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The Control of Actin Nucleotide Exchange by Thymosinβ_4 and Profilin. A Potential Regulatory Mechanism for Actin Polymerization in Cells

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We present evidence for a new mechanism by which two major actin monomer binding proteins, thymosinβ_4 and profilin, may control the rate and the extent of actin polymerization in cells. Both proteins bind actin monomers transiently with a stoichiometry of 1:1. When bound to actin, thymosinβ_4 strongly inhibits the exchange of the nucleotide bound to actin by blocking its dissociation, while profilin catalytically promotes nucleotide exchange. Because both proteins exchange rapidly between actin molecules, low concentrations of profilin can overcome the inhibitory effects of high concentrations of thymosinβ_4 on the nucleotide exchange. These reactions may allow variations in profilin concentration (which may be regulated by membrane polyphosphoinositide metabolism) to control the ratio of ATP-actin to ADP-actin. Because ATP-actin subunits polymerize more readily than ADP-actin subunits, this ratio may play a key regulatory role in the assembly of cellular actin structures, particularly under circumstances of rapid filament turnover.

INTRODUCTION

In this paper we explore the possibility that the exchange of nucleotide bound to actin monomers is regulated by two antagonistic actin binding proteins, profilin and thymosinβ_4. We propose that these reactions are part of a switch that control the assembly of actin into filaments in cells. As such there are interesting parallels with other cellular proteins that bind and hydrolyze a specific nucleotide, either ATP (i.e., HSP70) or GTP (i.e., $p21^{ras}$, G-proteins).

The interaction of actin with adenine nucleotides has been well characterized (Figure 1). Each actin molecule binds an adenine nucleotide in the cleft between the two major domains (Kabsch *et al.*, 1990). The nucleotide bound to actin monomers can exchange with nucleotides in the medium. Dissociation of the bound nucleotide is the rate limiting step ($k = 10^{-2}\ s^{-1}$ for ATP) when physiological concentrations (mM) of ATP are present in the medium. After assembly into a filament, the actin subunits hydrolyze irreversibly the bound ATP to ADP and inorganic phosphate. Noncovalent interactions retain these products. The phosphate dissociates slowly, whereas the ADP does not and therefore is essentially nonexchangeable (Korn *et al.*, 1987; Carlier *et al.*, 1988; Carlier 1989; Pollard, Goldberg, and Schwartz, unpublished data). ATP hydrolysis is not necessary for actin polymerization, but it confers asymmetry to the filament ends, making the critical concentration for polymerization one order of magnitude larger at the slowly growing pointed end than at the rapidly growing barbed end of filaments (Wegner, 1976; Wegner and Isenberg, 1983; Pollard, 1986). Depolymerization of actin filaments releases ADP-actin monomers. ADP-actin will also polymerize, but the critical concentration is 5–10 times higher than for ATP-actin (Pollard, 1986; reviewed by Korn *et al.*, 1987; Carlier, 1989; Pollard, 1990). Furthermore, ADP-actin monomers form the nuclei which initiate polymerization much slower than ATP-actin monomers (Lal *et al.*, 1984).

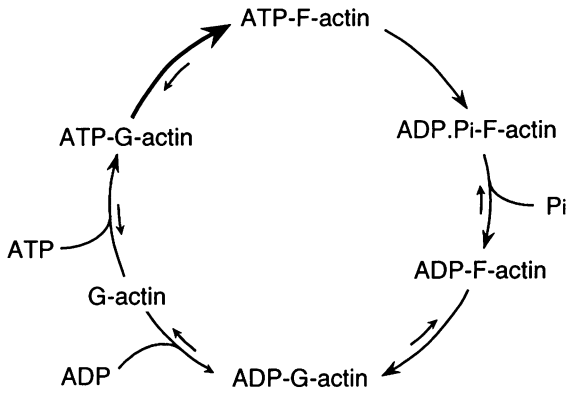

Figure 1. The actin cycle. G- and F-actin represents, respectively, the monomeric and filamentous conformation of actin.

The irreversible nucleotide hydrolysis step is thought to force the subunits to pass through the ADP-actin monomer intermediate, at one point of this cycle (Figure 1). Therefore, the exchange of the nucleotide bound to actin is a potential regulatory step in the assembly of the cytoskeleton. Actin filaments in nonmuscle cells turnover rapidly, with new subunits added to filaments at the leading edge of cells, and also throughout the cytoplasm (Wang, 1985; Omann *et al.*, 1989; Theriot and Mitchison, 1991). The rapid turnover of actin filaments should produce significant amounts of ADP-actin monomers and implies that cells must have a mechanism for recharging these monomers with ATP because ATP-actin monomers are better suited for fast elongation of filaments than ADP-actin (Pollard, 1986; Carlier, 1989).

Actin subunits released from filaments might reassociate with an actin filament but are likely to be captured by an actin monomer binding protein (Hartwig and Kwiatkowski, 1991). These binding proteins are thought to be responsible for keeping the concentration of unpolymerized actin in nonmuscle cells one to two orders of magnitude above the critical concentration for the assembly of purified actin. Inactivation of all the barbed ends by capping proteins and/or addition of ADP-actin monomers at barbed ends increase the actin critical concentration by ~10-fold, which enhances substantially the binding of actin monomers to binding proteins. Therefore, whether ADP or ATP is bound to actin monomers adding to elongating filaments may be crucial in controlling the extent of polymerization of the bulk of actin monomers, and the stability of existing actin filaments (Korn *et al.*, 1987).

We report that two of the most abundant monomeric actin binding proteins in platelets, thymosinβ_4 and profilin, (Hannappel and Van Kampen, 1987; Goldschmidt-Clermont *et al.*, 1991b; Safer *et al.*, 1991) compete for interaction with actin and have opposite effects on the rate of nucleotide exchange. Both form 1:1 complexes with actin and have micromolar affinities for actin exchanging between actin molecules on a second time scale.

