in ADP, 0.1 $\mu M$ in ATP), as well as for $K_F$ and $K_o$ for $K_s$ and $K_o$, for which best fit values to experimental curves were determined computationally.

In the absence of profilin, $V_o = k_{+2}[F_0]_0[A_0] - k_{-2}[F_0]$. In the presence of profilin, $[A_0] = [A] + [PA]; [P_0] = [P] + [PA]$ where $[F_0], [A_0]$, and $[P_0]$ represent the total concentrations of filament-barbed ends, G-actin, and profilin, respectively. $[PA] = (1/2)([P_0] + [A_0]) + [P] + [PA]$, $([A_0] + [P_0] + [PA])^2 = 4$. $V = k_{+2}[F]_0[A] - k_{-2}[F] + k_{-3}[F][PA] - k_{-3}[F]_0[A_0] + k_{-4}[FP_0] + k_{-7}[FP][PA] - k_{-7}[FP]_0$. The Madonna-Berkeley software was used to model $V/V_o$ using the above equations. The parameters were adjusted to fit the experimental data in Figs. 2 and 4 in ADP and CrATP, with free and formin-bound barbed ends. Constraints were brought by using the known values of the equilibrium dissociation constant $K_F = k_{-1}/k_{+1}$ for binding of profilin to G-actin ($K_1 = 4 \mu M$ in ADP and 0.2 $\mu M$ in MgATP or CrATP), and of the association and dissociation of G-actin to free barbed ends: $k_{+2} = 10 \mu M^{-1}$ s$^{-1}$ in MgATP or CrATP and 2.5 $\mu M^{-1}$ s$^{-1}$ in ADP; $k_{-3} = 1$ s$^{-1}$ in MgATP and 3.75 s$^{-1}$ in ADP, giving $C_{*} = 0.1 \mu M$ in ATP and 1.5 $\mu M$ in ADP. Other parameters were adjusted. The best fit values are given in Table 1. Whenever applicable, the range of values that equally well fitted experimental data were given.

FH1–FH2 (continuous lines and closed symbols). F-ADP-actin (3 $\mu M$) labeled with 2% pyrenyl-actin was assembled in the absence (black circles) or presence of 100 (blue squares) or 200 nm (red triangles) FH1–FH2. That the data reflect the behavior of 100% active ADP-actin was verified by adding 100 $\mu M$ ATP to ADP-actin and measuring the amount of F-actin at steady-state (dashed lines and open symbols) in the absence (black circles) or presence of 200 nm FH1–FH2 (blue squares). C, the rate of dilution-induced depolymerization of filaments was measured at the indicated profilin concentrations in the absence (blue circles) or presence of 50 nm FH1FH2 (red squares).
RESULTS

Profilin Blocks the Growth and Increases the Rate of Depolymerization of ADP-bound Barbed Ends in an FH1–FH2-enhanced Fashion—Under physiological conditions, ADP-G-actin polymerizes reversibly into ADP-F-actin with a critical concentration of 1.5 μM. Previous work has shown that FH1–FH2 binds ADP-F-actin at barbed ends with a $K_f$ of 3 nM (10, 19, 24). Consistently, we now find that FH1–FH2 nucleates ADP-actin, although less efficiently than ATP-actin (Fig. 1A).

Measurements of F-actin at equilibrium in ADP showed that profilin caused depolymerization of F-ADP-actin both in the absence and presence of FH1–FH2 (Fig. 1B). This result indicates that profilin-ADP-actin cannot participate in F-actin assembly, i.e. profilin sequesters ADP-actin, even if FH1–FH2 is bound to barbed ends. The derived binding constant ($K_d$) of profilin for ADP-G-actin is 4 μM, in agreement with previous data (29).

In dilution-induced depolymerization assays (Fig. 1C), profilin increased the rate of ADP-actin dissociation from free barbed ends, and even more from FH1–FH2-bound barbed ends, in agreement with previous reports (24, 36). These observations indicate that profilin actually associates with ADP-bound barbed ends, with equilibrium dissociation constants of 8 μM in the absence of formin and 2 μM in the presence of formin. These values are artificially higher than the actual binding constant of profilin for ADP-bound barbed ends because for sensitivity purposes, the depolymerization assays had to be carried out using filaments containing 50% pyrenyl-labeled actin to which profilin binds very poorly (37).

