Effects of Profilin and Thymosin β₄ on the Critical Concentration of Actin Demonstrated in Vitro and in Cell Extracts with a Novel Direct Assay*

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The free actin concentration at steady state, A°, is a variable that determines how actin regulatory proteins influence the extent of actin polymerization. We describe a novel method employing fluorescence anisotropy to directly measure A° in any sample after the addition of a trace amount of labeled thymosin β₄ or thymosin β₃ peptide. Using this assay, we confirm earlier theoretical work on the helical polymerization of actin and confirm the effects of actin filament-stabilizing drugs and capping proteins on A°, thereby validating the assay. We also confirm a controversial prior observation that profilin lowers the critical concentration of Mg²⁺-actin. A general mechanism is proposed to explain this effect, and the first quantitative dose-response curve for the effect of profilin on A° facilitates its evaluation. This mechanism also predicts the effect of profilin on critical concentration in the presence of the limited amount of capping protein, which is the condition often found in cells, and the effect of profilin on critical concentration in cell extracts is demonstrated for the first time. Additionally, nonlinear effects of thymosin β₁ on the steady state amount of F-actin are explained by the observed changes in A°. This assay has potential in vivo applications that complement those demonstrated in vitro.

Total cellular actin is equal to the sum of the concentration of free monomer (the critical concentration, A°), the concentration of unpolymerized actin sequestered by various actin-binding proteins, and the concentration of actin polymer or F-actin. Changes in A° induced by actin regulatory proteins influence actin polymer dynamics by an amplification mechanism that results in large changes in the amount of sequestered, unpolymerized actin. Quantitative evaluation of models of cytoskeletal function requires accurate knowledge of the value of A°. Unfortunately, A° has not yet been measured in cells and has only been estimated by very indirect methods in cell extracts (1). In vitro methods have been developed that allow for the measurement of F-actin using birefringence (2), viscosity (3), light scattering (4), centrifugation (5), binding of labeled phalloidin (6), or the fluorescence of pyrenyl-labeled actin (7).

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Methods such as the DNase I binding assay yield the sum of A°, and an indeterminate fraction of sequestered actin monomer (8). Clever uses of combinations of data have in some cases allowed investigators to subtract F-actin content from total actin and then fractionate the contributions of A°, and monomer sequestration (9, 10), but the results have proven controversial (11, 12).

Based on theoretical considerations, the critical concentration is expected to be equal to the ratio of the rate constants of association and dissociation of actin subunits from actin filaments. Measurement of these rate constants using a method such as electron microscopy can be used to indirectly evaluate A°, assuming the applicability of the theory (13, 14). However, the participation of actin bound to monomer-sequestering proteins in filament growth, such as occurs with profilin-actin (15, 16), greatly complicates this analysis, because such addition alters the observed rate constants but, in theory, can occur with or without (17) a change in A°.

The observation that actin monomer-sequestering proteins such as profilin (9) and members of the actobindin family of multirepeated thymosin β₄-like sequences (18) may alter A° is controversial (11), and moreover, postulated mechanisms for this effect have been disputed (11, 12, 19). The participation of profilin (or of proteins in the actobindin family) in barbed end elongation is an independent observation that may occur with or without an effect on A°. Two basic mechanisms were suggested during the last decade (9, 20).

The hypothesis that profilin could lower A° by formation of a copolymer of actin monomer and actin in complex with profilin was first suggested in Ref. 20 when it was found that the cross-linked profilin-actin complex could be polymerized into filaments. According to this hypothesis, free actin and profilin-actin complex would both contribute a partial critical concentration driving polymer elongation. Although there is tantalizing crystallographic evidence that a copolymer is possible (21), experimental evidence for copolymerization is mutually exclusive with evidence of barbed end capping by profilin (12, 22, 23). Although incorporation of profilin into actin filaments would be a condition required for the copolymerization model (24), no significant incorporation of profilin into actin filaments was found experimentally when profilin was not covalently cross-linked to actin (20). Instead, there are data that suggest that covalent or other high affinity profilin-actin complexes interfere with actin polymerization (22, 23). The addition of the profilin molecule to the barbed end of the actin filament is often called “capping” because most of the literature agrees that the profilin molecule is bound to the barbed end blocks further elongation, that is, profilin must dissociate from the end before the next actin subunit (free, or in complex with profilin) can be added. There is a disagreement in the literature on whether the
effect of capping by profilin is significant. Some researchers assume that the affinity of profilin to the barbed end is very low and can be neglected in the modeling of actin polymerization process. Capping by profilin is very different from the capping by gelsolin or Cap Z, because unlike capping proteins that block both elongation and depolymerization, profilin blocks elongation but increases the rate of depolymerization (12, 25).

A hypothesis for coupling ATP hydrolysis to the profilin pathway (9) has been suggested as a possible explanation of profilin effect on actin critical concentration. It is suggested that barbed end elongation by profilin-actin could lower $A_i$ if the free energy change upon the addition of an actin subunit to F-actin is different when monomer is directly added to the barbed end (9, 12). The thermodynamic difference does not have to be large (-2 kcal/mol) to explain the existing data but does require direct or indirect coupling of ATP hydrolysis to addition of the profilin-actin complex. Using a novel direct assay, we have obtained the first dose-response curve for the effect of profilin on actin critical concentration. That allowed us to evaluate the hypothesis of coupled ATP hydrolysis as well as an alternative hypothesis for the effect of profilin. We formulate a general description of actin polymerization in the presence of profilin. According to this mechanism, the main effect of profilin is the acceleration of actin polymerization dynamics.

