Filament Assembly from Profilin-Actin*

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§ The abbreviations used are: PA, profilin-MgATP-actin complex; NBD, 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole, EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; S1, myosin subfragment-1.

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Profilin plays a major role in the assembly of actin filament at the barbed ends. The thermodynamic and kinetic parameters for barbed end assembly from profilin-actin have been measured turbidimetrically. Filament growth from profilin-actin requires MgATP to be bound to actin. No assembly is observed from profilin-CaATP-actin. The rate constant for association of profilin-actin to barbed ends is 30% lower than that of actin, and the critical concentration for F-actin assembly from profilin-actin units is 0.3 μM under physiological ionic conditions. Barbed ends grow from profilin-actin with an ADP-Pi cap. Profilin does not cap the barbed ends and is not detectably incorporated into filaments. The EDC-cross-linked profilin-actin complex (PAcov) both co-polymerizes with F-actin and undergoes spontaneous self-assembly, following a nucleation-growth process characterized by a critical concentration of 0.2 μM under physiological conditions. The PAcov polymer is a helical filament that displays the same diffraction pattern as F-actin, with layer lines at 6 and 36 nm. The PAcov filaments bound phalloidin with the same kinetics as F-actin, bound myosin subfragment-1, and supported actin-activated ATPase of myosin subfragment-1, but they did not translocate in vitro along myosin-coated glass surfaces. These results are discussed in light of the current models of actin structure.

Profilin is a remarkable actin-binding protein. This small (15-kDa), ubiquitous, essential protein binds monomeric (G) actin in a 1:1 molar ratio (1). Although the profilin-actin complex is unable to spontaneously nucleate actin filaments, it can productively associate with the barbed ends specifically (2, 3). This unique property confers a dual function to profilin, depending on the capping of barbed ends. When barbed ends are blocked by capping proteins, profilin sequesters G-actin. In contrast, in motile regions of the cell where uncapped barbed ends are actively elongating, the profilin-actin complex can actively participate in filament growth. The profilin-MgATP-actin complex (PA1)1 can therefore be considered as an end-specific quasipolymerizable actin monomer. Recent results (4) show that this property of profilin is used to enhance the processivity of treadmilling (i.e. steady-state barbed end assembly) in the presence of actin depolymerizing factor. In the mechanism that was proposed based on thermodynamic data (5, 6), F-actin assembly from profilin-actin is possible only because of its coupling to ATP hydrolysis, as follows. Profilin-actin associates with the barbed end; the interaction of profilin with actin is weakened once the actin-bound ATP has been hydrolyzed; profilin then dissociates from that end, thus promoting the incorporation of one actin subunit in the filament and regenerating a free barbed end available for further growth from PA. Profilin is reused at each cycle and works as a catalyst of assembly. While polymerization of actin alone proceeds in a manner uncoupled from ATP hydrolysis (7, 8), these two reactions are proposed to occur in a compulsory order when actin filaments grow from profilin-actin units. In this paper, the proposed mechanism of barbed end growth from profilin-actin is challenged by kinetic experiments.

Understanding the kinetics of filament assembly from profilin-actin in depth also has implications concerning the structure of the filament. In the atomic model of the actin filament proposed by Holmes et al. (9) and refined by Lorenz et al. (10), the profilin interaction area on actin is exposed at the barbed end, accounting for the association of profilin-actin to a growing barbed end, but not to a pointed end. Hence, within Holmes’ model, it is anticipated that profilin can cap the barbed ends. On the other hand, examination of the actin-actin contacts in the crystals of the profilin-actin complex (11) shows evidence for a nonhelical ribbon structure of the profilin-actin complex, in which extensive actin-actin contacts, however different from those present in the Lorenz et al. atomic model of the actin filament (10) are involved. It was proposed (12, 13) that barbed end growth from profilin-actin could involve the transient extension of profilin-ATP-actin ribbons. The transition from ribbon to filament would be coupled to ATP hydrolysis and dissociation of profilin. According to this model, the profilin-actin ribbon would exist in solution only in the ATP- or ADP-Pi-bound form, and the orientation of the actin monomer in the filament derived from the ribbon would be different from the Holmes atomic model.

In the present work, we first determined the rate parameters for barbed end elongation from profilin-actin using a turbidimetric method. We next attempted to find conditions under which profilin-actin would undergo spontaneous self-assembly, with the goal to characterize the profilin-actin polymer. The EDC-cross-linked profilin-actin complex appears able to polymerize into filaments, which exhibit the same helical structure and same thermodynamic stability as native F-actin filaments. Profilin-actin filaments therefore provide a tool to probe the structure of the actin filament and the interface of actin with other actin-binding proteins.