Profilin is unique among actin binding proteins in catalytically accelerating the exchange of the nucleotide bound to actin monomers (Mockrin and Korn, 1980; Nishida, 1985; Goldschmidt-Clermont *et al.*, 1991b), while thymosinβ_4 strongly inhibits the nucleotide exchange. In platelets the concentration of thymosinβ_4 is 600 μM whereas profilin is 50 μM (Hannappel and Van Kampen, 1987; Goldschmidt-Clermont *et al.*, 1991b; Safer *et al.*, 1991), so the bulk of monomeric actin is likely to be bound to thymosinβ_4. However, the rapid protein exchange reactions and the very fast dissociation of the nucleotide from the transient actin-profilin complex allows profilin to catalytically accelerate nucleotide exchange even in the presence of thymosinβ_4.

We speculate that when cellular actin filaments turnover rapidly in the presence of physiological concentrations of ATP, ADP, and thymosinβ_4, profilin will determine the fraction of actin monomers charged with ATP. The availability of profilin may in turn be controlled by cellular activation through the metabolism of membrane phosphoinositides (Lassing and Lindberg, 1985; Goldschmidt-Clermont *et al.*, 1990, 1991a).

MATERIALS AND METHODS

Materials

Outdated human platelets were obtained from the Johns Hopkins Hospital and used within 15 d after phlebotomy. Saponin, Triton X 100, poly-L-proline, ATP, 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP), ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and dithiothreitol (DTT) were from Sigma Chemical (St. Louis, MO). *N*-hydroxylsulfosuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were from Pierce Chemicals (Rockford, IL). Centricon 10 filtration units were from Amicon (Beverly, MA). Bio-Gel P-10 gel filtration media were from Bio Rad Laboratories (Richmond, CA).

Protein Purification

Thymosinβ_4 was purified from either saponin (Safer *et al.*, 1990) or Triton X 100 (Goldschmidt-Clermont *et al.*, 1991b) platelet extracts. The extract (~200 ml) was clarified by centrifugation at 10 000 × *g* in a Beckman (Fullerton, CA) preparative centrifuge (JA14-rotor) for 30 min at 4°C. The supernatant in a 500-ml glass beaker was placed in a boiling water bath for 5 min. Further steps were performed at 4°C. Denatured material was cleared by centrifugation at 10 000 × *g* for 30 min (Beckman JA14-rotor), and the remaining extract was concentrated to a volume of 12 ml in a dialysis bag (3500 MW-cutoff) packed in Aquacide II for ~8 h at 4°C. The concentrated sample was filtered through a 10 000 MW cut-off Centricon unit. The filtrate was desalted by chromatography on a 2.5 × 20 cm column of Biogel P10 equilibrated and eluted with deionized water at a flow rate of 0.2 ml/min. Thymosinβ_4 concentration was estimated from absorbance at 205 nm with an extinction coefficient 27 mg^{-1} ml (Scopes, 1974). This empirical extinction coefficient was confirmed by a bicinchonic acid protein assay (Safer *et al.*, 1991). The absorbance at 280 was negligible. Profilin was purified from human platelets and actin from rabbit muscle as previously described (Goldschmidt-Clermont *et al.*, 1991b).

Chemical Cross-Linking Assay

We assayed binding of thymosinβ_4 and profilin to actin monomers by chemical crosslinking with a soluble carbodiimide that forms a

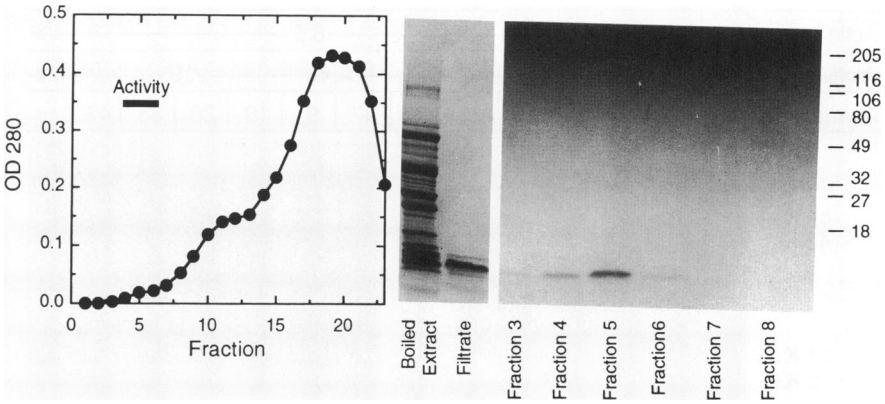

Figure 2. Purification of thymosinβ_4. Boiled platelet extract (first lane on SDS-PAGE, ~15 μg) was filtered on Centricon 10, and the filtrate (second lane, ~3 μg) was gel filtered on a column of Bio Gel P 10. The graph shows the absorbance of the 3-ml fractions. Activity indicates inhibition of actin polymerization by individual fractions. The samples were analyzed on 4–20% continuous gradient SDS-PAGE and stained with Coomassie Blue. Molecular weights (in kDa) of standard proteins are indicated.

zero length isopeptide bond between a lysine side chain and a carboxyl group (Vandekerckhove *et al.*, 1989). Individual proteins or protein mixtures in 100 μl samples were dialyzed against 2 mM potassium phosphate (pH 7.0) at room temperature, with a 3500 MW-cutoff membrane. After 2 h, EDC and NHS were added at a concentration of 1 mM to the protein mixture. The EDC and NHS concentrations were raised to 2 mM after 10 min. The reaction was terminated with 10 mM glycine after a total of 30 min. Samples were analyzed by gel electrophoresis in a 13.5% polyacrylamide gel containing Sodium dodecyl sulfate (SDS) and stained with Coomassie Blue.

Fluorescence Assay for Actin Nucleotide Exchange

The fluorescent signal provided by ϵATP bound to actin was used to measure the rate of exchange of actin nucleotide (Waechter and Engel, 1975) as described by Goldschmidt-Clermont *et al.*, (1991b) except for the buffer, which contained 2 mM tris(hydroxymethyl) aminomethane (Tris), pH 7.5; 1 μM ATP; 200 μM ϵATP; 0.5 μM $CaCl_2$; 1 mM EGTA; 1 mM $MgCl_2$; 50 mM KCl; and 2.5 μM DTT. Profilin and/or thymosinβ_4 were added to the buffer before the addition of actin to rule out interaction with ϵATP by observing fluorescence changes for ≥5 min. The reaction was initiated by adding actin.