We then examined how profilin affected the kinetics of filament growth from ADP-actin at pointed ends and at free or FH1–FH2-bound barbed ends. To avoid residual amounts of ATP on actin, assays were performed in the presence of 15 units/ml of hexokinase, 5 mM glucose, and 10 μM Ap5A, to avoid ATP synthesis from ADP by myokinase, which often contaminates actin preparations (Ref. 38 and “Experimental Procedures”).

Profilin inhibited pointed end growth from ADP-actin in a dose-dependent fashion (Fig. 2A) consistent with formation of a non-polymerizable profilin-ADP-actin complex and a binding constant of profilin for ADP-actin of 4 μM, identical to the value derived from measurements of F-actin at equilibrium (Fig. 1B). Altogether, kinetic and equilibrium measurements confirm that profilin has a 20-fold lower affinity for ADP-actin than for ATP-actin (29, 39 – 41).

Profilin inhibited barbed end growth from ADP-actin, but the inhibition was not consistent with sequestration of ADP-actin, because the inhibition curve at 9 μM actin (Fig. 2A, red curve) does not superimpose with the curve obtained at pointed ends at the same actin concentration (Fig. 2A, green curve). Half-inhibition of barbed end growth was observed at a total

![Figure 2. Profilin blocks barbed end growth from ADP-actin at either free or FH1–FH2-bound barbed ends and increases the rate of barbed end disassembly. A, inhibition of barbed end and pointed end growth of ADP-actin by profilin. The initial rate of pointed end elongation (green triangles) or of barbed end elongation (red circles) of ADP-actin (9 μM, 2% pyrenyl labeled) was measured at different concentrations of profilin. Curves are calculated using the model with the values of dissociation constants given in Table 1. B, barbed end growth initiated by spectrin-actin seeds (blue curves) or 200 nM FH1–FH2 (red curves) at 3 μM ADP-actin in the absence (thin lines) and presence (thick lines) of 1.36 μM profilin. C, the initial rate of barbed end growth from 3 μM ADP-G-actin was measured at different concentrations of profilin using spectrin-actin seeds (blue) or 100 nM FH1–FH2 (red) and normalized to the value of 1 in the absence of profilin. Curves are calculated using the model with the values of dissociation constants given in Table 1.](image-url)
How ATP Hydrolysis Controls Filament Assembly

TABLE 1
Equilibrium and rate parameters for profilin interaction with G-actin and free or formin-bound filament barbed ends in ADP, MgATP, and CrATP

The values of parameters are the ones that are used in the calculated curves that represent the best fit to experimental data (Figs. 2 and 4), within the model described under “Experimental Procedures.” The indicated range indicates the acceptable fit for a given parameter, keeping all other parameters at their indicated average value. + and − refer to presence and absence of profilin.

| Nucleotide | MgADP | MgADP | CrATP | CrATP | MgATP | MgATP |
|------------|-------|-------|-------|-------|-------|-------|
|            |       |       |       |       |       |       |
| Formin     |       |       |       |       |       |       |
| $K_i$, μM  |       |       |       |       |       |       |
| $K_{d1}$, μM | 1.5 | 4 | 1 | 0.2 | 0.2 | 0.2 | 0.2 |
| $K_{d2}$, μM | 3 ± 1 | 0.1 ± 0.05 | 0.1 ± 0.05 | 0.1 ± 0.05 | 0.1 ± 0.05 | 0.1 ± 0.05 | 0.2 ± 0.1 |
| $K_0$, μM | 5 ± 1 | 0.5 ± 0.1 | 0.09 ± 0.01 | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.6 ± 0.1 |
| $k_{i1}$ = $k_{a1}$, s$^{-1}$ | 10 ± 2 | 0.35 ± 0.05 | 0.12 ± 0.02 | 2 ± 0.2 | 2 ± 0.2 | 2 ± 0.2 | 2 ± 0.2 |