MATERIALS AND METHODS

Proteins—Actin was purified from rabbit skeletal muscle acetone powder (14) and isolated as CaATP-G-actin through Sephadex G-200 chromatography (15) in G buffer (5 μM Tris/Cl, 0.1 mM CaCl2, 0.2 mM ATP, 1 mM dithiothreitol, 0.01% NaN3, pH 7.8). MgATP-G-actin (~20
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μm) was prepared by incubation of CaATP-G-actin with 0.2 mM EGTA and 1 mol eq plus 10 μM excess MgCl₂ for 3 min and used immediately afterward. MgATP-G-actin was polymerized by the addition of 2 mM MgCl₂ and 0.1 mM KCl. CaATP-G-actin was polymerized by the addition of 0.1 mM KCl. Actin was NBD-labeled (16). Spectrin-actin seeds were prepared from human erythroid cells, and their molar concentration was determined as described (17) and by titration by gelsolin. Gelsolin-actin seeds were prepared at a 2 μM concentration by incubation of 2 μM human plasma gelsolin (a generous gift from Dr. Yukio Doi) with 4.2 μM CaATP-G-actin in G buffer. Profilm was isolated from bovine spleen by poly(t-proline) affinity chromatography (18). S₁(A₁) and S₁(A₂) isoforms of chymotryptic myosin subfragment-1 were resolved by SP-trisarcyl chromatography (19).

Fluorescence and Light Scattering Measurements—Fluorescence measurements were carried out at 20 °C with a Spex Fluorolog 2 spectrofluorometer at the following wavelengths: NBD-actin, λex 475 nm and λem 530 nm; rhodamine-phallolidin, λex 530 nm and λem 575 nm; tryptophan fluorescence, λex 285 nm and λem 330 nm.

Turbidity Measurements—Turbidity measurements were performed at 20 °C at 310 nm using a Cary 1 Varian spectrophotometer with 1-cm path cuvettes. All solutions were thoroughly filtered and degassed before the experiment.

Measurement of the Initial Rate of Filament Elongation—Initial rates of filament growth were measured using turbidity or NBD fluorescence. Seed-free seeds were prepared by polymerization at the barbed end (20), while gelsolin-actin seeds initiated elongation at the pointed end. At time 0, MgATP-G-actin (or CaATP-G-actin), in the absence or presence of profilin or profilin-actin covalent complex, was supplemented simultaneously with seeds and salts, and the time course of assembly was recorded. The actin concentrations were chosen so that the spontaneous nucleation could be neglected.

The rate of barbed end growth J at a given total concentration of G-actin ([A₀]) and in the presence of different total amounts [P₀] of profilin was monitored turbidimetrically. Data were analyzed as follows,

\[ J = [S]kₕ^-1[A₀] + k₇^[P₀] \]  
(Eq. 1)

with [A₀] = [A] + [PA].

In Equation 1, [S] is the concentration of spectrin-actin seeds; [A] and [PA] are the concentrations of free and profilin-bound G-actin; and kₕ^-1 and k₇^[P₀] are the corresponding barbed end association rate constants. The contribution of the off rates was neglected in equation 1 because measurements were carried out at actin concentrations well above the critical concentration. The change in J as the concentration [P₀] of profilin was increased reflected the increase in the contribution of PA to barbed end assembly as G-actin was gradually saturated by profilin. The concentration of PA was calculated as follows,

\[ [P₀] = \frac{[Pₐ] + [A] + Kₐ₆[Pₐ] - [Pₐ][A] + [A] + [Pₐ][A] - 4[Pₐ][A]}{2} \]  
(Eq. 2)

where \( Kₐ₆ \) represents the equilibrium dissociation constant for profilin-actin.

The contribution of J in absorbance units/s were converted in μm assembled F-actin/s using a critical concentration calibration curve as described previously (21), from which the polymerization of 1 μM F-actin led to an increase of 0.0017 absorbance units at 310 nm (1-cm optical path).

Combining Equations 1 and 2 led to the expression of J as a function of [Pₐ], [A], and \( Kₐ₆ \) of analysis of the data within this expression led to the determination of \( kₕ^-1 \) (from the measurement of J in the absence of profilin), \( k₇ \) (from the measurement of J in the presence of saturating amounts of profilin), and \( Kₐ₆ \), by adjustment of the theoretical curve to the data at different concentrations of profilin.

The rate of barbed end growth was also measured at different concentrations of G-actin and either in the absence or in the presence of saturating amounts of profilin (i.e. 1 mol eq of G-actin plus an excess of 5 μM bovine profilin or of 30 μM Arabidopsis thaliana profilin 3 (Δ)).

The resulting \( f(ψ) \) plots for actin and profilin-actin were used to derive the values of \( kₕ^-1 \) for actin or profilin-actin and of the critical concentrations \( Cₗ \) for polymerization, as follows:

\[ J = [S]kₕ^-1[Cₗ] \]  
(Eq. 3)

All experiments were performed using freshly prepared G-actin in G buffer containing 1 mM dithiothreitol to be sure that Cys³⁴ raised thoroughly, so that all of the actin in the preparation was able to bind profilin with high affinity. We reasoned that if a very small proportion of G-actin was oxidized, it would give rise to spontaneous polymerization at high actin concentrations even in the presence of an excess of profilin. Therefore, where appropriate, the spontaneous polymerization measured in the absence of seeds was subtracted from the data.
surrounding the filaments was subtracted from the images, which were floated as 12 arrays. Fourier transforms were calculated. They were characterized by the presence of three layer lines (110, 111, and 112), which can be indexed by the selection rule l = −6n + 13m.