**EXPERIMENTAL PROCEDURES**

*Proteins and Peptides—Pyrene-labeled and unlabeled rabbit skeletal muscle Ca$^{2+}$-actin, recombinant human profilin, and recombinant rat thymosin $\beta_4$ (identical in sequence to human) were purified as previously described (26). For labeling with tetramethylrhodamine-maleimide (T-6027; Molecular Probes Inc., Eugene, OR), the thymosin $\beta_4$ cDNA was modified by the addition of a C-terminal cysteine. A truncated e-amino rhodamine-labeled peptide (1–25 rhod-th$\beta_4$ peptide) was produced synthetically with a sequence that corresponds to the N-terminal 25 residues of thymosin $\beta_4$, SDKPDMAIEKFKDKNKKT TETQEK-rhodamine. Human recombinant gelsolin was truncated to the N-terminal 25 residues of thymosin $\beta_4$ peptide

Measurements of Critical Concentration in Cell Extracts—Calf brain cell extracts prepared from 200 grams of frozen bovine brain cut into very thin slices and placed in 100 ml of ice-cold extraction buffer (20 mM HEPES, 0.2 mM CaCl$_2$, 0.5 mM ATP, 1 mM dithiothreitol, 0.1 mM EGTA, 10% glycerol, pH 7.8) was generated with vertically excited polarized light at 546 nm in an L or T format steady state fluorimeter. The KCl concentration was 8.0 mM for the depolymerization assay was started immediately after polymerization was complete. The initial depolymerization rate was calculated from the ADP-actin for muscle actin is more than 5 min (19), and the depolymerization experiment (see Fig. 5A) and vertical (4, 10). The thermodynamic difference does not have to be large (-2 kcal/mol) to explain the existing data but does require direct or indirect coupling of ATP hydrolysis to addition of the profilin-actin complex. Using a novel direct assay, we have obtained the first dose-response curve for the effect of profilin on actin critical concentration. That allowed us to evaluate the hypothesis of coupled ATP hydrolysis as well as an alternative hypothesis for the effect of profilin. We formulate a general description of actin polymerization in the presence of profilin. According to this mechanism, the main effect of profilin is the acceleration of actin polymerization dynamics.

The equilibrium dissociation constant of thymosin $\beta_4$, $K_{d_0}$, so the observed anisotropy $r$ is calculated using

$$r = r_i + (r_o - r_i)(K_{rT} + A_i + T_o)$$

$$= r_i/((K_{rT} + A_i + T_o) + 1/2$$

$$= -r_i/((r_i - r_o)/(K_{rT} + A_i + T_o))$$

$$= -K_{rT}/((r_i - r_o)/(K_{rT} + A_i + T_o))$$

$$= -K_{rT}/((r_i - r_o)/(K_{rT} + A_i + T_o))$$

$$= -K_{rT}/((r_i - r_o)/(K_{rT} + A_i + T_o))$$

$T_o$ is the total concentration of labeled thymosin $\beta_4$ peptide, and $A_i$ is the total G-actin concentration for each value of $r$. Fitting parameters include only $r_o$, $r_i$, and $K_{rT}$. Calculation of the free actin concentration $A_i$ at a sample at steady state is straightforward using the measured anisotropy value and other parameters defined by calibration with a standard curve. Based on the equation $[A] = T_o/[A]/(K_{rT} + [A])$ with $r$ as defined above, $[A] = [\beta] + [\beta]_0/[r_i - r_o]$. For measurements at steady state, $A_i = [A]$. Assuming that thymosin $\beta_4$ and G-actin are at rapid equilibrium, the equations (and the assay) are also valid for calculation of [A] prior to steady state, for example, for determination of the free actin concentration as a function of time during actin polymerization (as in Fig. 3B).

Correlation for Possible Ternary Complexes of Profilin, Actin, and Thymosin $\beta_4$ in Calculation of $A_i$ Based on Anisotropy Data—The equation for $A_i$ when profilin, actin, and thymosin $\beta_4$ form a ternary complex

$$A_i = ([\beta] + [\beta]_0/[\beta]_0)^2 + ([\beta] + [\beta]_0/[\beta]_0)^2$$

$$= ([\beta] + [\beta]_0/[\beta]_0)^2 + ([\beta] + [\beta]_0/[\beta]_0)^2$$

We assume here, as supported by our previous data (26), that any ternary complex of actin, thymosin $\beta_4$, and another actin-binding protein, for example profilin (PAT), has the same value of $r_o$ as for the actin-thymosin $\beta_4$ complex. Then in the presence of actin and profilin $T_o = [A] + [\beta]_0$ and $T_o = [T] + [\beta]_0$ at equilibrium or steady state conditions, [AT] and [PAT] are defined by $A_i$, total concentrations of thymosin $\beta_4$ and actin, and equilibrium dissociation constants $K_{rT}$, $K_{T}$, and $K_{AT}$ for formation of ternary complexes $\beta_4$, $\beta_4$, and PAT, respectively. That means that $r$ is completely defined by $A_i$, $T_o$, $T_o$, $r_o$, and equilibrium constants. At relatively low concentrations of thymosin $\beta_4$ ($T_o < K_{rT}$), which is always the case for our experiments with both profilin and thymosin $\beta_4$, the ternary complex $T_o$ becomes independent of $T_o$. So, if $T_o > T_o$, and equilibrium constants are known, one can define $A_i$ from anisotropy measurements. The values for $r_o$, $r_i$, and the equilibrium constants are already defined for several ionic conditions in our previous papers (and these parameters do not depend strongly on conditions), but they were independently verified by additional calibrations for each condition employed in the current manuscript.