FIGURE 3. Fluorescence microscopy measurements of the inhibition of FH1–FH2-bound barbed end growth from ADP-actin by profilin. A, polymerization of 1.5 μM rhodamine-labeled ADP-actin was initiated with 1 μM ADP-actin filaments stabilized with Alexa 488-phalloidin in the presence of 100 nM FH1–FH2 in the absence or presence of 6 μM profilin. Filaments were stabilized at the indicated times with unlabeled phalloidin, diluted, and observed in fluorescence microscopy. B, the assay described in A was performed at different concentrations of profilin and the initial rate of barbed end growth measured in the presence of the indicated concentrations of profilin was normalized to the value of 1 in the absence of profilin.

Concentration of 3.5 μM profilin, indicating that ADP-bound barbed ends are blocked, by binding either free profilin or profilin–ADP-actin complex. A satisfactory fit was obtained to all curves at both ends by the model described under “Experimental Procedures,” using values of equilibrium and rate parameters summarized in Table 1.

In the presence of FH1–FH2, profilin inhibited assembly from ADP-actin much more efficiently at FH1–FH2-bound barbed ends than at free barbed ends (Fig. 2, B and C). Half-inhibition was observed at a total concentration of 0.21 μM profilin. In fluorescence microscopy measurements (Fig. 3, A and B) of individual formin-bound filaments growing from ADP-actin, profilin again blocked barbed end growth with high affinity ($K_i = 0.15$ μM).

In conclusion, in binding to terminal ADP-actin subunits at barbed ends, profilin blocks barbed end growth while facilitating depolymerization by destabilizing barbed ends. Consistently, in measurements of F-actin at equilibrium, profilin simply sequesters ADP-actin.

Profilin Blocks Barbed End Growth from CrATP-Actin in an FH1–FH2-enhanced Fashion—Our previous studies indicated that filament assembly from profilin-ATP-actin requires the rapidly hydrolyzable MgATP to be bound to actin. No assembly occurred when the slowly hydrolyzable CaATP was bound to profilin-actin (32). Both the cleavage of the γ-phosphate of ATP and the release of inorganic phosphate are slow on CaATP-actin, and occur at random on CaATP-actin (42, 43), whereas the cleavage of the γ-phosphate occurs vectorially on MgATP-actin (42). Finally, profilin prevents actin assembly at formin-bound barbed ends with the nonhydrolyzable ATP analog AMP-PNP (24).

Several experimental designs can be proposed to test whether the cleavage of the γ-phosphate of ATP, or the release of P$_i$, are required for profilin function. The use of BeF$_3$-, which binds to ADP-F-actin and reconstitutes the ADP-P$_i$-F-actin transition state was precluded in this type of growth kinetic measurements because of its very slow rate of association to ADP-F-actin (44). Inorganic phosphate is known to bind ADP-F-actin and reconstitute the ADP-P$_i$-F-actin state in which the tetracoordinated phosphate ion geometry differs from the bipyramidal pentacoordinated geometry of the ADP-P$_i$-F-actin transition state that precedes ADP-P$_i$-F-actin in the ATPase reaction. We challenged the ability of profilin to prevent barbed end growth from MgATP-actin when filaments are assembled in the presence of inorganic phosphate (supplemental Fig. S1). The slight inhibition caused by saturating amounts of inorganic phosphate was not greater than when sulfate was used as a control in place of phosphate. These data may indicate that the affinity of inorganic phosphate for terminal subunits at the barbed end is greatly decreased in the presence of profilin, or profilin does not cap barbed ends in the reconstituted ADP-P$_i$-F-actin state.

To determine whether the release of inorganic phosphate is required for profilin to dissociate from free or formin-bound ends, we used CrATP, an exchange-inert analog of MgATP (45), which binds to the nucleotide-metal ion binding site on actin and allows polymerization into CrADP-P$_i$-actin filaments in which cleavage of the γ-phosphate has occurred but the phosphate remains strongly bound to the β- and γ-phos-
phates of ATP (45), in an ADP·P·F-actin structural state. We first verified that profilin bound CrATP-actin with the same affinity as MgATP-actin, using the quenching of tryptophan fluorescence as a probe (Ref. 39, and supplementary Fig. S2). In the absence of profilin, the rate of filament growth was identical with MgATP-actin and CrATP-actin, as reported (45). FH1–FH2 nucleated assembly of CrATP-actin (Fig. 4A), as with MgATP-actin (45). On the other hand, nucleation of filaments by FH1–FH2 was allowed from profilin-MgATP-actin (10, 15, 24), but totally abolished from profilin-CrATP-actin (Fig. 4B).