**Sedimentation Assay for Binding of S1 to F-PA cov**—Actin or PA cov complex in G buffer was freed from ATP by Dowex-1 treatment (25), polymerized at 5 μM, and split into samples supplemented with 0, 4, 6, 8, 10, or 20 mM S1(A1). Samples were centrifuged 15 min later at 400,000 × g for 10 min at 20 °C in the TL 100 Beckman ultracentrifuge. Pellets were resuspended in the original volume of G buffer. Supernatants and resuspended pellets were submitted to SDS-polyacrylamide gel electrophoresis and Coomassie Blue-stained.

**ATPase Measurements**—Actomyosin MgATPase activity of myosin subfragment-1 was measured in the presence of 17 μM F-actin or F-P Ac, and 1 μM S1(A1) at 20 °C in a buffer containing 5 mM Tris/Cl pH 7.8, 0.1 M KCl, 2 mM MgCl2, 1 mM ATP, 0.1% methylcellulose, pH 7.6) supplemented with oxygen scavengers. Observations were conducted using whole myosin tethered to monoclonal antibodies (anti LMM 5C3–2) immobilized onto a nitrocellulose-coated glass coverslip, as described (26). The rhodamine-phalloidin filaments were diluted to 3–5 μM in motility buffer (25 μM imidazole, 25 mM KCl, 1 mM MgCl2, 5 mM 2-mercaptoethanol, 0.2 mM CaCl2, 7.5 mM ATP, 0.1% methylcellulose, pH 7.6) supplemented with oxygen scavengers. Observations were made on a Zeiss microscope using a 100X Plan Apochromat objective equipped with epifluorescence optics. Images of moving filaments were recorded with a SVHS video recorder.

**RESULTS**

**Filament Assembly at the Barbed Ends from Profilin-Actin Requires Mg2+ and Not Ca2+ as Divalent Metal Ion Tightly Bound to G-actin**—Since profilin binds derivatized actin very poorly (27), the conventional method using the increase in fluorescence of N-phenylcarboxamidomethyl- or NBD-labeled actin as a probe of polymerization is inadequate in its presence. Turbidimetry, which has proven useful to monitor actin polymerization in the case of ADF/cofilin (21), was used. The initial rate of barbed end assembly from spectrin-actin seeds was measured at a given G-actin concentration and in the presence of increasing amounts of profilin. Fig. 2a shows that when filament barbed ends elongated from CaATP-G-actin subunits, profilin inhibited the growth in a concentration-dependent fashion. Total inhibition of growth was observed at saturation by profilin. Data, analyzed as described under “Materials and Methods,” indicate that profilin binds CaATP-G-actin with an equilibrium dissociation constant of 1.2 ± 0.2 μM and that the profilin-CaATP-actin complex does not participate in barbed end assembly. Preassembled F-actin (5 μM) was also used to measure barbed end growth in the presence of 5 μM CaATP-G-actin, either in the absence or presence of 20 μM profilin. Turbidity increased in the absence of profilin, demonstrating active barbed end growth, but decreased when profilin was added together with G-actin. 70% depolymerization of the F-actin seeds was observed within 5 min. In conclusion, in the presence of CaATP-actin, profilin acts as a pure G-actin-sequestering protein. In particular, it does not cap the barbed ends.

**In contrast,** when barbed ends elongated from MgATP-G-actin subunits, profilin caused only a partial (40%) inhibition of growth. The data (Fig. 2a) are quantitatively consistent with the view that profilin binds MgATP-G-actin with an equilibrium dissociation constant of 0.1 μM, and the profilin-MgATP-actin complex associates productively with the barbed ends of actin filaments. At saturation by profilin, filaments grew from profilin-actin units exclusively, at a rate 40% lower than from MgG-actin alone. No further inhibition was observed at concentration of profilin as high as 100 μM, again indicating no detectable capping of the barbed ends by profilin.

In conclusion, in the presence of CaATP-actin, profilin acts as a pure G-actin-sequestering protein. In contrast, the complex of profilin with MgATP-actin participates in barbed end assembly. The different behaviors of profilin-Ca-actin and profilin-Mg-actin are in agreement with the conclusion of previous works (5, 6) that the effective participation of profilin-actin in barbed end growth required ATP hydrolysis to be coupled to polymerization, a condition that was fulfilled for profilin-Mg-actin but not for profilin-Ca-actin. The hydrolysis of ATP during polymerization of profilin-Mg-actin was measured at a high concentration (46 μM Mg2+ γ-32P]ATP-G-actin 1:1 complex plus 60 μM profilin). A short sonication was applied immediately after the addition of salt to enhance the rate of polymerization by fragmentation. Filament assembly showed a short lag followed by a rapid increase in light scattering (complete in less than 1 min). ATP was hydrolyzed in a manner that showed no detectable uncoupling from polymerization. However, a definite proof that elongation is coupled to hydrolysis requires the demonstration that the rate of elongation be kinetically limited at high concentration by the rate of ATP hydrolysis.