Depolymerization Experiment—0.5 μM 10% pyrene-labeled Mg$^{2+}$-actin polymerized from spectrin seeds was diluted immediately upon reaching the saturation level of polymerization to 0.1 μM in the same polymerization buffer (5 mM Tris-HCl, pH 7.9, 0.1 mM CaCl$_2$, 0.125 mM EGTA, 2 mM MgCl$_2$, 40 mM KCl); the predicted free Ca$^{2+}$ concentration for this buffer is 44.5 nM) containing various concentrations of profilin. Depolymerizing actin filaments should contain predominantly ADP-ATP or ADP-P, actin at the barbed end because the $t_o$ of phosphate release from ADP-actin for muscle actin is more than 5 min (19), and the depolymerization assay was started immediately after polymerization was complete. The initial depolymerization rate was calculated from the depolymerization time course and plotted against profilin concentration (see Fig. 5C).

Measurements of Critical Concentration in Cell Extracts—Calf brain cell extracts prepared from 200 grams of frozen bovine brain cut into very thin slices and placed in 100 ml of ice-cold extraction buffer (20 mM HEPES, 0.2 mM CaCl$_2$, 0.5 mM ATP, 1 mM dithiothreitol, 100 mM potassium acetate, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.5). A small amount of homogenizer (PESTLE B) was used to open the cells, and 0.6 mM diisopropyl fluorophosphate was added to the extract. The whole cell lysate was centrifuged at 35,000 × g for 1 h, and supernatant was frozen in liquid nitrogen. Fluorescence anisotropy was recorded as described above, except special attention was paid for control and correction of the effects of absorption and light scattering by the samples. To suppress the effect of light scattering, emission fluorescent was recorded at the wavelength of 575 nm rather than 568 nm. To control for light scattering, the base-line fluorescence of each sample was recorded before the addition of labeled thymosin $\beta_4$, and the initial reading was subtracted from the level of fluorescence in presence of thymosin $\beta_4$. Base-line fluorescence corresponded to 1–3% of the final sample reading. Fluorescence anisotropy was calculated for corrected and uncorrected fluorescence levels, and the results showed no significant difference. To control for the effect of light absorption in the samples, the G factor was separately recorded for each sample. Profilin was depleted from the extract by the addition of concentrated poly-
line beads equilibrated with the same extraction buffer used to prepare cell extracts. After incubation for 10 min, the beads were removed by low spin centrifugation. The dilution of the sample because of bead addition was calculated based on initial and final volumes, and the control sample was diluted by the same factor. Nonmuscle (platelet) actin was used for calibration of anisotropy measurements in cell extracts. The affinity of platelet actin to labeled thymosin β4 in cell extract buffer was two or three times higher than that of muscle actin.

**Nonmuscle Actin—**Lymphopilized platelet actin was purchased from Cytoskeleton (APHL99). 100 μl of H2O and 500 μl of G buffer were added to 1 mg of lymphopilized actin. After 30 min of incubation at room temperature 200 μl more of G buffer was added. Actin was centrifuged for 1 h at 4 °C and 65,000 rpm. 850 μl of supernatant was withdrawn, and the concentration was defined through optical density measurements at 290 nm with an extinction coefficient of 26 μg·cm⁻¹·cm⁻².

**RESULTS AND DISCUSSION**

**Validation of the Anisotropy Assay for Measurement of the Free Actin Concentration—**We and others have made frequent use of steady state fluorescence anisotropy measurements to determine the equilibrium binding kinetics of various fluorescently labeled actin-binding proteins to actin. Specifically, we have previously used this technique to assess binding of thymosin β4 to actin (26). The binding isotherm for this interaction, when plotted as anisotropy versus total actin for trace levels of rhodamine-labeled thymosin β4 (or synthetic 1–25 rhod-tβ4 peptide), provides a standard curve for determination of the free actin concentration in any sample in which the anisotropy is determined under the same standard conditions (Fig. 1, A and B). Full-length, recombinant thymosin β4 peptide interacts with actin with a $K_d$ of 0.30 ± 0.04 μM, and the synthetic peptide 1–25 rhod-tβ4 binds with a $K_d$ of 6.2 ± 0.5 μM. Because the assay is most accurate when the binding constant of the labeled thymosin β4 peptide is similar to the free actin concentration (or for $A_o$, when measuring at steady state), the use of 1–25 rhod-tβ4 peptide provides a standard curve with utility at higher free actin concentrations than does intact thymosin β4. We previously showed that thymosin β4 competes with rhodamine-labeled thymosin β4 in this anisotropy assay; with a dose response as expected if both have similar affinities for actin (26). Moreover, because labeled thymosin β4 also inhibits binding by profilin, either competitively (9) or noncompetitively (26), the use of thymosin β4 uniquely determines the free actin concentration in the presence of either or both of these actin monomer-sequestering proteins. Binding of 1–25 rhod-tβ4 peptide to actin is inhibited by profilin in a manner consistent with either competitive or noncompetitive inhibition, with the equilibrium dissociation constant for the ternary complex formation $K_{DT}$ ≈ 80 μM (Fig. 1A, inset). If noncompetitive, then the binding of peptide to profilin-actin is so weak as to make a correction for ternary complex formation unnecessary for the data reported here. We confirm that the fraction of thymosin β4 bound to F-actin is insignificant at concentrations up to 30 μM (Fig. 1C), in agreement with prior data that implied that if such binding occurred, the $K_a$ was several millimolar (10). This observation simplifies the analysis of the anisotropy data, because the anisotropy of labeled thymosin β4 will therefore only reflect binding to monomeric actin.

