Thymosin \( \beta_4 \) is acknowledged as a major G-actin binding protein maintaining a pool of unassembled actin in motile vertebrate cells. We have examined the function of \( \beta_4 \) in actin assembly in the high range of concentrations (up to 300 \( \mu \)M) at which \( \beta_4 \) is found in highly motile blood cells. \( \beta_4 \) behaves as a simple G-actin sequestering protein only in a range of low concentrations (<20 \( \mu \)M). As the concentration of \( \beta_4 \) increases, its ability to depolymerize F-actin decreases, due to its interaction with F-actin. The \( \beta_4 \) actin can be incorporated, in low molar ratios, into F-actin, and can be cross-linked in F-actin using 1-ethyl-3-(3-dimethylaminopropyl)carbo-dimide. As a result of the copolymerization of actin and \( \beta_4 \) actin complex, the critical concentration is the sum of free G-actin and \( \beta_4 \)-G-actin concentrations at steady state, and the partial critical concentration of G-actin is decreased by \( \beta_4 \)-G-actin complex. The incorporation of \( \beta_4 \) actin in F-actin is associated to a structural change of the filaments and eventually leads to their twisting around each other. In conclusion, \( \beta_4 \) is not a simple passive actin-sequestering agent, and at high concentrations the ability of \( \beta_4 \) actin to copolymerize with actin reduces the sequestering activity of G-actin-binding proteins. These results question the evaluation of the unassembled actin in motile cells. They account for observations made on living fibroblasts overexpressing \( \beta \)-thymosins.

It is generally thought that to elicit a motile response to extracellular signals, living cells regulate their content in F-actin, by spatially controlled changes in the steady state of filament assembly (1). Capping proteins and G-actin binding proteins are major players in this control. In the presence of ATP, capping proteins block the dynamics at the barbed ends of actin filaments and establish the high critical concentration of the pointed ends; when barbed ends are uncapped, the effective critical concentration is close to the critical concentration of the barbed end. The changes in critical concentration, however, represent a very small amount of actin in mass (less than 1 \( \mu \)M), hence by themselves they cannot elicit any massive assembly of actin. These changes, however, are largely amplified by G-actin binding proteins which maintain a pool of unassembled (sequestered) actin, used for site-directed actin assembly. The concentration of actin in complex with sequestering proteins is indeed determined by the concentration of free G-actin, i.e. the critical concentration. The concentration of actin in complex with sequestering proteins is high when barbed ends are capped, and decreases locally to yield F-actin upon creation and maintenance of available barbed ends, which is thought to occur upon stimulation. A major G-actin binding protein is thymosin \( \beta_4 \) (T\( \beta_4 \)) discovered in 1991 (2) in platelets and later found to be ubiquitous in vertebrate cells (see for review, Refs. 3 and 4). The function of \( \beta_4 \) as a simple passive sequestering protein was demonstrated in vitro (5–7) as well as in vivo (8–11). The equilibrium dissociation constant for the G-actin–\( \beta_4 \) complex lies in the 0.7–2.5 \( \mu \)M range, from measurements of its sequestering activity (5–7) as well as from direct binding studies (12, 13). However, in the above experiments, the concentrations of \( \beta_4 \) used were lower than the physiological concentrations, especially those found in motile blood cells (200–500 \( \mu \)M in platelets and neutrophils (5, 8)). In the present work, the function of \( \beta_4 \) is explored in greater detail in the higher concentration range found in motile living cells. The role of \( \beta_4 \) appears more complex than previously thought, because actin filaments fail to totally depolymerize in the presence of high concentrations (100–200 \( \mu \)M) of \( \beta_4 \) due to incorporation of very low amounts of \( \beta_4 \)-actin in the filaments. The consequences of this property of \( \beta_4 \) on the structure of filaments and on the regulation of actin assembly in living cells is examined.

