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Modulation of the interaction between G-actin and thymosin β_4 by the ATP/ADP ratio: Possible implication in the regulation of actin dynamics

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ABSTRACT **The interaction of G-actin with thymosin β_4 ($T\beta_4$), the major G-actin-sequestering protein in motile and proliferating cells, has been analyzed *in vitro*. $T\beta_4$ is found to have a 50-fold higher affinity for MgATP–actin than for MgADP–actin. These results imply that in resting platelets and neutrophils, actin is sequestered by $T\beta_4$ as MgATP–G-actin. Kinetic experiments and theoretical calculations demonstrate that this ATP/ADP dependence of $T\beta_4$ affinity for G-actin can generate a mechanism of desequestration of G-actin by ADP, in the presence of physiological concentrations of $T\beta_4$ ($\approx$0.1 mM). The desequestration of G-actin by ADP is kinetically enhanced by profilin, which accelerates the dissociation of ATP from G-actin. Whether a local drop in the ATP/ADP ratio can allow local, transient desequestration and polymerization of actin either close to the plasma membrane, following platelet or neutrophil stimulation, or behind the *Listeria* bacterium in the host cell, while the surrounding cytoplasm contains sequestered ATP–G-actin, is an open issue raised by the present work.**

In nonmuscle cells, especially motile and proliferating cells, a high concentration of actin ($\approx$0.1 mM) is maintained unpolymerized by interaction with sequestering proteins (1). Polymerization of actin is induced upon appropriate stimulation, which implies that some unknown desequestration mechanism controls the formation, within seconds, of short filaments in well-localized areas of the cell; typical examples include the explosive polymerization of actin upon stimulation of platelets (2–5), neutrophils (6), or chemotactic amoebae (7), and in the lamellipodia of motile keratocytes (8), as well as the propulsive movement of the *Listeria* bacterium in a host cell (9–11).

In vertebrate tissues at least, the main G-actin-sequestering protein is not profilin, as thought initially (12), but thymosin β_4 ($T\beta_4$), a ubiquitous 43-amino acid peptide, which was first shown to sequester G-actin in resting platelets, where it is present at 500–600 μM (13–15) and binds G-actin with an equilibrium dissociation constant in the micromolar range (16). $T\beta_4$ appears to be present in high amounts (0.1–1% of total protein) in most tissues (17, 18), where it is synthesized at high rates (19) but essentially not secreted (20, 21); its expression is regulated at the translational level during the cell cycle (21) and is enhanced upon thymocyte stimulation (22). The N-terminal tetrapeptide of $T\beta_4$ is a regulator of the proliferation of hematopoietic cells (23, 24). All the above observations are in support of $T\beta_4$ being a regulatory component of the cytoskeleton (25). In contrast to $T\beta_4$, profilin has an enigmatic role in the regulation of actin dynamics; it appears precisely in regions of the cell where actin filaments are nucleated—i.e., at the plasma membrane (ref. 26; see ref. 27 for a recent review) and at the rear of *Listeria* (11), where it is assumed to interact with the polyproline-rich bacterial protein ActA (28). The double function of profilin as a G-actin-sequestering and an F-actin-stabilizing element is supported by cell injection experiments (29). An attractive model has been proposed (30, 31) to account for the involvement of $T\beta_4$ and profilin in the reactions leading to actin polymerization. According to this model, most cellular G-actin is in the ADP form, due to rapid filament turnover, and nucleotide exchange on G-actin is blocked by $T\beta_4$ (31). Exchange of ATP present in cellular medium for bound ADP would be catalyzed by profilin, which is known to have the opposite property of increasing the rate of nucleotide exchange on G-actin (32–34). Profilin would therefore rapidly promote the local formation of ATP–G-actin in amounts high enough to generate spontaneous nucleation of actin filaments. The issue of the possible regulation of G-actin–$T\beta_4$ interaction by nucleotides is addressed in this work.