Assay for Actin Polymerization

The concentration of polymerized actin was measured by 90° light scattering at 400 nm (Wegner and Engel, 1975). The solutions were degassed to minimize spurious light scattering and not mixed during assembly. Conditions: 22°C; 2.0 mM Tris, pH 7.5; 0.1 mM $CaCl_2$; 0.2 mM ATP; 0.5 mM DTT; 1.0 mM EGTA; 1.0 mM $MgCl_2$; and 50 mM KCl.

Nondenaturing PAGE

Mixtures of platelet profilin, thymosinβ_4, and muscle actin were analyzed on polyacrylamide gel electrophoresis (PAGE) without SDS, stained with Coomassie Blue (Safer *et al.*, 1990).

Computer Modeling

The kinetic simulation program KINSIM (Barshop *et al.*, 1983) was used to model the nucleotide exchange data (Goldschmidt-Clermont *et al.*, 1991b). This program allows us to generate time courses starting with known concentrations of reactants and with the use of selected rate constants. The original KINSIM program was modified for Macintosh computers (HOPKINSIM, now available from Daniel Wachsstock).

RESULTS

Purification of Thymosinβ_4

We purified thymosinβ_4 from human platelets by a new method. The key steps are boiling a soluble extract to precipitate most of the proteins and filtration through a membrane with a cutoff of 10 kDa, then through a desalting column. After gel filtration the thymosinβ_4 is homogeneous by the criterion of gel electrophoresis in SDS (Figure 2). Only the gel filtration fractions containing the 5-kDa thymosinβ_4 band inhibited spontaneous polymerization of actin as described by Safer *et al.* (1990). Because thymosinβ_4 absorbs minimally at 280 nm (Safer *et al.*, 1990; Figure 2), we used absorbance at 205 nm with an extinction coefficient determined by two methods to measure the concentration.

Profilin and Thymosinβ_4 Compete for Binding to Actin

Using chemical cross-linking as an assay for binding to actin, we found that profilin and thymosinβ_4 compete with each other for binding to actin. The formation of a 48 kDa 1:1 complex of thymosinβ_4 and actin increases with the concentration of thymosinβ_4 (Figure 3). The 1:1 complex of profilin and actin migrates as a doublet at 57 kDa (Figure 3). In mixtures of actin, thymosinβ_4, and profilin, increasing the concentration of thymosinβ_4 or profilin decreases the formation of the crosslinked complex of the other protein with actin (Figure 3). We never observed a 62-kDa product in reactions containing profilin, actin, and thymosinβ_4, so we have no evidence for a trimolecular complex.

Analysis of mixtures of platelet profilin, thymosinβ_4, and muscle actin by nondenaturing gel electrophoresis (Safer *et al.*, 1990) confirmed the results of the cross-linking assay. On native gels, the complex of thymosinβ_4 and actin is more stable than the profilin-actin complex. Mixtures of profilin, thymosinβ_4, and actin did not contain extra-bands that might correspond to a trimolecular complex.

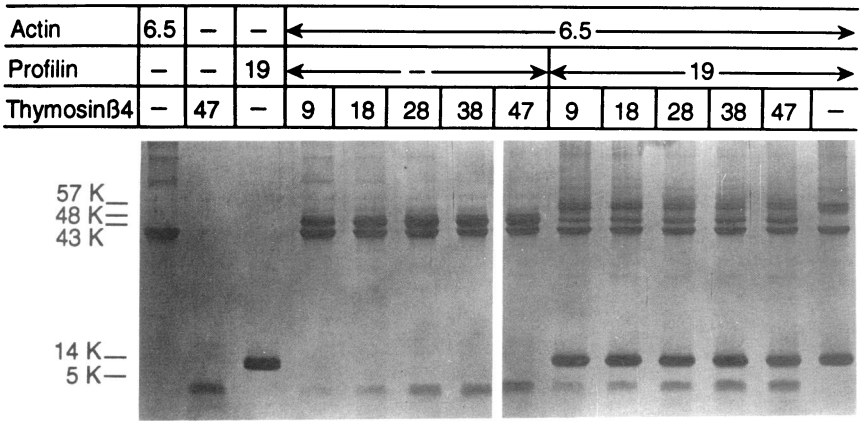

Figure 3. SDS-PAGE analysis of the products of chemical crosslinking. Individual proteins and protein mixtures containing the indicated concentrations (in μM) of actin, thymosinβ_4 and profilin were covalently cross-linked with EDC-NHS. Identical volumes from each sample were electrophoresed on 13.5% polyacrylamide gel and stained with Coomassie Blue. The molecular weights for the various protein species are indicated in kDa: 5 thymosinβ_4, 14 profilin, 43 actin, 48 thymosinβ_4-actin complex, and 57 profilin-actin complex.

Effect of Profilin and Thymosinβ_4 on the Exchange of Nucleotide Bound to Actin Monomers

We analyzed the effects of profilin and thymosinβ_4 on the exchange of the nucleotide bound to actin not only to learn how mixtures of the two proteins influence nucleotide exchange but also to evaluate the rate constants for the association and dissociation of the complexes of these proteins with actin. Kinetic simulation of the full time course of the exchange reaction over a range of protein concentrations provided us with detailed quantitative information about both the dynamics of the nucleotide exchange and of the reactions of profilin and thymosinβ_4 with actin.

Profilin accelerates the exchange of the ATP bound to actin monomers (Mockrin and Korn, 1980; Nishida, 1985) and our analysis of new data (Figures 4 and 5; Table 1) shows that the effect of platelet profilin on muscle actin under physiological salt conditions can be explained by the same catalytic mechanism that we proposed for platelet actin in a low salt buffer (Goldschmidt-Clermont *et al.*, 1991b). In this mechanism the nucleotide dissociates much more rapidly from the actin-profilin complex than from an actin monomer because the profilin rapidly exchanges between actin molecules.