**Kinetic Parameters for Barbed End Assembly from Profilin-Actin Complex**—The rate constants for profilin-MgATP-actin association to and dissociation from the barbed ends can be derived from the analysis of the dependence of the rate of
filament growth on profilin-actin concentration ($J(c)$ plot; Ref. 8). The $J(c)$ plots for Mg-actin and profilin-Mg-actin (Fig. 2b) were derived from growth rate measurements using spectrin-actin seeds. The values found for the association rate constants were $4.9 \pm 0.5 \, M^{-1} \, s^{-1}$ for Mg-actin, in agreement with Pollard and Mooseker (28), and $3.5 \pm 0.4 \, M^{-1} \, s^{-1}$ for profilin-Mg-actin. The rate constants for MgATP-actin and profilin-MgATP-actin dissociation from the barbed ends were derived from the ordinate intercepts of the plots. Values of $0.6 \pm 0.2 \, s^{-1}$ and $1 \pm 0.3 \, s^{-1}$ were found for actin and profilin-actin, respectively. In other words, when barbed ends are actively elongating from profilin-actin as well as from G-actin subunits, terminal subunits dissociate at a rate 1 order of magnitude lower than the rate of dissociation of ADP-F-actin ($8 \, s^{-1}$). In conclusion, the present kinetic data rule out a simple model for barbed end growth from profilin-actin, according to which actin incorporation and profilin release from the barbed end would be tightly coupled to $P_i$ release, as described in Fig. 7 (Scheme I).

In a range of high concentrations of profilin-actin, the $J(c)$ plot deviated from linearity and curved downward (Fig. 2b, inset), indicating that the association of profilin-actin units to barbed ends was rate-limited by another reaction. We propose this reaction to be ATP hydrolysis, since this feature appears to be characteristic of the barbed end growth from profilin-actin and was not observed when the elongation from G-actin was measured. Quantitatively identical data were obtained with bovine profilin and Arabidopsis profilin 3 (compare open circles and closed diamonds in Fig. 2b, inset).

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**Fig. 2.** Polymerization properties of profilin-actin complex. a, profilin-MgATP-actin, but not profilin-CaATP-actin, participates in filament growth at the barbed ends. Barbed end growth off spectrin-actin seeds was initiated by adding 2.8 mM spectrin-actin seeds, 2 mM MgCl$_2$, and 0.1 M KCl to a solution containing 3.5 mM MgATP-G-actin and profilin at the indicated concentrations in G buffer (closed circles). The same experiment was carried out with CaATP-actin at 5.1 mM in the presence of 4.6 mM seeds (open circles) and at 7.5 mM (open squares) in the presence of 3.7 mM seeds. Turbidity at 310 nm was monitored to determine the initial rate of elongation. The rate of elongation in the absence of profilin was normalized to 1. Solid lines were calculated using Equations 1 and 2 with $K_{PA} = 0.1 \, mM$ and $k_{PA}^{\text{on}} = 0.6 \, k_1^{A}$ for MgATP-actin, and $K_{PA} = 1.2 \, mM$ and $k_{PA}^{\text{on}} = 0$ for CaATP-actin. The inset represents typical raw data for 3.5 mM MgATP-actin in the presence of 0 and 6 mM profilin. b, kinetic parameters for filament barbed end assembly from profilin-MgATP-actin. The initial rate of barbed end elongation was monitored turbidimetrically at the indicated concentrations of MgATP-actin in the presence (open circles) or absence (closed circles) of a saturating amount of bovine profilin, 2.8 mM spectrin-actin seeds, 2 mM MgCl$_2$, and 0.1 M KCl. Inset, the $J(c)$ plot deviates from linearity at high profilin-actin concentration. Open circles, bovine profilin-actin; closed diamonds, Arabidopsis profilin-actin. Values of the control without seeds (closed triangles) have been subtracted from the data shown.
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Efforts were made to detect nonhelical ribbon-like extensions of filaments that might be transiently formed at the barbed ends in regimes of rapid growth. At very high G-actin concentration, ATP hydrolysis is known to be more largely uncoupled from ATP-actin stretches, possibly in a ribbon-like structure (12). Specimens undergoing rapid assembly at high concentrations of profilin-actin were rapidly observed in the initial stages of the reaction. No structural difference was seen between the core and the end-proximal regions of the filament, which remains bound to actin, a purification of the covalently cross-linked profilin-actin complex, was elaborated (see “Materials and Methods”).