MATERIALS AND METHODS

Proteins. Muscle actin was purified as described (35) and isolated as CaATP–G-actin by Sephadex G-200 chromatography (36) in buffer G (5 mM Tris Cl, pH 7.6/0.1 mM $CaCl_2$/0.2 mM dithiothreitol/0.2 mM ATP/0.01% NaN_3). Actin was routinely 80–92% pyrenyl labeled as described (37). CaATP–G-actin 1:1 complex was prepared by rapid Dowex-1 treatment (32). MgATP–G-actin was prepared as described (38) by addition of 50 μM $MgCl_2$ and 0.2 mM EGTA to 10–20 μM CaATP–G-actin and incubation for at least 5 min at 0°C before use. ADP–G-actin was prepared as described (39). Briefly, CaATP–G-actin 1:1 complex (40 μM, 3–5% pyrenyl labeled) was polymerized by addition of 1 mM $MgCl_2$ and 0.2 mM EGTA. Following the complete hydrolysis of actin-bound ATP (as judged by the overshoot polymerization kinetics), F-actin was depolymerized by 10-fold dilution with ice-cold buffer G' [5 mM Tris Cl/0.1 mM $CaCl_2$/0.2 mM EGTA/0.2 mM dithiothreitol/80 μM ADP/10 μM diadenosine pentaphosphate (Ap_5A), pH 7.5], and three sonications of 3 sec were given to the solution. The MgADP–G-actin solution was concentrated to $\approx$10 μM with a Centriprep 30 (Amicon) apparatus and centrifuged at 400,000 × *g* for 40 min at 4°C. The solution of MgADP–G-actin, obtained within 90 min, was stored on ice and used within 3 hr.

Profilin was purified from sheep spleen by poly(L-proline) affinity chromatography (40), eluted by 7 M urea (41), and then renatured by dialysis against buffer G without ATP. Profilin purified by this procedure bound CaATP– and MgATP–actin with K_d values of 1.2 and 4.5 μM, in agreement with reported values (42–44).

$T\beta_4$ was purified from sheep spleen essentially as described (45). The perchloric acid extract was chromatographed on Lichroprep RP-18 (40–63 μm; Merck). The 33% propanol

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Abbreviation: $T\beta_4$, thymosin β_4.

eluate was concentrated under vacuum and purified by reversed-phase HPLC using a Ultrasphere ODS C_{18} column (1 cm × 25 cm; Beckman) monitored at 220 nm. Solvent A was 5% acetonitrile/0.1% trifluoroacetic acid; solvent B was 60% acetonitrile/0.1% trifluoroacetic acid. $T\beta_4$ was eluted with a gradient of 5–36% B in 43 min, at 3 ml/min. The purity of the material was checked by analytical HPLC using a C_{18} Deltapak column, before and after oxidation of Met^6 by 3% H_2O_2 at 37°C. $T\beta_4$ present in a platelet saponin extract (13) was eluted at the same position as spleen $T\beta_4$, as described (14). The choice of sheep spleen guaranteed a more straightforward purification and higher yield than bovine spleen, because sheep spleen, like pig spleen, possesses the $T\beta_9^{Met}$ variant (in which Leu^6 is replaced by Met^6), which is clearly separated from $T\beta_4$. Amino acid analysis of purified $T\beta_4$ was performed in duplicate (Pico-Tag system, Waters) and showed a composition (data not shown) identical to that reported for bovine $T\beta_4$ (46).

Polymerization Measurements. The critical concentration for polymerization of actin was derived from pyrene fluorescence measurements of serially diluted samples. Two methods were used. In the first method, samples were incubated overnight prior to fluorescence measurements. In the second method, the samples were briefly sonicated (three times for 3 sec) to accelerate the dilution-induced depolymerization; fluorescence was read within 1 hr (i.e., when stable equilibrium values were reached). The two methods gave identical values in the presence of ATP. In the presence of ADP, only the second method was used due to the lability of ADP–actin.

Kinetics of polymerization under controlled periodic fragmentation were measured as described (47). Polymerization was started by adding 0.1 M KCl to the 3–5% pyrenyl-labeled G-actin solution. Sonication was applied periodically for 0.2 sec every 3 sec by using a time controller attached to the sonicator.

The initial rate of filament elongation from F-actin seeds was also measured (39) from the change in pyrenyl fluorescence upon addition of pyrenyl-labeled F-actin (0.5–1 μM coming from a 20 μM F-actin solution) to a solution of identically labeled G-actin supplemented with 0.1 M KCl.