We estimated the rate constants for the reactions by kinetic simulation, a method where one tests the validity of a mechanism and the rate constants for each step by their ability to fit full time courses of reactions over a range of reactant concentrations (Barshop *et al.*, 1983). First, we measured the rate of exchange of ϵATP for bound ATP. Assuming an association rate constant of $\sim 10^7\ M^{-1}\ s^{-1}$ (Nowak *et al.*, 1988) and that the affinity of actin for ATP is two to three times higher than for ϵATP (Waechter and Engel, 1975; Pollard *et al.*, 1992),

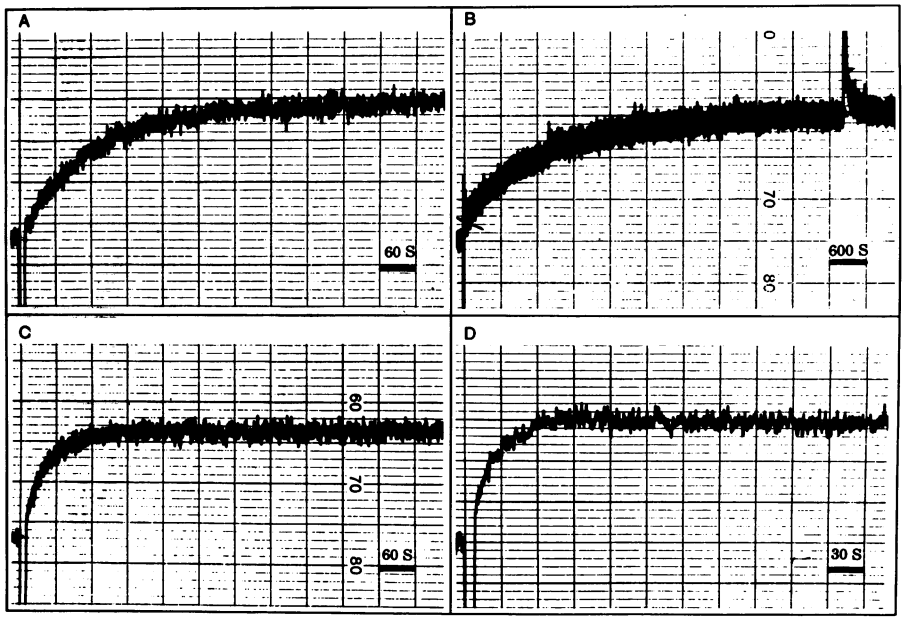

Figure 4. Effect of profilin and thymosinβ_4 bound separately and mixtures of the two proteins on the time course of the exchange of ϵATP for ATP bound to actin monomers. At time zero, 0.3 μM ATP-actin was added to 2 mM Tris, pH 7.5; 0.2 mM ϵATP; 0.5 μM $CaCl_2$; 1 μM ATP; 1 mM EGTA; 1 mM $MgCl_2$; 50 mM KCl; and 2.5 μM DTT at 22°C. The samples contained the following concentrations of profilin and thymosinβ_4: (A) zero profilin and thymosinβ_4; (B) zero profilin, 8.0 μM thymosinβ_4; (C) 0.14 μM profilin, 8.0 μM thymosinβ_4; (D) 0.14 μM profilin, zero thymosinβ_4 (time scale in seconds is indicated by the bars).