The effect of profilin on barbed end growth from CrATP-actin was assayed with free or FH1–FH2-bound barbed ends (Fig. 4C). Profilin blocked barbed end growth in both cases, in a substoichiometric ratio with respect to G-actin, and in a range of even lower concentrations of profilin when FH1–FH2 was bound to barbed ends. The effect of profilin in CrATP therefore was strikingly different from the one observed in MgATP (dashed lines in Fig. 4C). We conclude that by capping CrATP- or CrADP-P·bound barbed ends, profilin prevents CrATP-actin or profilin-CrATP-actin assembly. The experimental data were accounted for using the model described under “Experimental Procedures” and parameter values in Table 1.

In conclusion, the release of P_i appears indispensable for profilin dissociation from barbed ends and sustained barbed end growth from profilin-ATP-actin. Moreover, FH1–FH2 enhances this requirement. The higher affinity of profilin to cap formin-bound barbed ends is likely due to its interaction with the FH1 domain of formin.

CrATP Arrests Processive Growth from FH1–FH2 Beads in the Presence of Profilin—The above solution studies suggest that as long as P_i is not released, FH1–FH2 remains strongly bound to profilin-actin at arrested barbed ends. To confirm that release of P_i following ATP hydrolysis on actin is required for FH1–FH2-induced processive assembly of profilin-actin, beads coated with FH1–FH2 at low density were placed in a solution of profilin/F-actin at steady-state in physiological ionic conditions, in the presence of MgATP or CrATP. In the presence of MgATP, single filaments nucleated at the bead surface grew at a constant rate of 0.40 μm/min, in agreement with previous results (24). In the presence of CrATP instead of MgATP, no filament processive assembly was recorded, all beads remained bare for at least 40 min (data not shown). In a medium containing 10% CrATP and 90% MgATP, single filaments grew at an average rate of 0.32 μm/min for transient periods of time that were interrupted by pauses of several minutes during which the filament barbed end remained stably

![Figure 4](Image)

**Figure 4.** Profilin blocks filament growth from CrATP-actin at either free or FH1–FH2-bound barbed ends. **A**, FH1–FH2 nucleates polymerization of CrATP-actin in a dose-dependent fashion. ADP-actin (3 μM, 5% pyrenyl-labeled) was supplemented with 300 μM CrATP and immediately polymerized by addition of 100 mM KCl in the presence of FH1–FH2 as indicated. **B**, FH1–FH2 induces polymerization from CrATP-actin, as well as from MgATP-actin, but profilin blocks barbed end growth in the presence of CrATP-actin and allows productive growth from MgATP-actin. ADP-actin (1.5 μM, 5% pyrenyl-labeled) was supplemented with either 100 μM MgATP (black curves) or 300 μM CrATP (magenta curves) and polymerized in the presence of 50 nM FH1–FH2 and in the absence (thin curves) or presence (thick curves) of 20 μM profilin. C, profilin caps formin-bound CrATP-barbed ends with a higher affinity than free CrATP-barbed ends. The initial rate of elongation of barbed end growth from 1.5 μM actin (5% pyrenyl-labeled) was measured in the presence of 300 μM CrATP (solid lines) at free barbed ends (spectrin-actin seeds, cyan) and at FH1–FH2-bound barbed ends (50 nM FH1–FH2, magenta) in the presence of profilin as indicated. Solid curves are calculated using the model with the values of dissociation constants given in Table 1. Dashed curves (cyan, free barbed ends; magenta, FH1–FH2-bound barbed ends) represent the effect of profilin in MgATP (from Refs. 24 and 31).
attached to formin at the surface of the bead (Fig. 5A). Pauses shorter than 40 s were not visually detectable. Typical pauses of up to 380 s (blue curve) between two periods of growth are shown in Fig. 5B. Consistent with the biochemical data described above, the arrest of growth is interpreted within the capping of filaments, and induces bundling of filaments (Fig. 7C). Low speed centrifugation assays showed that FH1–FH2 co-sedimented with PACov filaments, not with filaments assembled from noncovalent profilin-actin, in agreement with light scattering data (Fig. 7D). This result confirms that FH1–FH2 interacts with the sides of PACov filaments, inducing formation of bundles.