RESULTS AND DISCUSSION

Thymosin β_4 Binds to ADP–G-actin with an Affinity 2 Orders of Magnitude Lower Than to ATP–G-actin. The sequestering of ATP–G-actin by $T\beta_4$ causes a concentration-dependent inhibition in the kinetics of polymerization of ATP–G-actin (data not shown). The equilibrium dissociation constant for $T\beta_4$ binding to ATP–G-actin was derived from the change in the apparent critical concentration for polymerization in the presence of a given concentration of $T\beta_4$. The data shown in Fig. 1 are consistent with the formation of a nonpolymerizable 1:1 complex (TG) between $T\beta_4$ (T) and MgATP–G-actin (G). The equilibrium dissociation constant, $K_d = ([T_0] - [TG])[G]/[TG]$, where $[T_0]$ represents the total concentration of $T\beta_4$, and [G] is the critical concentration for polymerization of actin, determined in a control in the absence of $T\beta_4$. In the presence of 5 μM $T\beta_4$ ($T_0 = 5$), the concentration of unpolymerized actin was 0.6 μM; since the critical concentration, [G], was 0.16 μM, a value of 0.44 μM was derived for [TG], leading to $K_d = (4.56 \times 0.16)/0.44 = 1.7$ μM, in reasonable agreement with ref. 14. In an independent experiment carried out with 3 μM $T\beta_4$, values of 0.12 μM and 0.18 μM were found for G and TG, leading to $K_d = 2$ μM. The slope of the critical-concentration plots was slightly higher in the presence than in the absence of $T\beta_4$, consistent with a slightly lower affinity of $T\beta_4$ for pyrenyl-labeled G-actin than for unlabeled G-actin. A similar experiment done with CaATP–actin yielded a K_d value of 9 μM

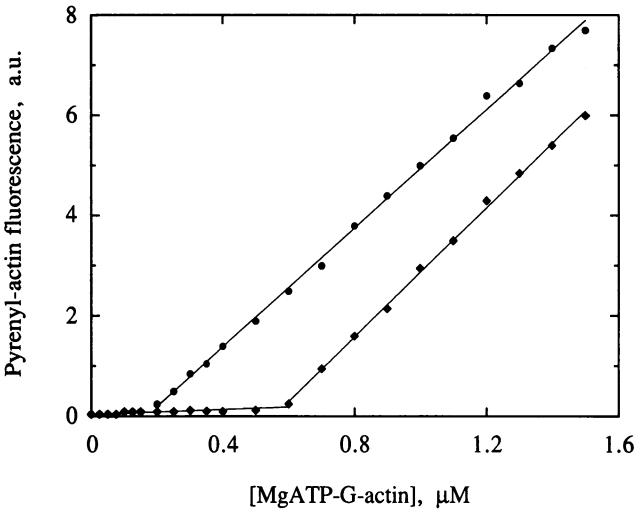

FIG. 1. Change in apparent critical concentration for polymerization of MgATP–actin in the presence of $T\beta_4$. Serial dilutions of Mg–F-actin (5% pyrenyl labeled) were made in 5 mM Tris, pH 7.5/0.1 mM $CaCl_2$/0.2 mM EGTA/0.2 mM ATP/50 μM $MgCl_2$/0.1 M KCl with (◆) or without (●) 5 μM $T\beta_4$. In all figures, fluorescence is shown in arbitrary units (a.u.).

(data not shown). Therefore binding of $T\beta_4$ to G-actin is sensitive to the ATP-bound divalent metal ion.

The binding of $T\beta_4$ to MgADP–G-actin was assayed as above by measuring the inhibition in the kinetics of spontaneous polymerization and the change in apparent critical concentration. Fig. 2 demonstrates that $T\beta_4$ binds very poorly to MgADP–G-actin under physiological ionic conditions. Analysis of polymerization curves (*Inset*) and of the change in apparent critical concentration of ADP–actin in the presence of 35 μM and 50 μM $T\beta_4$ led to K_d values of 85 μM and 80 μM, respectively, for the MgADP–G-actin–$T\beta_4$ complex. A value of 100 μM was obtained in an independent experiment, in the presence of 25 μM $T\beta_4$. $T\beta_4$ thus far appears to be the only protein that exhibits such a large difference in affinity (≈50-fold) for ATP- and ADP–G-actin.