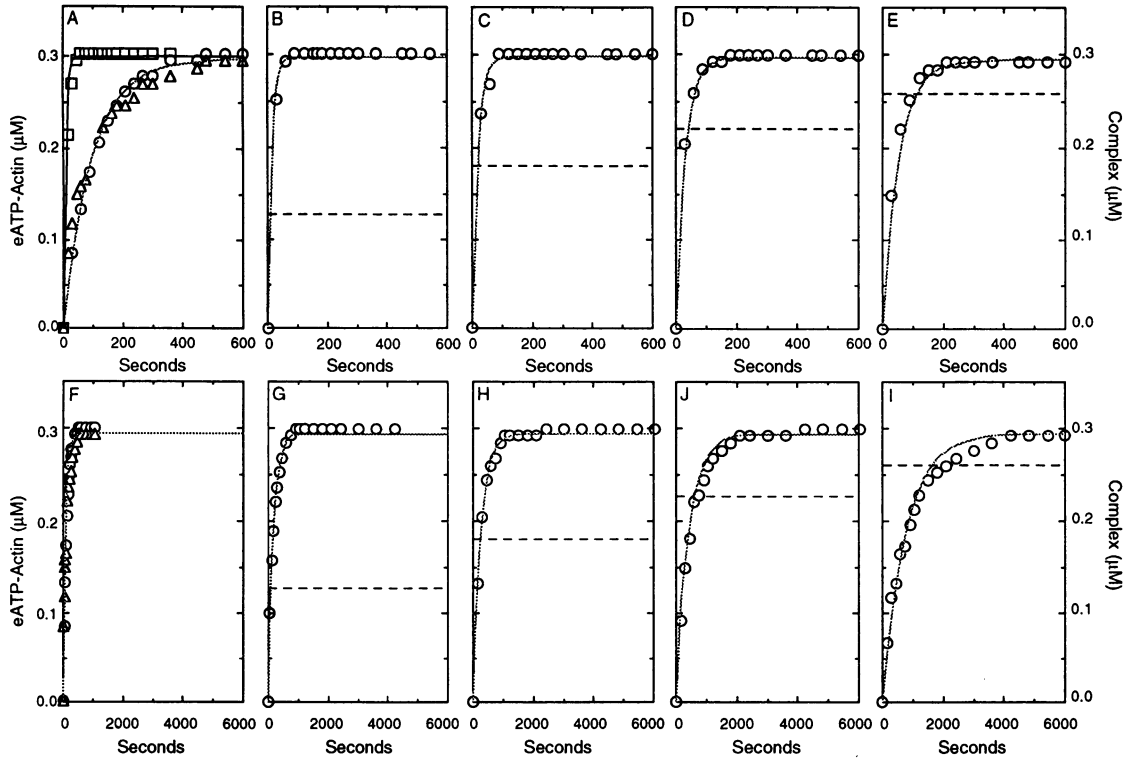

Figure 5. Comparison of theoretical curves (continuous lines) from kinetic simulation of actin nucleotide exchange with the experimental data (open symbols). Fluorescence was converted to the concentration of actin with bound ϵATP (left axis) with the fluorescence of 0.3 μM actin saturated with ϵATP in the absence of profilin and thymosinβ_4. The grey lines were calculated with the mechanism and rate constants from Table 1. The dashed lines show the calculated steady-state concentrations of all actin species (ATP-actin and ϵATP-actin) bound to thymosinβ_4 (right axis). The concentrations of actin bound to profilin are too low to be distinguished from baseline. Conditions: same as for Figure 4. (A) Actin alone (0.3 μM, two separate runs: ○, Δ) or in the presence of profilin (0.14 μM, □); (B–E) Actin (0.3 μM), profilin (0.14 μM), and various concentrations of thymosinβ_4: (B) 1.0 μM, (C) 2.0 μM, (D) 4.0 μM, and (E) 8.0 μM. (F–I) Actin (0.3 μM) in the presence of various thymosinβ_4 concentrations: (F) zero (same two runs as in A), (G) 1.0 μM, (H) 2.0 μM, (G) 4.0 μM, and (I) 8.0 μM.

the kinetic simulation tightly constrained the values of the dissociation rate constants for ATP and ϵATP (Table 1). Second, we considered the binding of profilin to the muscle actin monomers and the binding of the nucleotides to the complex of actin and profilin. To simplify the analysis, we assumed that profilin has the same affinity for ATP-actin and ϵATP-actin and that ATP and ϵATP have the same affinity for the actin-profilin complex. This left 4 variables: the association and dissociation rate constants for profilin binding to the nucleotide-actin complex; and the association and dissociation rate constants for nucleotide from the actin-profilin complex. By trial and error we found values that account for the full time course of the nucleotide exchange reaction over a range of profilin concentrations (Table 1). We determined that the association rate constant for profilin and muscle actin is the same in a low salt buffer for profilin and platelet actin (Goldschmidt-Clermont *et al.*, 1991b), but the dissociation rate constant for the complex of profilin with muscle actin is 16 times higher. The affinity of profilin for muscle actin may be somewhat lower than for platelet actin, but the solution conditions are an important factor. The K_d of 31 μM for the profilin-muscle actin complex (Table 1) is similar to that determined independently for profilin and muscle actin (22.5 μM) in the same buffer containing KCl and $MgCl_2$ with the *Limulus* acrosomal process elongation assay (Goldschmidt-Clermont *et al.*, 1991b). The rate constant for dissociation of nucleotide from the muscle actin-profilin complex is very large, with a lower limit of 100 s^{-1}. Thus profilin binds to muscle actin weakly and exchanges rapidly between actin monomers. During the brief time that profilin is bound to an actin monomer, the dissociation of nucleotide is 10^4 times faster than from actin monomers.

Thymosinβ_4 strongly inhibits the exchange of nucleotide bound to actin monomers (Figures 4 and 5), and analysis by kinetic simulation indicates that the dissociation of nucleotide is exceedingly slow (Table 1). In this analysis we assumed that thymosinβ_4 has the same affinity for ATP-actin and ϵATP-actin and that ATP and ϵATP have the same affinity for the complex of actin and thymosinβ_4. Given the values for the binding of ATP and ϵATP to actin (Table 1), this left four

Table 1. Reactions

	k_+ (μM^{-1} s^{-1})	k_- (s^{-1})
1. T + A ⇌ TA	7.0	0.01
2. ϵT + A ⇌ ϵTA	7.0	0.024
3. A + P ⇌ AP	—	—
4. TA + P ⇌ TAP	1.3	40
5. ϵTA + P ⇌ ϵTAP	1.3	40
6. T + AP ⇌ TAP	7.0	≥100
7. ϵT + AP ⇌ ϵTAP	7.0	≥100
8. A + Y ⇌ AY	—	—
9. TA + Y ⇌ TAY	0.65	0.75
10. ϵTA + Y ⇌ ϵTAY	0.65	0.75
11. T + AY ⇌ TAY	$\leq 10^{-10}$	$\leq 10^{-10}$
12. ϵT + AY ⇌ ϵTAY	$\leq 10^{-10}$	$\leq 10^{-10}$

A mechanism for the exchange of the nucleotide bound to actin (A) in the presence of thymosinβ_4 (Y) and profilin (P) consisting of 12 reactions and the rate constants that provide the best fit to the experimental data. Other abbreviations: T, ATP; ϵT, ϵATP. The initial values for the rate constants were selected from previous reports (Goldschmidt-Clermont *et al.*, 1991b) when available and then varied by at least two orders of magnitude in each direction until we found absolute values or limits that best fit the experimental data (Figure 5). Rate constants for reactions 3 and 8 could not be determined because they have no effect on the simulated time course under our experimental conditions. Rate constants for reactions 1, 2, 4, 5, 9, and 10 and association rate constants for reactions 6 and 7 could not be changed by >50% without altering substantially the quality of the fit at one or more thymosinβ_4 concentrations. The dissociation rate constants for reactions 6 and 7 are minimal rates, and rate constants for reactions 11 and 12 correspond to maximal values for these reactions. Further refinement of these rate constants could not be obtained with available experimental data.

variables: the association and dissociation rate constants for thymosinβ_4 binding to the nucleotide-actin complex; and the association and dissociation rate constants for nucleotide binding to the complex of actin and thymosinβ_4. To fit the full time course for nucleotide exchange over a range of thymosinβ_4 concentrations, the dissociation and association rate constants for the nucleotides from the complex of actin and thymosinβ_4 must be very small. We estimate an upper limit of 10^{-10} s^{-1} and 10^{-4} M^{-1} s^{-1}, respectively. The data tightly constrain the values of the rate constants for binding and dissociation of the complex of actin and thymosinβ_4 to $\sim 10^6$ M^{-1} s^{-1} and 1 s^{-1} (Table 1). The conclusion is that thymosinβ_4 binds to muscle actin monomers with a dissociation equilibrium constant of about 1 μM, exchanges between actin monomers on a second time scale, and when bound to actin very strongly inhibits nucleotide dissociation. Note that the thymosinβ_4 exchange rate is much faster than the dissociation of nucleotide from actin monomers, so that even though the complexes are transient, little nucleotide will exchange on either the free actin or the actin complex with thymosinβ_4.