Self-assembly of PA cov—The covalent profilin-actin complex was assayed for its nucleotide binding properties. ATP was found bound to the complex following gel filtration on Sephadex G-25 (PD10; Amersham Pharmacia Biotech) in G buffer containing no ATP. To assess whether profilin retained the property to enhance the rate of nucleotide exchange in the PA cov complex, [α-32P]-ATP was mixed with either noncovalent profilin-ATP or PA cov-ATP 1:1 complex (Ca-ATP-actin, in G buffer, with 20 μM free Ca 2+), in the stopped flow, and the increase in fluorescence of e-ATP associated with the exchange of e-ATP for bound ATP was monitored. Comparison of the nucleotide exchange kinetics on the noncovalent and covalent complexes, shown in Fig. 3a, demonstrates that, in the presence of 20 μM free Ca 2+ ions, the rate constants of nucleotide exchange kinetics on the noncovalent complex (CA-ATP-actin) were 0.085 s⁻¹ and 0.025 s⁻¹ for the noncovalent and covalent complex, respectively, while a rate constant of 0.0006 s⁻¹ was measured for nucleotide exchange on unliganded actin under the same conditions. Nucleotide exchange therefore was

Fig. 3. Functional properties of the PA cov complex. a, enhancement of the rate of nucleotide exchange. Noncovalent profilin-actin complex (obtained by mixing 2 μM ATP-G-actin 1:1 complex with 5 μM bovine profilin) or covalent profilin-actin-ATP 1:1 complex (2 μM) was mixed with 10 μM e-ATP in the stopped flow. The final concentrations were 1 μM profilin-actin, 5 μM e-ATP, and 20 μM free Ca 2+. The increase in fluorescence due to nucleotide exchange was monitored. Top curve, noncovalent profilin-actin complex (k e = 0.084 s⁻¹); bottom curve, covalent profilin-actin (k e = 0.025 s⁻¹). Note that the extent of fluorescence change is the same in both samples, indicating that 100% of the covalent complex is active for nucleotide exchange. b, effect of covalently cross-linked profilin-actin on F-actin assembly. MgATP-actin (15 μM) was polymerized by the addition of 2 mM MgCl2 and 0.1 M KCl in the absence (actin) or in the presence (actin + PA cov) of 1.5 μM PA cov. Polymerization was monitored turbidimetrically. Curve PA cov is the curve observed for polymerization of PA cov alone at 1.5 μM (the final turbidity level was 0.063 absorbance units). c, spontaneous assembly of profilin-actin covalent complex. Spontaneous aggregation of Mg-PA cov following the addition of 2 mM MgCl2 and 0.1 M KCl at concentrations (in μM) indicated on the curves is monitored by turbidimetry. The assembly of 8 μM Ca-PA cov was initiated by adding 0.1 M KCl. At time 70 min (arrow) the solution was sheared by pipetting, d, critical concentration plot for self-assembly of the covalent profilin-actin complex. PA cov was polymerized at 8 μM in F buffer for 8 h at room temperature and diluted in the same buffer to the indicated concentrations. Samples were kept at room temperature for 18 h before being centrifuged for 45 min at 400,000 × g. Closed circles represent the concentration of PA cov in the supernatant measured by the Bio-Rad assay. Open circles are the calculated amounts of polymerized PA cov derived from the difference between the concentrations of total PA cov and of PA cov in the supernatant.
accelerated 150- and 45-fold on noncovalent and covalent profilin-actin, respectively. The fact that the covalent profilin-actin complex is purified by poly-L-proline chromatography also indicates that the poly-L-proline binding property of profilin (and of profilin-actin) is not altered in the covalent complex. In conclusion, the covalent complex is biochemically very similar to the noncovalent complex.

PA$_{cov}$ was able to self-assemble upon the addition of salt. The time courses of spontaneous polymerization of actin alone, PA$_{cov}$ alone, and mixed actin and PA$_{cov}$ were compared (Fig. 3b). The turbidity curve observed when actin and PA$_{cov}$ were mixed together could not be described as the sum of the time courses recorded for actin alone and PA$_{cov}$ alone, which suggests that PA$_{cov}$ can copolymerize with F-actin. The effect of PA$_{cov}$ on the rates of filament growth at the barbed and pointed ends confirmed this conclusion. PA$_{cov}$ had no effect on the rate of growth at the pointed ends and very slightly slowed down the rate of G-actin assembly onto spectrin-actin seeds monitored by the change in fluorescence of NBD-actin, in agreement with the data shown in Fig. 3b. Notably, no high affinity capping of barbed ends by PA$_{cov}$ was observed. Altogether, these results indicate that PA$_{cov}$ can copolymerize with F-actin.