**DISCUSSION**

Models proposed so far for the function of profilin in actin polymerization rely on an energy square according to which filament growth occurs via either association of one profilin-actin (PA) complex to a barbed end or association of free actin followed by profilin binding to the terminal subunit at barbed ends. Measurements of F-actin assembly at steady state in the presence of profilin showed that the energy square is not balanced in ATP, and profilin caused a decrease in the critical concentration of ATP-actin (29). These results led to the view that productive barbed end growth from profilin-actin occurred with direct coupling of ATP hydrolysis on the terminal subunit (29). In contrast, polymerization kinetic assays of filament growth from profilin-ATP-actin led to the opposite proposal that an isoenergetic square model could well account for the effect of profilin, and that profilin did not lower the critical concentration of ATP-actin (49). The controversy was clarified in a recent review (27) explaining that in the polymerization of ATP-actin, (i) the nature of barbed end-bound nucleotide, when filaments are either growing or in a dynamic steady-state in ATP, may be a mixture of ATP, ADP-Pγ, and ADP, which has to be considered in the energy square; (ii) the critical concentration of ATP-actin, meant as free ATP-G-actin concentration at steady-state, appears lowered by profilin both in steady-state (29) and in polymerization assays (49); and (iii) the possibility of an indirect coupling of ATP hydrolysis also existed (27).

The present work brings novel data supporting the direct coupling of ATP hydrolysis to profilin-actin assembly (27, 29,
We further demonstrate that the release of Pi is required for profilin to dissociate from the barbed end and allow sustained growth. Because release of inorganic phosphate on F-actin is known to be slow, these results suggest, but do not demonstrate, that the rate of Pi release is increased by profilin. Finally, we find that the properties of profilin are enhanced by barbed end-bound formin and used for the processive walk of formin at barbed ends.

Profilin binds to barbed ends in ADP and CrATP, thus preventing barbed end growth. The capping of barbed ends by profilin is enhanced by FH1–FH2, consistent with the simultaneous binding of FH2 to actin and FH1 to profilin at barbed ends. The finding that profilin caps barbed ends when terminal subunits are in the intermediate states of ATP hydrolysis (ATP/ADP-Pi and ADP) contrasts with the established fact that profilin, even at a very high concentration, allows filament growth when regular ATP hydrolysis is associated with filament elongation. These results cannot be accounted for by the simple model used so far, in which profilin binds only to the barbed end terminal actin subunit on which ATP is hydrolyzed in ADP, resulting in profilin dissociation.

The simplest alternative model is fully described under "Experimental Procedures" and illustrated in supplemental Fig. S3. A simplified version is displayed in Fig. 8. The model considers that during barbed end assembly in the presence of actin and profilin, the two terminal subunits at barbed ends make an elongating site that can bind actin, profilin, and profilin–actin in different ways. Barbed end configurations can be F_n, F_nP, F_nPS, and F_nPPS representing a filament that has n subunits and either no profilin or profilin bound to the terminal subunit (P) or subterminal subunit (P_s) or both. ATP is hydrolyzed at the terminal position but not at the terminal position.

In the presence of ADP or a non-hydrolysable ATP, the square model describing interaction of profilin with actin filaments is truly isoenergetic, hence it does not allow effective polymerization from profilin–actin, and barbed ends are capped by profilin, both with or without formin. In the presence of fully hydrolysable ATP, the square model is no longer isoenergetic, supporting polymerization from profilin–ATP–actin. In a regime of elongation, a barbed end constantly displays ATP and profilin bound to the terminal subunit. Association of a profilin–ATP–G–actin complex to this end triggers ATP hydrolysis on the penultimate actin subunit (F_nP_s and F_nPP_s), leading to release of profilin from this subunit. Further growth occurs via recycling filaments F_n and F_nP.