In spite of strong inhibition of actin bound nucleotide exchange by thymosinβ_4, the addition of low concentrations of profilin can reverse this inhibition completely (Figures 4 and 5)! Kinetic curves calculated from the mechanism in Table 1 and the same set of rate constants determined for nucleotide exchange by actin, the complex of actin and profilin, and the complex of actin and thymosinβ_4, and for the binding of profilin and thymosinβ_4 to actin (Table 1) fit the full time course of nucleotide exchange over a range of profilin and thymosinβ_4 concentrations (Figure 5). This result provides strong support for the mechanism and the rate constants in Table 1.

DISCUSSION

Our main new insight is that under physiological conditions in the presence of the major actin sequestering protein thymosinβ_4, the rate of exchange of nucleotide bound to actin monomers can be varied over orders of magnitude by small changes in the concentration of profilin. The interaction of profilin with polyphosphoinositides and its ability to catalyze nucleotide exchange on actin provides potential mechanism to couple the membrane phosphoinositide pathway to the assembly of the actin cytoskeleton. This interplay between the effects of profilin and thymosinβ_4 on actin is probably one of many complex relationships among the numerous proteins that influence the assembly and organization of the actin cytoskeleton. Other examples include the combined effects of the severing/capping proteins and crosslinking proteins on the properties of actin gels (Janmey *et al.*, 1990) and the effects of the severing protein actophorin and the crosslinking protein alpha-actinin on the formation of actin filament bundles (Maciver *et al.*, 1991).

Validity of the Analysis

Our conclusions are based on rate constants derived from a kinetic simulation of the time course of nucleotide exchange over a range of concentrations of thymosinβ_4 and profilin. The simplest model that fits the experimental data consists of 10 reactions (Table 1) many drawn from previous work on the nucleotide of actin and mixtures of actin and profilin (Goldschmidt-Clermont *et al.*, 1991b). Inclusion of additional reactions such as concurrent binding of profilin and thymosinβ_4 to actin would allow even better fit to the data, but the minimal model in Table 1 is consistent with the crosslinking and native gel experiments, showing that profilin and thymosinβ_4 do not bind actin simultaneously. Most of the constants are either measured independently or are well constrained by the kinetic data. Two additional reactions (Eqs. 3 and 8 in Table 1) had no effect on the simulation, and their rate constants must remain indeterminate until they can be evaluated separately. Without these reactions, we cannot be sure that the energy balance of the overall mechanism is correct. In the

following discussion, we assume that the effects of the thymosinβ_4 and profilin on ADP exchange will be similar to those documented for ATP exchange. This assumption seems reasonable because the affinity of actin for ATP is only ≤5 times higher than for ADP (Pollard *et al.*, 1992). However, it is possible that the bound-nucleotide affects the affinity of thymosinβ_4 for actin.

Mechanism of Thymosinβ_4 Interaction with Actin

Our new data and analysis confirm that thymosinβ_4 forms a 1:1 complex with actin monomers with a dissociation equilibrium constant of ~1 μM and revealed that thymosinβ_4 is a potent inhibitor of actin nucleotide binding and dissociation. Given that thymosinβ_4 dissociates from actin on a second time scale, some nucleotide exchange occurs even in high concentrations of thymosinβ_4, but while the thymosinβ_4 is bound to an actin monomer the rate of nucleotide dissociation is nearly zero (Table 1). Understanding the mechanism of this inhibition will require additional information about the site where thymosinβ_4 binds to actin. In this regard, we expect that it binds to a site which does not overlap exactly with the binding site of DNase I, another inhibitor of nucleotide exchange (Mannherz *et al.*, 1980), because thymosinβ_4 but not DNase I (Carlsson *et al.*, 1977) competes with profilin for binding to actin.

Mechanism of Profilin Interaction with Actin

The current work establishes that under physiological ionic conditions, profilin catalytically accelerates the exchange of the nucleotide bound to actin. It works catalytically by exchanging rapidly between actin monomers and during the <1 s that it is bound to each actin molecule, it promotes the rapid dissociation of the bound nucleotide. Due to the rapid exchange between actin molecules, low concentrations of profilin, well within the physiological range, promote nucleotide exchange of a large excess of actin. In a buffer with physiological concentrations of salt, the profilin binds weakly to actin due to rapid dissociation. Nevertheless, it is an effective catalyst because nucleotide dissociates more rapidly from these transient complexes than the profilin.

Effects of Mixtures of Thymosinβ_4 and Profilin on Actin

Because thymosinβ_4 binds to actin more strongly than profilin and prevents the dissociation of the nucleotide bound to the actin, it was surprising to find that low concentrations of profilin can completely reverse the effect of thymosinβ_4 (Figures 4 and 5). Nevertheless this unanticipated behavior follows directly from the individual mechanisms of interaction of these proteins with actin. In particular, the mechanism and rate constants in Table 1 not only account for the effects of either profilin or thymosinβ_4 on actin, but they also predict accurately the effects of mixtures of the proteins in varying ratios (Figure 5). The ability of profilin to overcome inhibition of the nucleotide exchange by thymosinβ_4 results from differences in the rates of dissociation of nucleotide and the two monomer binding proteins from actin compared with the rates of dissociation of the nucleotide from the complex of actin with each protein as follows. ATP dissociates slowly (k = 0.01 s^{-1}) from monomeric actin but very rapidly (k ≥ 100 s^{-1}) from the actin-profilin complex. Profilin binds transiently to actin due to a dissociation rate constant of ~40 s^{-1}, so it "hops" from one actin monomer to another. Nevertheless it binds long enough to dissociate the nucleotide from most actins that it encounters. Thymosinβ_4 binds tighter to actin because its dissociation rate constant is ~50 times smaller than that of profilin. However, it dissociates often enough for profilin to bind and efficiently catalyze the dissociation of the nucleotide, even when on average most actin molecules are associated with thymosinβ_4 as illustrated by the dashed lines in Figure 5.