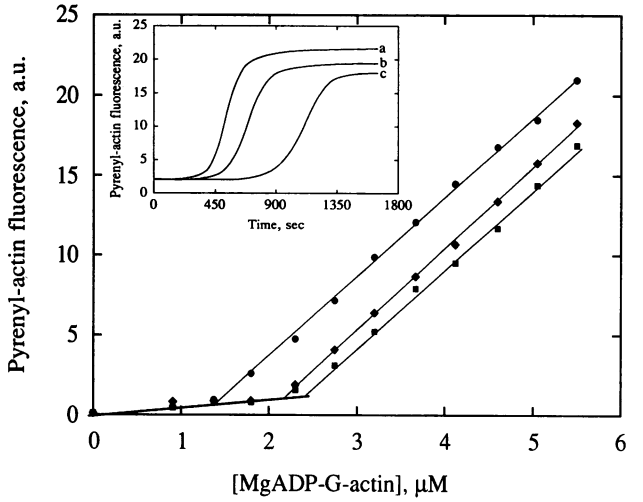

FIG. 2. Change in apparent critical concentration for polymerization of MgADP–actin in the presence of $T\beta_4$. MgADP–G-actin (5.5 μM, 5% pyrenyl labeled) was polymerized in the absence or presence of $T\beta_4$ at 35 or 50 μM. When the polymerization plateau was reached (see *Inset*), the samples were serially diluted in polymerization buffer without $T\beta_4$ (●) or with 35 μM (◆) or 50 μM (■) $T\beta_4$. (*Inset*) Polymerization time course with continuous fragmentation of 5.5 μM MgADP–G-actin (5% pyrenyl labeled) in the presence of a = 0; b = 35; c = 50 μM $T\beta_4$.

Profilin has been reported to have only ≈3-fold higher affinity for ADP–G-actin than for ATP–G-actin (42); gelsolin (48) and myosin subfragment 1 (49) show similar affinities for ATP- and ADP–G-actin. This result indicates that the conformations of MgATP–G-actin and MgADP–G-actin are very different, at least in the region of the $T\beta_4$ binding site. The implication of the modulation of the G-actin–$T\beta_4$ interaction by nucleotides is examined below.

The pronounced preference of $T\beta_4$ for MgATP–G-actin versus MgADP–actin is a result opposite to the one expected in the model proposed (30, 31) to account for the regulation of actin dynamics in cells via the concerted action of $T\beta_4$ and profilin. In this model, actin would be sequestered by $T\beta_4$ as ADP–G-actin, and desequestered as polymerizable ATP–G-actin via the action of profilin, which would catalyze exchange of ATP for bound ADP. This mechanism implies that $T\beta_4$ has a higher affinity for ADP–G-actin than for ATP–G-actin. Our data show that the contrary is true and lead to the conclusion that in resting cells, G-actin must be sequestered as ATP–G-actin by $T\beta_4$.

The intriguing issue raised by the large difference in affinity of $T\beta_4$ for ATP- and ADP–actin is, could the desequestration of G-actin be promoted by a large change in the ATP/ADP ratio in the living cell? The data in Figs. 1 and 2 let us anticipate that if the ATP/ADP ratio decreases abruptly in some cell compartments following stimulation, the population of G-actin molecules will be shifted from the ATP–G-actin–$T\beta_4$ (sequestered) state to the free ADP–G-actin (unsequestered) state. If the local concentration of ADP–G-actin were high enough above its critical concentration for polymerization, ADP–G-actin should polymerize into filaments.