As expected based on polymer theory, (28), the critical concentration of rabbit muscle skeletal actin of 0.16 ± 0.03 μM is shown to be independent of total actin concentration (Fig. 2). Capping the barbed end of actin filaments with gelsolin increases the $A_o$ value to 0.8 ± 0.2 μM, a value consistent with previous reports (29). Calcium actin in 100 mM KCl is shown to have $A_o$ of 0.7 ± 0.2 μM, as previously reported (30). The actin-filament stabilizing drug jasplakinolide decreases $A_o$ to 0.08 ± 0.02 μM as speculated by us based on indirect evidence (31). The monomer sequestering drug latrunculin A does not lower $A_o$ either in the presence (1.0 ± 0.3 μM) or in the absence (0.22 ± 0.04 μM) of gelsolin, as expected for an agent that only sequesters monomeric actin (32).

**Effects of Profilin on the Critical Concentration—**Using the anisotropy assay, profilin is seen to lower $A_o$ when the barbed ends of Mg²⁺-F-actin are free, but not when they are capped by

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**Fig. 1. Anisotropy assay measures concentration of free actin.**

A, rhodamine-labeled thymosin β4 binds to Mg²⁺-actin. The buffer contains 40 mM KCl and 2 mM MgCl₂ with 0.1 μM labeled thymosin β4. Anisotropy was measured after conversion to Mg²⁺-actin but before polymerization occurred. Full-length thymosin β4 binds to actin (circles), with the line showing the best fit to $K_d = 0.30 ± 0.04 μM$. The 1–25 thymosin β4 fragment (1–25 rhod-tβ4) binds more weakly to actin (squares) ($K_d = 6.2 ± 0.5 μM$). Inset, profilin displaces 1–25 rhod-tβ4 fragment from actin (open triangles). The actin concentration is 5.0 μM, and the line is the best fit to the data using the same $K_a$ (6.2 μM) for the fragment, $K_{DT} = 0.2 μM$ for profilin and actin, and $K_{DT} = 80 μM$ for the ternary complex of actin, profilin, and the fragment. B, linearization of data in A shows no systematic deviation (function B is defined under “Experimental Procedures”). C, the fraction of rhodamine-labeled thymosin β4 bound to F-actin is insignificant at concentrations up to 30 μM of F-actin. There is no significant variation in anisotropy of samples of varying F-actin concentration with 0.1 μM labeled thymosin β4 (closed circles, left axis), consistent with binding of labeled thymosin β4 only to free actin monomer. Similarly, a pelleting assay shows no dose-dependent decrease in the amount of thymosin β4 found in the supernatant fraction after pelleting of F-actin with increasing actin concentration (open circles, right axis). Moreover, pelleting of F-actin in the anisotropy samples had no effect on the observed anisotropy, confirming that the measured binding activity was to G-actin (data not shown). The error bars represent 2 σ.
Relative to sequestered actin, and when F-actin concentration is large (0.1 mM CaCl₂ and 100 mM KCl (open squares)), gelsolin segments 1–3 at a fixed ratio of 1:100, gelsolin segments 1–3 to actin (closed circles), and gelsolin as before with 1.5 μM latrunculin A (closed triangles). For calcium actin, A, was measured in 0.1 mM CaCl₂ and 100 mM KCl (open diamonds).

gelsolin (Fig. 3A). The free actin concentration, followed as a function of time, demonstrates a unique end point when steady state is approached from either G- or F-actin (Fig. 3B). Although both assays adequately track F-actin concentration (Fig. 3B, inset), relative to the measurement of F-actin by pyrenyl fluorescence, the anisotropy assay does not require labeled actin, yields data for A₀ and not the sum of A₀ and sequestered actin, and when F-actin concentration is large relative to A₀, does so more precisely. Only small amounts of profilin are necessary to alter A₀, and A₀ varies continuously as a function of profilin concentration (Fig. 4A). When barbed ends are capped by gelsolin, the assay does not reveal any change of A₀ at profilin concentrations up to 6 μM (Fig. 4B).

The Mechanism of the Effect of Profilin on Actin Critical Concentration—The data shown in Figs. 3 and 4A provide a basis for a quantitative test of theories regarding the mechanism by which profilin decreases A₀. A derivation of the equations for the effect of profilin on A₀ is provided in the "Appendix," and the results are as follows. The rate of change in the concentration of F-actin subunits, A₀, is,

\[ dA₀/dt = f₀(k_p + w_0 h_0)([A] - A₀) \]  

(Eq. 3)

where

\[ A₀ = (k_p + w_0 h_0)(k_p + w_0 h_0) \]  

(Eq. 4)

\[ w = (1 + (k_p h_0)(P)K_{p0} + (1 + [P]K_{p0}) \]  

(Eq. 5)

\[ w_0 = (1 + (k_p h_0)(P)K_{p0} + (1 + [P]K_{p0}) \]  

(Eq. 6)

At steady state, \( dA₀/dt = 0 \), and

\[ [A] = A₀ \]  