To illustrate the antagonistic effects of these proteins on actin nucleotide exchange under physiological conditions, we used the mechanism and rate constants in Table 1 together with the concentrations of ATP, ADP, actin, and thymosinβ_4 in human platelets to simulate the effect of various concentrations profilin on the time course of the exchange of ADP for ATP bound to actin (Figure 6). For demonstration purposes, we initiated the reaction with 300 μM ADP-actin and no ATP-actin. At a physiological concentration of thymosinβ_4, most of the actin monomers are bound to thymosinβ_4 and in the absence of profilin, the ADP-actin pool is very stable. A physiological concentration of profilin (50 μM) markedly increases the rate of exchange of ADP for ATP on the actin monomers. Even low concentrations of profilin accelerate nucleotide exchange. Note that under conditions where profilin increases the rate of nucleotide exchange, the concentration of actin-profilin complexes is low, and most of the actin is bound to thymosinβ_4.

Regulation of Actin Assembly in the Cell by Thymosinβ_4 and Profilin

We think that exchange of ADP for ATP bound to unpolymerized actin may be an important control point in the assembly of the actin cytoskeleton. Under steady state conditions in vitro with a large excess of ATP over ADP (as it is the case in most cells), nucleotide exchange is fast enough that any ADP-actin dissociating from filaments is quickly converted to ATP-actin (Oosawa and Asakura, 1975). However, under some experimental conditions in vitro such as continuous sonication (Pantaloni *et al.*, 1984), many new polymer ends containing ADP-actin can form so that the bulk rate of ADP-actin dissociation leads to a high fraction of ADP-actin monomers. Under these conditions, the nucleotide ex-

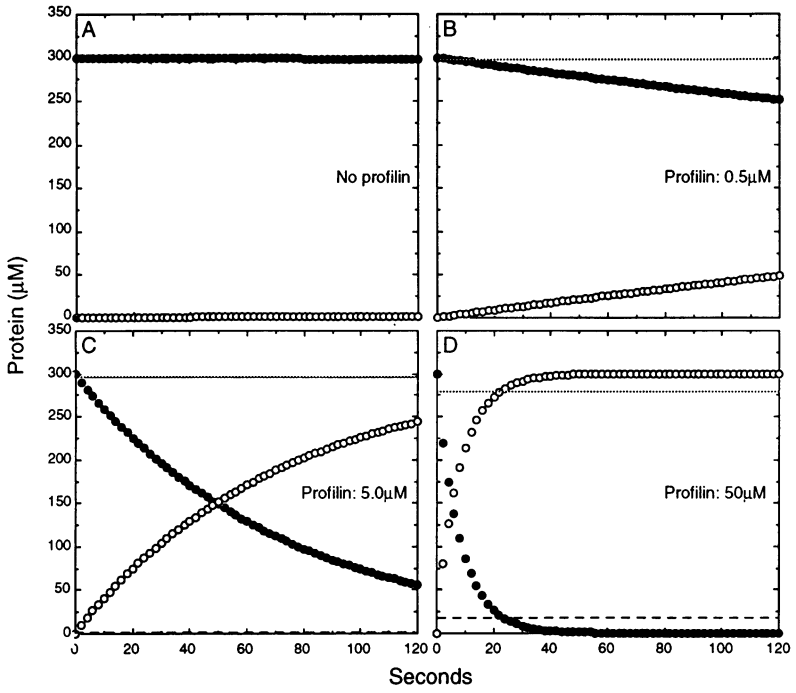

Figure 6. Simulation of the effect of profilin concentration on the time course of the exchange of ATP for ADP bound to actin under the conditions in platelets. The calculations used the model in Table 1, except that ϵATP was replaced by ADP. The initial conditions were 2 mM ATP, 20 μM ADP, 500 μM thymosinβ_4, 0 μM ATP-actin, and 300 μM ADP-actin. Theoretical curves show the time course of changes in the concentration of ADP-actin (●), ATP-actin (○), thymosinβ_4-actin complex (grey line) and profilin-actin complex (– – –). The concentration of profilin was varied: (A) zero, (B) 0.5 μM, (C) 5 μM and (D) 50 μM. The following rate constants were used (units: k^+: $\mu M^{-1}\ s^{-1}$; k^-: s^{-1}; D represents ADP): reaction 1 (T + A $\rightleftharpoons$ TA) k_+:7.0, k_-:0.01 (this paper and Goldschmidt-Clermont *et al.*, 1991); reaction 2 (D + A $\rightleftharpoons$ DA) k_+:0.036, k_-:0.01 (Neidl and Engel, 1979; Goldschmidt-Clermont *et al.*, 1991); reaction 3: nonrelevant; reactions 4 and 5 (TA + P $\rightleftharpoons$ TAP and DA + P $\rightleftharpoons$ DAP) k_+:1.0, k_-:2.5 (for platelet actin, Goldschmidt-Clermont *et al.*, 1991b); reaction 6 (D + AP $\rightleftharpoons$ DAP) k_+:0.036, k_-:13 (Neidl and Engel, 1979; Goldschmidt-Clermont *et al.*, 1991b); reaction 7 (T + A $\rightleftharpoons$ TAP) k_+:7.0, k_-:13 (Goldschmidt-Clermont *et al.*, 1991b); reaction 8: nonrelevant; Reactions 9 and 10 (TA + Y $\rightleftharpoons$ TAY and DA + Y $\rightleftharpoons$ DAY) k_+:0.65, k_-:0.75 (Table 1); reactions 11 and 12 (D + AY $\rightleftharpoons$ DAY and T + AY $\rightleftharpoons$ TAY) k_+10^{-10}, $k_-:^{-10}$ (Table 1). We assumed in these simulations that the interaction of thymosinβ_4 with actin is similar for all actin isoforms.