Interestingly, this model accounts for the fact (not accounted for so far) that profilin–MgATP–actin polymerizes with a barbed end critical concentration as low as unliganded MgATP–G–actin (32). The low value of the actin critical concentration at barbed ends is due to the persistent terminal ATP–or ADP–P_i–bound subunit at steady state, which the previous model (reviewed in Ref. 27) could not accommodate.

This model quantitatively accounts for all data globally (calculated curves in Figs. 2 and 4) using the values of equilibrium ATP–actin. In a regime of elongation, a barbed end constantly displays ATP and profilin bound to the terminal subunit. Association of a profilin–ATP–G–actin complex to this end triggers ATP hydrolysis on the penultimate actin subunit (F_nP_s and F_nPP_s), leading to release of profilin from this subunit. Further growth occurs via recycling filaments F_n and F_nP.
and rate parameters shown in Table 1. Although the model is formally simple, considering the role of two terminal subunits of the filament introduces 4 new equilibrium dissociation constants that could not be evaluated individually.

Several steric clashes occur, in particular between profilin bound to the penultimate subunit and the hydrophobic plug of the terminal subunit, if the two profilin-actins at the barbed end are assumed to adopt the same structure and orientation as all F-actin subunits in the body of the filament (50). A similar clash between the hydrophobic plug of the terminal subunit and the α-tentacle of Capping Protein has recently been observed (51). To resolve the conflict between biochemical and structural data, we propose that subunits at the barbed end may adopt a different structure, schematized by a tilted geometry in Fig. 8, which would allow the binding of profilin to the penultimate actin subunit. This possibility is supported by the fewer actin-actin bonds made by these terminal subunits. The fact that formin enhances the affinity of profilin for barbed ends suggests that this different structure/orientation of the terminal subunits is stabilized by formin. In this respect, in the crystal structure of FH2-actin (25), subunits are arranged in a pseudo-filament that is structurally different from the regular F-ADP filament, and may represent the packed repeat of a filament-barbed end. In support to our proposal, docking of profilin on the FH2-actin-barbed end structure (25) can be done with modest steric clashes.4 The proposed modified interaction between the two terminal profilin-actin subunits is consistent with the profilin-induced increase in the depolymerization rate (36). It is also possible that the change in orientation of the hydrophobic plug is in relation with ATP hydrolysis on the penultimate subunit. Sterical constraints would be relaxed upon ATP hydrolysis and dissociation of profilin from the penultimate ADP-actin subunit, restoring the regular ADP-F-actin orientation.

4 L. Renault, personal communication.
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FIGURE 8. Simplified model for actin assembly and ATP hydrolysis. This is a simplified version of the complete model described under "Experimental Procedures," which is illustrated in supplementary Fig. S3 and has been used in the fit to the experimental curves in Figs. 2 and 4 with parameters in Table 1. The two strands of a growing filament barbed end are represented. ADP-actin subunits are in gray, ATP- and ADP-Pi, actin are in yellow. Profilin is a red circle. The barbed end is capped in the intermediate state with two profilins bound to the terminal and penultimate subunits. A, self-assembly of actin alone is not coupled to ATP hydrolysis. B, filament assembly from profilin-actin occurs with coupled ATP hydrolysis and P_i release from the penultimate subunit. C, processive barbed end assembly of profilin-actin by formin occurs with coupled ATP hydrolysis and P_i release.

The elementary steps of this model are formally unaffected by formin, except for an increased affinity of profilin for formin-bound barbed end terminal subunits, allowing cycles of processive assembly (Fig. 8), in which ATP hydrolysis modulates the affinity of the transient complexes formed in the processive cycle (26, 27, 52). The FH1–FH2 makes a ternary complex with profilin and ATP-actin, stabilized by profilin-actin, FH1–profilin, and FH2-actin bonds. Hydrolysis of ATP and release of P_i from the penultimate subunit weakens profilin-actin and FH2-actin bonds, resulting in a global weakening of the ternary complex. Note that the structural change of the FH1–FH2-actin at barbed ends that allows processive growth is very similar to the postulated structural change that relieves the capped conformation of FH2-bound barbed ends in the crystal structure (25). In our model, this structural change is fostered by profilin. Profilin is the crucial coupling device in the formin machinery and is uniquely responsible for its processivity.