(Eq. 7)

In these equations, \( f₀ \) is the concentration of actin polymer (i.e. the concentration of filaments). The constants \( k_p, k_p, k_p, h_0, h_0, h_0 \) are the respective elongation and dissociation rate constants from the pointed and barbed ends of F-actin, respectively. The primed rate constants are for the addition of profilin-actin and the loss of profilin-actin occurring at the barbed end. The constants \( K_{p0} \) and \( K_{p0} \) are equilibrium dissociation constants for profilin for monomers and barbed ends, respectively, and \([A]\) and \([P]\) are the concentrations of free actin and free profilin. Parameters \( w_0 \) and \( w \) provide relative weighting factors for the barbed end off and on rates, respectively. Parameter \( R \), characterizing the misbalance of the apparent energy square, is defined in the "Appendix," and \( R = 1 \) when the energy square is balanced; in this case \( w_0 = w \). At saturation with profilin, \( w = w_{sat} = (k_p h_0 P)K_{p0} \) and equals \( -100 \) according to published values for the rate and equilibrium constants. The value of \( w_0 \) varies from 1 to \( w_{max} = w_{sat}/R \). Fig. 4A (inset) demonstrates the dependence of \( w \) on profilin concentration when the energy square is assumed to be balanced and when the rate constants are equal to those used in Fig. 4A in the fit to the data corrected for formation of ternary complex.

As can be seen from Equation 4, the measured values of \( A₀ \) yield a weighted average of the critical concentration at each end of an actin filament. Profilin, by accelerating the addition and removal of actin subunits from the barbed end, would be predicted to increase the effective weight of the barbed end, lowering \( A₀ \) (Fig. 5, A–C). Acceleration of removal is because of a difference in off rates for profilin-actin and actin (Refs. 12 and 25 and Fig. 5C), whereas the acceleration of addition is because of the increased abundance of profilin-actin relative to free actin, with similar on rates for both species (Ref. 16 and Fig. 5B). The data from the two independent experiments shown on Figs. 4A and 5C are fit with the same sets of parameters.

Critical Concentration Changes Induced by Profilin with a Balanced Energy Square—According to the published values of rates constants (33), the effect of pointed ends cannot be neglected in the absence of profilin, and either \( h_0 - h_0 \) or \( k_p - h_0 \). Then in the absence of profilin \( A₀ = (k_p - h_0)(k_p - h_0) \), the value intermediate between the pointed end critical concentration, \( A_{cp} = k_p - h_0 \), and the barbed end critical concentration, \( A_{cb} = k_p - h_0 \), is introduced by guest on July 18, 2018
critical concentration, $A_{cb} = k_{b\rightarrow h}k_{b}$. When the energy square is balanced, $w_g = w$, and the change of critical concentration with increasing profilin concentration occurs through the change of relative contributions of the barbed and pointed ends in polymerization and depolymerization events. In the absence of profilin $w = 1$ and $A_c > A_{cb}$. At saturating profilin concentration, $w$ reaches 100, and $k_{b\rightarrow h} = \frac{w}{w_h}, k_{b\rightarrow p} = \frac{w}{w_p}$, and $A_c$ decreases to $A_{cb}$. The data in Fig. 4A are fit with parameters: $R = 1$, $A_{sp} = 0.8 \mu M$, $k_{b\rightarrow h} = k_{b\rightarrow p} = 10 \mu M^{-1} s^{-1}$, $K_{pb} = 13.8 \mu M$, $k_{p\rightarrow p} = 2.24 \mu M^{-1} s^{-1}$, $A_{ob} = 0.02 \mu M$, and $K_{pb} = 0.15 \mu M$ for the data corrected for the formation of a ternary complex of actin, profilin, and thymosin $\beta_4$ (closed symbols), and $k_{p\rightarrow p} = 2.0 \mu M^{-1} s^{-1}$, $A_{ob} = 0.04 \mu M$, and $K_{pb} = 0.07 \mu M$ for the uncorrected data (open symbols). The range of predicted values for the critical concentration of the barbed end (0.02–0.04 $\mu M$) is very low, but previously reported experimental values have been in this range (9, 34).

Contribution of an Energy Imbalance to the Effect of Profilin on Critical Concentration—If the effect of pointed end dynamics on critical concentration is negligible, i.e. $k_{p\rightarrow b} \ll k_{b\rightarrow h}, k_{b\rightarrow p} \ll k_{b\rightarrow p}$, then $A_c = k_{h\rightarrow p}A_{ob}$, then saturating profilin concentrations $A_c = (w_R k_{b\rightarrow p})(w_k k_{b\rightarrow p}) = A_{cb}/R$. In this case profilin affects critical concentration only through the use of ATP hydrolysis coupled with the profilin pathway. A physical interpretation of this result is that profilin lowers $A_c$ to that of a barbed end with terminal subunits containing ATP, and there is evidence that the nucleotide content of terminal subunits influences $A_c$ (35). With a balanced energy square and insignificant contribution from pointed ends, $A_c = A_{cb}$. Importantly, this means that the fact that the profilin-actin complex can add to the barbed end, by itself, cannot explain the effect of profilin on critical concentration. The data in Fig. 4A can be fit indistinguishably from the pictured theoretical curve, assuming only energy imbalance with parameters, $k_{p\rightarrow b} = k_{b\rightarrow h} = 0$, $k_{b\rightarrow p} = k_{b\rightarrow p} = 10 \mu M^{-1} s^{-1}$, $A_{ob} = 0.16 \mu M$, $K_{pb} = 0.20 \mu M$, and $K_{pb} = 15.4 \mu M$, $R = 7.5$. The fit to the data in Fig. 4A is very sensitive to increasing $R$ and becomes poor when $R$ exceeds 7.5.