The kinetics of spontaneous polymerization of pure PA$_{cov}$ at different concentrations was examined by turbidimetry. The polymerization curves shown in Fig. 3c consisted in a lag phase followed by an exponential increase, suggestive of a nucleation-growth process similar to the polymerization of actin itself. The lag phase was much more pronounced for CaATP-PA$_{cov}$ than for MgATP-PA$_{cov}$. This feature again is strongly reminiscent of the slower nucleation of filaments from CaATP-actin than from MgATP-actin (31). Shearing of the polymers by pipetting accelerated the polymerization process of PA$_{cov}$, consistent with the view that fragmentation of the PA$_{cov}$ polymers increases the number of elongation sites, as observed for F-actin filaments. The extent of turbidity change at the end of the polymerization process increased linearly with the concentration of PA$_{cov}$ in the polymerizing sample. Samples of PA$_{cov}$ polymerized at different concentrations in the range 1.5–10 M were sedimented at 400,000 × g for 45 min when the turbidity plateau was reached. The concentration of unassembled PA$_{cov}$ present in the supernatants was 0.2 M for all samples. From these data, the specific increase in turbidity per M assembled PA$_{cov}$ was found to be 0.048 cm$^{-1}$ at 310 nm, a value 2-fold higher than the one (0.0017 ± 0.0002) measured for F-actin (21), indicating that the size of the PA$_{cov}$ polymer that scattered light was much larger than the size of the actin filament.

A conventional critical concentration plot was derived from measurements of the amounts of unassembled PA$_{cov}$ present in the supernatants of sedimented samples prepared by serial dilutions of a preassembled solution of 8 M PA$_{cov}$ followed by 18-h incubation at room temperature. Data shown in Fig. 3d demonstrate that PA$_{cov}$ polymerized with a critical concentration of 0.2 M.

The ATP bound to actin in the PA$_{cov}$ complex was hydrolyzed during the polymerization of PA$_{cov}$. When 7.7 M PA$_{cov}$ was polymerized in the presence of γ$^32$P-labeled ATP, no $^32$P was found in the pellet of the sedimented material at the end of the polymerization process. 75 M P$_i$ were found in the supernatant after 16 h, indicating that PA$_{cov}$ filaments turn over.

In conclusion, the polymerization of PA$_{cov}$ shares mechanistic similarities with the polymerization of F-actin.

**PA$_{cov}$ Polymerizes into Helical Filaments—**The structure of the PA$_{cov}$ polymer was examined by electron microscopy. Observation of negatively stained specimens of assembled PA$_{cov}$ at steady state showed a homogeneous population of bundled filaments. The ends of the bundles often appeared blunt, suggesting that the filaments that composed the bundle grew together in a synchronous fashion. The kinetics of formation of these bundles was examined in electron microscopy and turbidity simultaneously, at a concentration of PA$_{cov}$ low enough (1.5 μM) for the different steps of nucleation and growth to be clearly time-resolved. Electron micrographs of the polymerizing sample at different times of the polymerization process shown in Fig. 4 indicate that during the lag time only short individual filaments were formed, which gradually interacted with each other in bundles. Essentially, bundles of ~4–6 filaments were visible on the grid at the end of the lag phase (t = 6 min) when the change in turbidity was less than 1% of the total change. Bundles became thicker with time. The Fourier transforms of the PA$_{cov}$ filaments displayed clear layer lines at spacings of 36 and 6 nm, corresponding to the first and sixth layer lines of F-actin. These results indicate that PA$_{cov}$ polymerizes into helical filaments that have the same helical periodicities as F-actin. Fourier transforms of the bundles observed at later stages of assembly show an identical pattern. Bundling most likely results from the change in charge of filaments due to profilin, which is a basic protein. Native filaments contain repulsive charges that maintain a distance between each polymer in solution. Binding of ligands can abolish the repulsion between filaments and favor their mutual interactions (32).

The poly-L-proline binding site exposed at the surface of profilin may also mediate hydrophobic contacts between the PA$_{cov}$ filaments and favor the formation of bundles. Accordingly, bundling was less extensive at low ionic strength.

To understand whether the bundling resulted from the EDC/NHS and/or dimethyl sulfoxide treatment of actin, in a control experiment the uncross-linked actin recovered from the poly-L-proline affinity column at the end of the PA$_{cov}$ preparation (see “Materials and Methods”) was supplemented with 30% Me$_2$SO and processed like the covalent complex. Turbidity measurements showed that this G-actin material polymerized at the same rate and to the same extent as standard actin and did not form bundles (data not shown).

Binding of phalloidin to the PA$_{cov}$ filaments was monitored by the increase in fluorescence of tetramethylrhodamine-phalloidin (33, 34). The time courses for binding 1 mol eq of tetramethylrhodamine-phalloidin to 1.6 M either F-actin or F-PA$_{cov}$ were superimposable (Fig. 5), indicating that the binding site of phalloidin is very similar in the two structures.