Due to permanent tethering of the actin filament, the formin profilin machinery differs from the N-WASP/Arp2/3 machinery that promotes movement via insertional actin association to fluctuating free ends, transiently tethered during branching (see Ref. 53 for a review). The formin profilin motor thus allows faster polymerization, and we expect it should also produce a higher force per growing filament than the few piconewtons produced by the growth of a free fluctuating end.

Our conclusion and model are at variance with other works on three points, discussed below. First, Kinosian et al. (34) concluded that filaments could be assembled from profilin-ADP-actin. However, the discrepancy is only apparent, because the data (Fig. 5 in Ref. 33) actually show that profilin inhibits assembly of ADP-actin. To reach the conclusion that profilin-ADP-actin polymerizes, data were modeled assuming that profilin bound ADP-actin with a high affinity. The same data would be fully consistent with our conclusions if they were modeled using a value of the K_d of profilin for ADP-actin in the micromolar range, as derived from the two independent methods presented here.

Second, filament assembly from profilin-ATP-actin was proposed not to require ATP hydrolysis (35), because a slight delay was recorded between polymerization of profilin-actin and release of P_i (Fig. 4B in Ref. 35); however, in that experiment, the initiation of rapid polymerization of [γ-32P]ATP-actin by ADP-F-actin seeds at equilibrium with ADP-G-actin resulted in 20% of the polymerizing actin that was not bound to [γ-32P]ATP, thus biasing the analysis of the coupling of actin assembly and ATP hydrolysis by generating an artifactual delay.

Third, Kovar and co-workers (23) reported processive growth of HMM-attached filaments from immobilized formin in ADP. The striking discrepancy with our results may have diverse origins, among which the production of ATP by myokinase that contaminates actin preparations (38) and may contaminate the HMM as well. In addition, due to the high surface: volume ratio in microscopy assays, myokinase may be concentrated by adsorption to the glass surfaces of the flow chamber. To test the possible effect of myokinase, we repeated all the experiments performed in ADP (Figs. 1 and 2) but omitting the myokinase inhibitor Ap5A. A transient overshoot polymerization was observed, indicating that ATP is present under these conditions and that after hydrolysis of residual ATP, profilin blocks ADP-actin polymerization (supplemental Fig. S4). The possible presence of ATP may explain the internal difficulties of the model proposed by Vavylonis et al. (54) to accommodate processive growth independent of ATP hydrolysis reported by Kovar et al. (54). The data were accounted for only if a value of 5 μM was used for the K_d of the profilin-ATP-actin complex, incompatible with the consensus values of K_d ranging from 0.05 to 0.2 μM. The model of coupled hydrolysis was considered as a valid alternative by Vavylonis et al. (54) who stated that they “tested the approximate effects of a coupled hydrolysis mechanism by violating detailed balance and were able to obtain a good fit to the elongation rate by using K_d = 0.1 μM (supplemental Fig. S2).”

The FH1–FH2 domain of mDia1 does not bind to the sides of actin filaments assembled from either actin or profilin-actin, in contrast to other formins like FRL, mDia2, and AFH1 (10, 48, 55, 56). On the other hand, FH1–FH2 remains bound to F-actin assembled from the covalently cross-linked profilin-actin complex, indicating that the flexibility of FH1–FH2 is sufficient to maintain the FH2-actin and FH1-profilin contacts even though the profilin-actin interface is disrupted following ATP hydrolysis. In regular processive assembly of noncovalent profilin-actin
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by FH1–FH2, profilin dissociation from actin following ATP hydrolysis causes translocation of a protomer of FH1–FH2 from the filament end, a step that is required for processivity. Within this view, formins like FRL, which bundle filaments by remaining bound to the sides of filaments may need an additional regulatory element to retain a processive function.

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