A “mixed mechanism” resulting from the combination of increased barbed end weighting and energy imbalance could explain the effect of profilin on critical concentration. When the contributions of the pointed ends are non-negligible and $R > 1$, then profilin may not only lower $A_c$ to that of the barbed end but also further lower $A_c$ to that of a barbed end with terminal subunits containing ATP. The data in Fig. 4A can also be well fit with these assumptions, with the best fit obtained with parameters of $k_{p\rightarrow b} = 1 \mu M^{-1} s^{-1}$, $A_{sp} = 0.8 \mu M$, $k_{b\rightarrow p} = k_{b\rightarrow p} = 10 \mu M^{-1} s^{-1}$, $A_{cb} = 0.1 \mu M$, $K_{pb} = 0.18 \mu M$, and $K_{pb} = 14.7 \mu M$, $R = 4.7$.

Increased Dynamics at the Barbed End—The steady state subunit flux on the barbed end increases with concentration of profilin (Fig. 5B). The on flux (Equation 8) and off flux (Equation 9) are as follows (see “Appendix”).

$$f(k_{b\rightarrow p}[A] + k_{b\rightarrow p}[PA]) - f w_h k_{b\rightarrow p}[A]$$

$$f w_h k_{b\rightarrow p}[k_{b\rightarrow p} + w_h k_{b\rightarrow p}]/(k_{b\rightarrow p} + w_h k_{b\rightarrow p}) - f w_h k_{b\rightarrow p}$$

The increase of the on flux occurs because both actin and profilin-actin complex add to the barbed end, and even if $[A]$ decreases with profilin concentration, the total sum of unpolymerized actin $[A] + [PA]$ still exceeds the critical concentration in the absence of profilin. The increase of the off flux is demonstrated in the experimental data shown in Fig. 5C. Note that the same sets of parameters used to fit the data on Fig. 4A also fit these depolymerization data. Fig. 5B shows the relative contributions from different terms at steady state conditions corresponding to that of Fig. 4A. The set of parameters corresponding to a mixed mechanism is demonstrated here, although any of the sets of parameters show that the barbed end dynamics at 10 $\mu M$ profilin is greatly enhanced compared with the dynamics in the absence of profilin.

Importance of Depolymerization Term for Actin Polymerization Dynamics—Fig. 5D shows relative contributions from different terms at elongation conditions when the total actin concentration is 3 $\mu M$ and the profilin concentration is 0 or 10 $\mu M$. It is clearly seen that the total depolymerization rate is about 35% of the total polymerization rate, and the term corresponding to profilin pathway contributes to about 80% of the net depolymerization. Although it is commonly assumed that this term is negligible and therefore ignored, this result shows that term should not be neglected in models describing actin polymerization.
polyproline-treated extracts, $A_c$ is three times higher than the $A_c$ in untreated extracts, and both are relatively low. The effect of profilin on $A_c$ is consistent with predictions based on the assumption that cell extracts have limited amounts of capping protein. The inset to Fig. 6 shows the predicted dependence of critical concentration on profilin when $95\%$ of the barbed ends are capped. The lines correspond to the parameter sets with $R/H_{11005}$ 1 (dotted line), 4.7 (dashed line), and 7.5 (solid line) used to fit the data on Figs. 4A and 5C. At these conditions the predicted dependences of critical concentration on profilin differ significantly for different values of $R$. This is in contrast to the data in Figs. 4A and 5C, for which three sets of parameters were defined that fit all of those data equally well. The two data points (circles) correspond to the average values received in the two experiments shown on the main panel for the treated and untreated extracts and to our best estimate for the profilin concentration in these extracts.

The effect of profilin on $A_c$ is consistent with predictions based on the assumption that cell extracts have limited amounts of capping protein. The inset to Fig. 6 shows the predicted dependence of critical concentration on profilin when $95\%$ of the barbed ends are capped. The three different sets of parameters used to fit the data in Fig. 4A (with $R = 1, 4.7$, or 7.5) were used to generate the theoretical curves. Numerical computer simulation done in Ref. 36 with the set of parameters corresponding to $R/H_{11005}$ 14 gives similar results. The two data points on the inset correspond to the average values received in polyproline-treated and untreated extracts and to our best estimate for the profilin concentration in these extracts.

First, note that in these conditions of subtotal capping, profilin now is expected to have a marked effect on $A_c$. Note that in contrast to the data in Fig. 4A, which do not distinguish between energy balance and imbalance because those data can be fit by a range of values for $R$, the dependence of $A_c$ on profilin with partial capping should be highly sensitive to the value of $R$. Thus, the data obtained in cell extracts are consistent with the predictions of the kinetic parameters used to fit the data in Fig. 4A, which were determined in an earlier study. Because profilin alters the filament number by sequestration of actin monomer, the fraction of capped filaments will be different in samples that differ only in their