Possible Regulation of Actin Sequestration–Desequestration by a Change in the ATP/ADP Ratio: Role of Profilin. The above-mentioned possibility that a large decrease in the ATP/ADP ratio, in a medium in which ATP–G-actin is sequestered by $T\beta_4$, could trigger actin polymerization was tested as follows. In the first experiment (Fig. 3*A*), the rate of filament elongation from MgADP–actin (10 μM) was 40% inhibited by 50 μM $T\beta_4$ (compare curves 1 and 2). When ADP–actin was converted to ATP–actin, 50 μM $T\beta_4$ caused a 98% decrease in the rate of growth (compare curves 3 and 4). Note that when ATP was added to a solution of filaments elongating from ADP–G-actin monomers in the presence of 50 μM $T\beta_4$, the elongation process was stopped immediately (within 2 sec), consistent with the known rapid spontaneous exchange of ATP for ADP (50, 51). Therefore, in the presence of high amounts of $T\beta_4$, filaments grow at a faster rate from ADP–actin than from ATP–actin (compare curves 2 and 4), a behavior opposite to the one that is observed in the absence of $T\beta_4$, where filaments grow at a faster rate from ATP–G-actin than from ADP–G-actin subunits (39, 52–54). In brief, ATP causes sequestration, and ADP causes desequestration of actin.

In a second experiment (Fig. 3*B*), the effect of ADP on filament elongation from ATP–G-actin 1:1 complex was examined in the presence or absence of 50 μM $T\beta_4$ and of 0.5 μM profilin. ADP was added together with F-actin seeds, at time zero, to the G-actin/$T\beta_4$/profilin solution. In the absence of $T\beta_4$, profilin accentuated the inhibition of filament elongation caused by ADP (curves 1–3); in contrast, in the presence of 50 μM $T\beta_4$, profilin accentuated the desequestrating effect of ADP, and promoted a faster rate of elongation (curves 4–6). Both observations are consistent with the reports (32–34) that profilin increases the rate of ATP dissociation from G-actin, which is known to be slow and which is kinetically limiting in the production of ADP–G-actin. In conclusion, in the presence of high amounts of $T\beta_4$, ADP can desequester G-actin, and the rate of production of ADP–G-actin—and hence the rate of polymerization—is regulated by profilin.