change may be rate limiting, even in the presence of excess ATP. Thus it is possible that the rapid turnover of actin in nonmuscle cells (Wang, 1985; Omann *et al.*, 1989; Theriot and Mitchison, 1991) might generate large pools of ADP-actin monomers. Furthermore, this ADP-actin pool could be stabilized by thymosinβ_4 even in the presence of millimolar concentrations of ATP. Because the polymerization of ADP-actin is less favorable than for ATP-actin, exchange of ADP for ATP on this pool of ADP-actin monomers might be a key factor limiting the repolymerization of actin subunits. The observation that *Drosophila* nurse cells from *chickadee* mutants that do not contain enough profilin (Cooley *et al.*, 1992) and profilin-deficient yeast cells (Haarer *et al.*, 1990) fail to synthesize normal actin networks is consistent with profilin controlling a limiting step in actin assembly. Further biochemical characterization of drosophila and yeast profilins will be necessary to determine if this control is mediated by the effect of profilin on the nucleotide exchange.

Under these conditions, profilin is an excellent candidate to regulate the pool of ADP-actin in cells, because its concentration determines the rate at which the ADP-actin is converted to ATP-actin, even in the presence of physiological concentrations of thymosinβ_4. For profilin to control this reaction, it would be desirable for its concentration to depend on the state of activation of the cell, providing a link to other cellular activities (Hartwig *et al.*, 1989). For example, many cells respond to agonists with an increase in the concentration of polymerized actin (Cooper, 1991). This polymerization could be accomplished by creating new nuclei (Shariff and Luna, 1992), uncapping filaments (Stossel, 1989), and/or by increasing the concentration of free actin monomers or actin molecules charged with ATP.

Release of profilin from membrane binding sites as a part of cell activation may play a role in the polymerization of the actin. It is possible that high concentrations of profilin are maintained near the membrane where the actin turnover is most rapid (Theriot and Mitchison, 1991) as a result of profilin interaction with membrane lipids (Lassing and Lindberg, 1985; Hartwig *et al.*, 1989; Goldschmidt-Clermont *et al.*, 1990; Machesky *et al.*, 1990). The flux through the membrane polyphosphoinositide pool in response to agonists is expected to dissociate profilin from the membrane. Even low concentrations of profilin can have a profound positive effect on the exchange of actin nucleotides and could promote the formation of actin subunits charged with ATP and ready for elongation of newly formed nuclei and uncapped filaments (Stossel, 1989; Goldschmidt-Clermont *et al.*, 1991b; Goldschmidt-Clermont and Janmey, 1991; Shariff and Luna, 1992).

Thymosinβ_4 and profilin have similar stoichiometry and affinity for actin, but have opposite effects on the nucleotide exchange, and may also have opposite effects on actin polymerization. Much more work is needed, but thymosinβ_4 looks like a much better candidate than profilin to sequester actin monomers in an unpolymerized state. At least in platelets, its concentration is high enough to account for most of the unpolymerized actin pool in addition to suppressing nucleotide exchange.

On the other hand, profilin may even promote actin polymerization by shuttling actin to the barbed end of elongating filaments (Tilney *et al.*, 1983; Pollard and Cooper, 1984; Pring *et al.*, 1992).

The fine regulation of the interaction between actin and its nucleotide described in this paper probably represents a paradigm for other nucleotide triphosphatases. Actually, exchange of the guanine nucleotide bound to p21^{ras} is also accelerated by a specific cytoplasmic factor (Bourne *et al.*, 1991). For example, in the sevenless protein tyrosine kinase pathway of R7-photoreceptor cells of *Drosophila*, the son of sevenless protein stimulates the exchange of GDP for GTP bound to p21^{ras}, which in this system may represent the switch used by sevenless to activate p21^{ras}. (Simon *et al.*, 1991). It is possible that another protein, yet unidentified, plays the role of thymosinβ_4 in the p21^{ras} pathway. Further biochemical analysis of the p21^{ras} system may in turn provide information relevant to the organization of actin cytoskeleton.

Open Questions for Future Investigation

The reactions summerized in Table 1 not only provide new insight into fundamental actin biochemistry but also raise a number of fascinating questions about the cell biology of the actin system. The present work has focused our attention on the following issues: 1) What is the nucleotide content of actin in the unpolymerized pool in cells? Although it is known that in platelets most actin subunits within filaments are bound to ADP (Daniel *et al.*, 1986), the nature of the nucleotide bound to monomeric actin (ATP vs. ADP) has yet to be identified. 2) How do the pools of ATP- and ADP-actin turn over with various cellular activities? 3) How do profilin and thymosinβ_4 bind to ADP-actin, and how do they affect dissociation of ADP? 4) How does thymosinβ_4 inhibit the polymerization of ADP- and ATP-actin? 5) Is the activity of thymosinβ_4 regulated? Expression of the thymosinβ_4 gene varies during cell differentiation (Shimamura *et al.*, 1990; Gondo *et al.*, 1987), and thymosinβ_4 levels increase rapidly in thymocytes activated with concanavalin A (Schobitz *et al.*, 1990). Regulation at the level of synthesis is crude but may be appropriate for a protein with a global effect on the concentration of free monomeric actin in cells. It is also possible that the activity of thymosinβ_4 may be regulated by post-translational mechanism(s). In the case of profilin, we have speculated that cellular activation through the phosphoinositide pathway may control a profilin gradient between membranes and cytoplasm (Goldschmidt-Clermont *et al.*, 1991a; Goldschmidt-Clermont and Janmey, 1991), but this hypothesis has to be tested directly within cells. All of these issues need to be resolved to confirm the proposal we have presented in this paper.

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