**Interaction of the Myosin Head with the Profilin-Actin Filament—**Several assays were carried out to understand how the cross-link of profilin to actin affects the actin-myosin interface and its functional properties. The binding of myosin subfragment-1 to standard filaments and PA$_{cov}$ filaments in the absence of ATP was examined in parallel in a sedimentation assay. Data shown in Fig. 6 show that S$_1$(A$_1$) binds tightly to PA$_{cov}$ filaments; however, the stoichiometry was lower than the 1:1 value confirmed here for S$_1$ binding to F-actin. A maximum of 0.4–0.5 S$_1$ was bound per PA$_{cov}$ subunit.

In the electron microscope, filaments assembled from PA$_{cov}$ and partially decorated (as above) by S$_1$ failed to display the conventional arrowhead decoration by S$_1$. The myosin heads were attached irregularly to the PA$_{cov}$ filament with randomly distributed orientations. No diffraction pattern could be derived from the images (data not shown).

In an ATP-free low ionic strength buffer, S$_1$(A$_1$) is known to induce the rapid formation of G-actin-S$_1$ oligomers, followed by their slower condensation into arrowhead-decorated F-actin-S$_1$ filaments. These two reactions can be monitored by light scattering (19, 35). Under those ionic conditions, no oligomer nor any assembly process could be detected within 1 h when S$_1$(A$_1$) at concentrations up to 20 μM was added to PA$_{cov}$-ATP 1:1.
On the other hand, the MgATPase of S$_1$(A$_1$) was clearly enhanced by PA$_{cov}$ filaments. The rate of ATP hydrolysis, under different ionic conditions (presence versus absence of 0.1 mM KCl), was 2.6-fold lower than the ATPase rate measured in the presence of the same amount of F-actin (17 μm) in a parallel sample.

The sliding movements of actin and PA$_{cov}$ filaments over myosin-coated glass surfaces were assayed in parallel. While actin filaments moved at a speed of 6.6 ± 1.2 μm/min, the profilin-actin filaments remained immobile and firmly attached to the myosin-coated surface. Filaments containing 50% actin and 50% PA$_{cov}$ moved almost as well as standard actin filaments with an average speed of 3.9 ± 1.0 μm/min and displayed a discontinuous, somewhat stick-slip motion. Filaments containing 10% PA$_{cov}$ moved at the same speed and displayed the same regular motile behavior as standard filaments.

Overall, these data suggest, but do not prove, that the actin-S$_1$ interface remains functional in the profilin-actin filament, but some steric hindrance due to the presence of profilin inhibits the movement of the myosin head, which is necessary for its translocation along actin filaments. Similarly, the fact that S$_1$ fails to induce oligomer and F-acto-S$_1$ assembly from PA$_{cov}$ indicates that S$_1$ cannot interact with 2 PA$_{cov}$ molecules as it does with G-actin.

**DISCUSSION**

Under physiological ionic conditions (MgATP-actin, 2 mM MgCl$_2$, 0.1 mM KCl), filaments actively elongate from profilin-actin units, at a rate that is only 30–40% lower than from G-actin subunits. These results are in agreement with an earlier report (36), which used Acanthamoeba profilins and the Limulus acrosomal processes as seeds; however, we find no evidence for a weak capping activity of profilin even at physiologically high concentrations. Profilin is not incorporated into the filaments, even in the ATP- or ADP-P$_i$-bound state, at variance with the expectations from a ribbon-to-helix model.

Kinetic data show that filament growth from noncovalent profilin-actin, although mechanistically coupled to ATP hydrolysis, is not kinetically coupled to P$_i$ release. Evidence for this conclusion is provided by the low critical concentration for barbed end assembly from profilin-actin and extrapolation of the J(c) plot to a low value of the dissociation rate constant $k_-$, which is inconsistent with the presence of rapidly dissociating ADP subunits at the barbed ends in a regime of growth. In the proposed alternative scheme (Fig. 7), growth from profilin-actin and ATP hydrolysis are tightly coupled, and a stabilizing ADP-P$_i$ cap (37) prevents the occurrence of rapidly dissociating ADP subunits at the ends of elongating barbed ends. The downward curvature of the plot at high profilin-actin concentration (with both bovine and plant profilins) is due, within this scheme, to ATP hydrolysis, which kinetically limits profilin release and subsequent filament growth. The rate of ATP hydrolysis would be about 80 s$^{-1}$ under physiological ionic conditions and would be consistent with the value measured here for the association rate constant (4.9 μM$^{-1}$s$^{-1}$). If a higher value of the association rate constant is used, e.g. 10 μM$^{-1}$s$^{-1}$ (38), then the corresponding ATPase rate would be 163 s$^{-1}$. J(c) measurements carried out at lower ionic strength (1 mM MgCl$_2$, no KCl) yielded a limit value of J(c) of 25 ± 5 s$^{-1}$ (data not shown). This value is in reasonable agreement with results showing that the rate of hydrolysis accompanying filament elongation reached a limit of 13 s$^{-1}$ in the presence of 1 mM MgCl$_2$ (29).