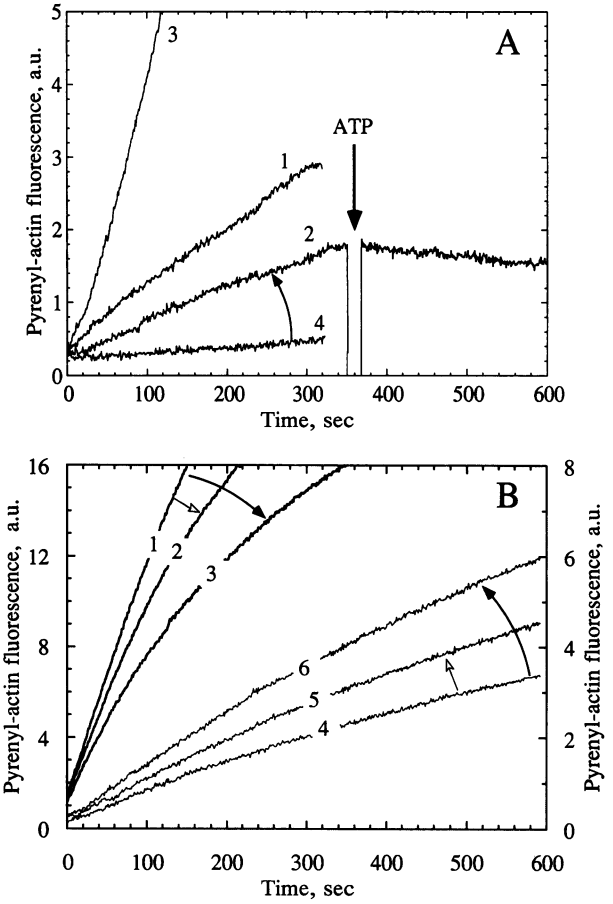

FIG. 3. Desequestration of G-actin in complex with $T\beta_4$. (*A*) Time course of filament growth from MgADP–G-actin. MgADP–G-actin (10.7 μM, 5.4% pyrenyl labeled) in buffer G′ was supplemented at time zero with 0.1 M KCl and 1 μM F-actin seeds. Curve 1, ADP–G-actin alone; curve 2, ADP–G-actin plus 50 μM $T\beta_4$ (at the time indicated by the thick arrow, 100 μM ATP was added to the cuvette); curve 3, ADP–G-actin plus 100 μM ATP added at time zero with seeds; curve 4, ADP–G-actin plus 100 μM ATP and 50 μM $T\beta_4$. The thin arrow schematizes the desequestering effect of ADP. (*B*) Desequestering effect of ADP: Possible role of profilin. MgATP–actin 1:1 complex (10 μM, 5.4% pyrenyl labeled) was supplemented at time zero with 0.1 M KCl and 1 μM F-actin seeds. Curve 1, ATP–G-actin alone (control); curve 2, 2 mM ADP was added at time zero; curve 3, 2 mM ADP was added at time zero to ATP G-actin containing 0.5 μM profilin; curve 4, ATP–G-actin plus 50 μM $T\beta_4$; curve 5, 2 mM ADP was added at time zero to ATP–G-actin plus 50 μM $T\beta_4$; curve 6, 2 mM ADP was added at time zero to ATP–G-actin plus 50 μM $T\beta_4$ and 0.5 μM profilin. Identical data were obtained with 1 μM profilin. Arrows with open and filled heads schematize the effect of ADP in the absence or presence of profilin. Note: left ordinate refers to curves 1–3, right ordinate to curves 4–6. Data were directly transferred from the Spex fluorimeter to an Apple computer.

In a third experiment (data not shown), the shift from ATP to ADP was exerted by the hexokinase/glucose system. The addition of hexokinase (100 units/ml) to the ATP–G-actin/$T\beta_4$/2 mM glucose/F-actin seeds solution promoted ATP exhaustion, actin desequestration, and filament elongation from ADP–G-actin subunits following a 40-sec delay.

The above experiments have been performed at total concentrations of actin (10 μM) and $T\beta_4$ (50 μM) that are feasible in the laboratory; however, the desequestering effect of ADP will be more pronounced in a range of higher, physiological concentrations of $T\beta_4$, as demonstrated by the following calculations and Fig. 4.

The rate of filament elongation is a function of [ATP]/[ADP], and of total concentrations of G-actin (G_0) and of $T\beta_4$

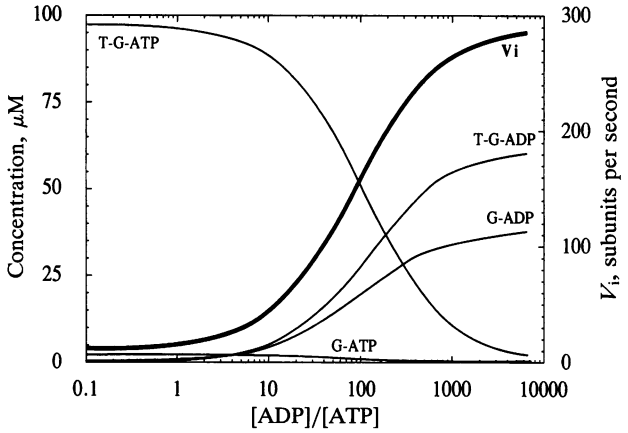

FIG. 4. Distribution of the different G-actin species and change in the rate of filament growth as a function of the [ADP]/[ATP] ratio. The values of [G–ATP], [G–ADP], [T–G–ATP], and [T–G–ADP] and the rate of filament growth (V_i) were calculated from Eqs. 2–4 and the following parameter values: $K_D/K_T = 3.5$ (55); $[G_0] = 100$ μM; $[T_0] = 200$ μM; $L_T = 1.9$ μM; $L_D = 85$ μM; critical concentrations for polymerization of ATP- and ADP-actin, 0.1 μM and 1.5 μM (56); $k_T = 12$ $\mu M^{-1}\cdot sec^{-1}$ and $k_D = 8$ $\mu M^{-1}\cdot sec^{-1}$ as reported (53) under the same ionic conditions (100 mM KCl, 1–5 mM $MgCl_2$, 1 mM EGTA). The increase in V_i was qualitatively similar, with $k_T = 5$ $\mu M^{-1}\cdot sec^{-1}$, and $k_D = 0.9$ $\mu M^{-1}\cdot sec^{-1}$ (54) found under slightly different conditions (100 mM KCl, 1 mM $MgCl_2$, no EGTA).