**Fig. 5. Relative contributions of barbed and pointed ends to filament dynamics in presence and absence of profilin.**

**A**, cartoon demonstrating the effect of profilin on the rates of elongation and dissociation at the barbed and pointed ends at steady state. The top diagram illustrates reactions at each end in the absence of profilin, and the bottom diagram illustrates reactions in the presence of profilin (P). The width of the arrow indicates the relative rate of the reaction at steady state. Saturation by profilin accelerates the dissociation of subunits from the barbed end (12, 25) and accelerates the association of subunits in proportion to the formation of profilin-actin complex and the fraction of filaments not capped by profilin. **B**, contribution of barbed ends and pointed ends to the rates of subunit addition and loss (as free monomer or as profilin-actin) in the absence and presence of profilin at steady state conditions. The inset to Fig. 5C demonstrates reactions at each end in the absence of profilin, and the bottom diagram illustrates reactions in the presence of profilin, at steady state conditions. The inset to Fig. 5C illustrates reactions at each end in the absence of profilin, and the bottom diagram illustrates reactions in the presence of profilin, at steady state conditions.
allow these complexes to polymerize, then thymosin $\beta_4$ may deplete gelsolin available for capping and lower $A_c$. But this hypothesis does not explain why thymosin $\beta_2$ also decreases critical concentration for uncapped filaments. Also, nucleotide exchange on G-actin seems to be irrelevant to the effect of thymosin $\beta_4$ on $A_c$ because the presence of profilin in the experiment with thymosin $\beta_2$ and completely capped filaments does not change the $A_c$ when compared with that determined for thymosin $\beta_4$ alone (data not shown).

**Implications for Actin Dynamics**—Rapid growth of actin filaments in local regions of a cell with dynamic filament assembly is assumed to occur through the addition of the profilin-actin complex to the barbed end (38). Profilin has relatively high affinity for globular actin (G-actin), and at moderate concentrations of profilin a large amount of un polymerized actin is present in a form of profilin-actin complex. The concentration of free actin, $A_c$, defines the concentration of profilin-actin complex in any given intracellular region in which other factors, such as VASP, may bind profilin so as to increase local profilin concentration (39). Thus, the ability to determine the effect of actin regulatory proteins on $A_c$ is absolutely essential to predicting how these proteins work in cells. Toward this end, our preliminary data show that the anisotropy technique described here works well in cell extracts, yielding results within the limits of those previously reported (1) and consistent with theoretical predictions. Most promisingly, fluorescence anisotropy methods are suitable for microscopic applications in live cells, with measurements of both local and whole cell anisotropy being feasible (40).

The concentration of free actin is a critical parameter not only at steady state conditions but also during polymerization. As seen from Equation 3, the polymerization rate is proportional to the value of $(A_c - A_s)$, and this value may become very small under conditions when almost all unpolymerized actin is present in the form of complex with actin-binding proteins. With increasing concentration of profilin, the term $(k_{+} + w_{b,c})$ may become large, but the value of $(A_c - A_s)$ would decrease faster and at some conditions may even become negative, which would lead to fast depolymerization of actin. Acceleration of actin polymerization dynamics by profilin may play an important regulatory role in cells when rearrangements of the cytoskeleton occur within a short period of time. Also, in conditions in which temporal or spatial concentration of capping protein becomes insufficient to cap all of the barbed ends, changes in profilin concentration may switch actin filaments from depolymerization to fast polymerization, and fine regulation of actin polymerization by profilin in ensemble with capping proteins is possible.

The value of $R$ depends on a combination of various factors that include, but are not limited to, the energy of ATP hydrolysis, the energy of the phosphate release, and the rate of the phosphate release. Also, in general, $R$ may be a function of profilin concentration. The importance of phosphate release is supported by the fact that the critical concentration is very low in the presence of inorganic phosphate (35). Of interest, the observed difference in the values of $R$ for muscle and nonmuscle actin correlates with the difference in the phosphate release rates. For muscle actin $R$ varies between 1 and 14 (9, 11) and is as high as 33 for nonmuscle actin (12). Published data confirm that the rate of phosphate release for yeast actin is much faster than for muscle actin (41). The faster rate of release may lead to less energy dissipation and therefore to a larger potential difference in free energy between the pathways for addition of profilin-actin and actin alone. These differences may reflect differences in the physiological function of actin derived from various sources.

**Fig. 7. Steady state effects of thymosin $\beta_4$ on gelsolin-capped Mg$^{2+}$-F-actin are explained by alteration of $A_c$.** A, pyrenyl-actin assay for monomer sequestration by thymosin $\beta_4$ has a nonlinears dependence on thymosin $\beta_4$ so that the data cannot be explained with any single $K_d$ (dashed lines). However, the data are well fit assuming a single $K_d$ and a variable $A_c$ determined by fluorescence anisotropy (solid lines). Samples from the left have 0 (squares), 1 (circles), 2 (triangles), 4 (inverted triangles), 8 (diamonds), 16 (leftward triangles), and 24 $\mu$M (rightward triangles) thymosin $\beta_4$. B, fluorescence anisotropy confirms that thymosin $\beta_4$ causes a dose-dependent decrease in $A_c$ (closed circles). The line provides values for critical concentration as a function of thymosin $\beta_4$ that are used in $A_c$. Assuming a $K_d$ of 0.12 $\mu$M, the best fit to the data in A for $A_c$ for each concentration of thymosin $\beta_4$ (shown as open triangles) corresponds well to the experimental data in B.