The noncovalent profilin-actin complex can, to some extent, be considered as a polymerizing monomer with a low critical concentration of 0.3 μM. This view corroborates in vivo observations of stabilization of actin filaments in cells overexpressing profilin (39). When filaments are assembled at steady state in the presence of profilin, the partial critical concentrations of
The positions of S1 volume of loaded pellets corresponds to a 2 times higher amount of the consistent with previous results (5). The excess profilin does polymerization of G-actin and profilin-actin, respectively; [PO] (not shown) showed that in the F-actin plus S1 sample, S1 increases linearly in the supernatant above a total concentration of 5 μM, consistent with a maximum binding ratio of 0.4 S1 to TMR-phalloidin at time indicated by the arrow.

The experimental facts do not fully meet the behaviors expected from either model. The data show that the covalent profilin-actin complex was expected to be unable to self-assemble, due to steric conflict between subdomain 2 and profilin along the long pitch helix. On the other hand, PAcov was expected to efficiently cap the barbed ends of actin filaments. Within Schutt’s hypothesis, the covalent profilin-actin complex was expected to possibly polymerize into a nonhelical ribbon polymer.

The huge loss in affinity of profilin for actin following cleavage of actin-actin contacts are similar in F-actin and in F-PAcov filaments (40) described by following equations,

\[
\frac{[A_c]}{C_1} + \frac{[PA]}{C_2} = 1 \quad \text{(Eq. 4)}
\]

\[
[PA_c] = [P_c] + \frac{[A_c]}{K_F} \quad \text{(Eq. 5)}
\]

where C1 and C2 are the intrinsic critical concentrations for polymerization of G-actin and profilin-actin, respectively; [Pc] is the total concentration of profilin; and KF is the equilibrium dissociation constant of the profilin-actin complex. Within the above equations, [Ac] decreases from 0.1 μM to 0 and [PAc] increases from 0 to 0.3 μM as Pc increases. This evolution is consistent with previous results (5). The excess profilin does not cap the barbed ends up to 100 μM.

The covalently cross-linked profilin-actin complex provides a tool to probe the structure of the actin filament. The EDC-cross-link made here between a carboxylate of G-actin, preactivated by EDC, and an NH2 group of bovine spleen profilin is thought to be the homolog of the EDC cross-link between Glu364 of actin and Lys125 of profilin in the Acanthamoeba profilin-actin complex. According to Schutt et al. (12), this corresponds to a cross-link between Glu364 of actin and Lys125 of profilin in the vertebrate profilin-actin complex. The covalent profilin-actin complex retained the divalent metal ion/nucleotide binding properties of the noncovalent profilin-actin complex. However, its assembly properties were very surprising.

Considering the two current models of actin assembly of Holmes et al. (9, 10) and of Schutt et al. (11), two alternative behaviors were expected. Within the Holmes/Lorenz model of the filament, the covalent profilin-actin complex was expected to be unable to self-assemble, due to steric conflict between subdomain 2 and profilin along the long pitch helix. On the other hand, PAcov was expected to efficiently cap the barbed ends of actin filaments. Within Schutt’s hypothesis, the covalent profilin-actin complex was expected to possibly polymerize into a nonhelical ribbon polymer.

The huge loss in affinity of profilin for actin following cleav-
age of ATP is a very strong assumption that has to be made to interpret the present data within Holmes' model, in particular to account for the low critical concentration for PA\textsubscript{cov} assembly. It would explain why profilin does not cap the barbed ends. It implies that the simple cleavage of ATP causes a very large structural change of subdomains 1 and 3, which does not appear in the atomic model built from the structure of CaATP-G-actin. This implication needs to be challenged by further experiments. The loss in affinity of profilin for actin might also tentatively be explained by the unfolding of profilin following the covalent cross-link; this explanation, however, seems unlikely, since the native properties of profilin-actin (poly-L-proline binding, enhancement of nucleotide exchange) appear to be conserved in the PA\textsubscript{cov} complex, indicating that cross-linked profilin is not unfolded.

Another possible interpretation of our data, which would be inconsistent with Holmes' model, is that both the profilin-G-actin interface and the actin-actin bonds that build the F-actin filament are conserved upon polymerization of the covalent profilin-actin. In the absence of covalent link, the loss in affinity of profilin for F-actin would not allow the profilin to remain bound to F-actin. This interpretation also accounts for the failure of profilin to cap the barbed ends, but it implies that the orientation of actin in the filament is different from both the Holmes and Schutt models. High resolution analysis of the structure of the F-PA\textsubscript{cov} filament and image reconstruction are now under way to solve those issues.

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**Fig. 7. Possible mechanisms for filament barbed end assembly from profilin-actin complex.** The experimental $J(c)$ plots corresponding to barbed end growth from G-actin (thin line) and profilin-actin (thick line) are drawn in comparison with the theoretical $J(c)$ plot expected for profilin-actin within Scheme I (dashed line). Scheme II accounts for the experimental $J(c)$ plot for profilin-actin.