(T_0); let us call K_T and K_D the equilibrium dissociation constants of the ATP–G-actin (G_T) and ADP–G-actin (G_D) complexes, respectively, and call L_T and L_D the equilibrium dissociation constants for the complexes of Tβ4 (T) with ATP–G-actin and ADP–G-actin. The mass conservation equations are (we assume that the concentration of G-actin with no nucleotide bound is negligible)

$$G_0 = G_T + G_D + TG_T + TG_D$$
$$T_0 = T + TG_T + TG_D, \quad [1]$$

leading to

$$[G_D] = \frac{[G_0]}{1 + ([ATP]/[ADP])K_D/K_T(1 + [T]/L_T) + [T]/L_D} \quad [2]$$

and

$$[T] = \frac{[T_0]}{1 + [G_D]\{([ATP]/[ADP])(K_D/K_T)/L_T + (1/L_D)\}}. \quad [3]$$

The value of $[G_D]$ can be derived from the combination of Eqs. 2 and 3. The rate of filament elongation, V_i, can then be calculated, if one knows the values of the bimolecular association rate constants of ATP–G-actin (k_T) and ADP–G-actin (k_D) to filament ends.

$$V_i = k_D([G_D] - Cc_D) + k_T([G_T] - Cc_T), \quad [4]$$

where Cc_D and Cc_T are the critical concentrations for polymerization of ATP–G-actin and ADP–G-actin. Fig. 4 shows the decrease in TG_T and G_T, the increases in TG_D and G_D and the concomitant increase in the rate of polymerization, V_i, upon increasing [ADP]/[ATP], at total concentrations $[G_0] = 100$ μM and $[T_0] = 200$ μM.

Conclusion and Perspectives. The results indicate that the combined effects of Tβ4, profilin, and a decrease in the [ATP]/[ADP] ratio can promote, under physiological conditions, the desequestration of G-actin leading to filament assembly from ADP–G-actin subunits. This mechanism operates in a manner opposite to the one proposed (30, 31). The present model accommodates well the known properties of profilin, which accelerates the rate of dissociation of ATP from G-actin.

In the conventional scheme for filament assembly, the polymerizable form of actin is ATP–G-actin, and ATP hydrolysis accompanies polymerization and regulates actin–actin interactions in the filament (56, 57). The present model for actin desequestration and polymerization relies on the unusual polymerization of ADP–G-actin. However, energy is necessary for a cycle of actin sequestration–desequestration–resequestration to operate: ATP is necessary to ensure sequestration of G-actin by Tβ4, and a local source of ADP created by an associated ATPase is necessary to deplete ATP locally and allow actin desequestration. Once away from the source of ATP hydrolysis, filaments are in an ATP-rich medium and depolymerize, leading to sequestered ATP–G-actin–Tβ4, and a cycle has been completed. Interestingly, the possible function of profilin in catalyzing exchange of ADP for bound ATP on G-actin is the reverse of the classical function of guanine nucleotide-releasing factors, which promote exchange of GTP for bound GDP on G proteins (58).

The crucial issue to be examined is whether the mechanism proposed here actually operates *in vivo*; i.e., whether local and transient medium ATP depletion occurs in the regions of the cells where rapid polymerization of actin occurs, upon platelet or neutrophil stimulation, or at the leading edge of locomoting cells, or at the rear of *Listeria* bacteria. It is known that a large number of ATP-consuming reactions (e.g., in the phosphatidylinositol cycle) occur at the internal leaflet of the membrane immediately upon stimulation; the pool of cytoplasmic (metabolic) ATP in platelets decreases by 20% in the first seconds following stimulation by thrombin (59–61); this number corresponds to total cytoplasmic ATP; however, the drop in free ATP in the vicinity of the membrane might be larger. The fact that up to 91% of platelet myosin cosediments with F-actin when analyzed 30 sec following thrombin addition, and this amount then decreases to ≈60% in the following minutes (4), is suggestive of the transient formation of rigor F-actin–myosin complexes due to ATP depletion. The absence of bound ATP in the high-affinity profilactin complex (40) transiently formed in platelets is consistent with ATP depletion and with the known decrease in affinity of ATP for G-actin upon binding of profilin (42). The agonist-induced oscillations of actin polymerization in neutrophils (62) may be due to cycles of ATP depletion–synthesis. A recent report (63) showed that Tβ4 was not modified following stimulation of neutrophils, a result consistent with the regulation of the affinity of Tβ4 for G-actin by transient changes in ATP.

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