**Effects of Thymosin $\beta_4$ on the Critical Concentration**—At high concentrations, thymosin $\beta_4$ has been observed to cause nonlinear effects on the steady state amount of F-actin (Ref. 10 and Fig. 7A). Proposed explanations have included the possible formation of copolymers of actin and thymosin $\beta_4$-actin complex or an effect on $A_c$ (10). Here we demonstrate that thymosin $\beta_4$ decreases the $A_c$ value of capped actin filaments (Fig. 7B). Thymosin $\beta_4$ has a very similar effect on $A_c$ when filaments are uncapped (data not shown). The effect of dose response on $A_c$ is much different for profilin and for thymosin $\beta_4$, and no detectable change in $A_c$ is induced at low concentrations such as that used for rhodamine-labeled thymosin $\beta_4$ in the anisotropy assay. Steady state data for the fluorescence of pyrenyl-labeled actin cannot be fit by a single $K_d$, assuming constant $A_c$ (Fig. 7A). However, using values for $A_c$ as a function of thymosin $\beta_4$ as determined by a fit to the fluorescence anisotropy in Fig. 7B, the single $K_d$ of 0.12 $\mu$M (determined as in Fig. 1) is shown to fit all of the data reasonably well. Similarly, assuming a single $K_d$ of 0.12 $\mu$M, the critical concentrations required to explain the data in Fig. 7A are shown to correspond to the experimental values determined by anisotropy in Fig. 7B. Although the data are empirically consistent, we know of no satisfactory explanation that accurately predicts the dependence of $A_c$ on thymosin $\beta_4$ concentration. An effect of thymosin $\beta_4$ caused by an interaction with F-actin is ruled out by the data in Fig. 1C. Thymosin $\beta_4$ may bind to the complex of gelsolin with actin dimers or small oligomers, thereby sequestering the gelsolin from solution. If thymosin $\beta_4$ binds to gelsolin-actin complexes with affinity of ~5–10 $\mu$M and does not
of F-actin subunits, the absence of profilin, the rate of change in the concentration of actin complex. Assumptions that equilibrium between free profilin and both G-actin and barbed ends establishes much faster than the net rate of polymerization were used here. These assumptions are consistent with the agreement in literature that the dissociation rate of profilin from the barbed end (which is the measure of the establishment of equilibrium) is very fast. Then 

\[
d_A/\text{d}t = f_0(h_p + h_b, \lambda) \left( k_p + k_p^c \right) \text{d}P \left( \lambda \right) \text{d}A \]

where \( f_0 \) is the concentration of actin polymer (i.e. the concentration of filaments) and \( k_p^c \) and \( k_p^d \) are the elongation and dissociation rate constants from the pointed end of F-actin.

When profilin is present,

\[
d_A/\text{d}t = f_0(h_p, \lambda) \left( \frac{1}{k_d^\text{actin}} + \frac{1}{K_d^\text{GDP}} \right) \text{d}P \left( \lambda \right) \text{d}A \]

with

\[
A = (k_p + h_b) \left( h_p + h_b \right)
\]

**APPENDIX**

**Thermodynamic Constraints**—The thermodynamic energy square (9, 12) describes the interdependence of the binding and/or rate constants for two different pathways for filament elongation (Fig. 8). Without ATP hydrolysis involved, when all reactions are reversible, as for example, in the case for ADP actin, the two pathways are energetically identical and the ratio \( R = \left( \frac{k_b^+}{k_b^-} \right) \left( K_{d}^\text{actin} \right)^2 \left( \frac{k_b^d}{k_b^c} \right) \) is 1, where \( k_b^+ \) and \( k_b^- \) are the respective elongation and dissociation rate constants from the barbed end of F-actin, \( K_{d}^\text{actin} \) and \( K_d \) are the equilibrium dissociation constants of profilin for monomers and the barbed end, respectively, and the primed rate constants are for the addition of profilin-actin and the loss of profilin-actin occurring at the barbed end. \( R \) is the factor of misbalance that is equal to 1 if the energy level is satisfied.

In the case when ATP hydrolysis is involved, \( R \) may no longer be equal to 1, and, in general, a function of profilin concentration. According to data by different authors (9, 11, 12), \( R \) varies between 1 and 33. There is also evidence that hydrolysis of actin is not fast enough to be coupled with profilin dissociation (19), that implies that hydrolysis of ATP is not directly involved in the profilin pathway. A factor of 33 for \( R \) would correspond to free energy input of about 2 kcal/mol, corresponding approximately to the energy of ATP hydrolysis without dissociation of inorganic phosphate. At conditions existing in cells, the complete energy of ATP hydrolysis with the inorganic phosphate dissociation corresponds to 11.5 kcal/mol. That would correspond to \( R = 1.6 \times 10^8 \) if the total energy of ATP hydrolysis was utilized for polymerization. However, there is an agreement in the literature that the whole energy of ATP is not used for actin polymerization and that only a small part of it, if any, might be involved in the profilin pathway.

**Equations for the Interaction of Actin and Profilin-Actin**—In the absence of profilin, the rate of change in the concentration of F-actin subunits, \( A_\text{act} \) is

\[
d_A/\text{d}t = -d[A]/\text{d}t = f_0(h_p^\text{act} + h_b^\text{act}) \left( \frac{1}{k_d^\text{actin}} + \frac{1}{K_d^\text{GDP}} \right) \text{d}P \left( \lambda \right) \text{d}A \]

with

\[
A = (k_p + h_b) \left( h_p + h_b \right)
\]

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Effects of Profilin and Thymosin β4 on the Critical Concentration of Actin Demonstrated in Vitro and in Cell Extracts with a Novel Direct Assay
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