MATERIALS AND METHODS

Proteins—Actin was purified from rabbit skeletal muscle (14) and isolated as calcium ATP-G-actin by Sephadex G-200 chromatography (15) in G buffer (5 mM Tris-Cl, pH 7.8, 0.1 mM CaCl\(_2\), 0.2 mM ATP, 0.2 mM dithiothreitol, 0.01% NaN\(_3\)). Actin was pyrenyl-labeled as described (16). Thymosin \( \beta_4 \) was purified from bovine spleen as follows. All operations were done at 4°C. 400 g of frozen spleen (cut into 2 × 2 × 2-cm cubes and frozen on dry ice at the slaughterhouse, then stored at −80°C) were homogenized with 4 volumes of cold 0.625 N HClO\(_4\) in a Waring blender for 2 min, then centrifuged at 20,000 × g for 15 min. The supernatant was brought to pH 4 by dropwise addition of 5 N KOH, and filtered to remove KClO\(_4\). The solution was loaded onto a 6 × 12-cm column of Lichroprep RP18 (40–63 \( \mu \)M, Merck). Following a 2000-mL H\(_2\)O wash, elution was performed with 400 mL of 33% 1-propanol in water. The eluted material was concentrated to ~50 mL by rotary evaporation, brought to pH 7.8 with KOH, filtered over 0.22- \( \mu \)m nitrocellulose filters, and submitted to anion exchange chromatography on Q-Sepharose (2.5 × 25 cm) equilibrated in 40 mM ammonium acetate, pH 7.8. Elution was performed using a gradient from 40 mM ammonium acetate, pH 7.8, to 0.2 M ammonium acetate, 0.2 M acetic acid, pH 5.0 (400 mL × 2). Elution was monitored by absorbance at 214 nm following 10-fold dilution of an aliquot of the fractions in H\(_2\)O. The peak of \( \beta_4 \) was identified by analytical HPLC\(^1\) on RP18 Select B (Merck, 4 × 125

\(^{1}\) The abbreviations used are: HPLC, high performance liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GBP, G-actin binding protein.
mm) using a gradient from 5 to 50% acetonitrile. Fractions containing Tβ4 were pooled, concentrated, and final purification was achieved by preparative HPLC on RP18 Select B as described (7). About 40 mg of pure Tβ4 were obtained. The purity of Tβ4 was checked by mass spectrometry. The concentration of Tβ4 was determined by the biocinchonic acid assay, using bovine serum albumin as a standard.

Tβ4 was chemically synthesized on a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) with an additional Gly-Cys at the extreme C terminus. Previous studies have indicated that the nature of the C terminus of Tβ4 is not important for actin binding (17). The C-terminal cysteine residue was then labeled with iodo-[1-14C]acetylimidate (Amersham) using a 1:1.5 molar ratio of thymosin β4 to label. The resulting [14C]thymosin β4 had a specific activity of 20,000 cpm/mmol.

Tβ4 was oxidized into Metα-sulfoxide-Tβ4 by incubation for 1 h at room temperature in the presence of 2 N HClO4 (6% v/v), immediately followed by lyophilization. Profilin was purified from bovine spleen as described (18). Gelsolin was purified from pig plasma as described (19). Recombinant CapG was expressed and purified as described (20).

steady state Measurements of F-actin—The amount of actin assembled at steady state in the presence of different amounts of Tβ4 or profilin was monitored by pyrene fluorescence. Actin (1% pyrenyl labeled) was polymerized at the indicated concentration in G buffer supplemented with 2 mM MgCl2 and 0.1 M KCl. Samples of 300 μl containing Tβ4 or profilin, gelsolin, or cap, at the indicated concentrations were incubated for 3 h at room temperature in the dark. Pyrene fluorescence was measured in a Spex Fluorolog instrument. Excitation and emission wavelengths were 366 and 387 nm, respectively.

Barbed ends were capped by either gelsolin or CapG. Gelsolin was added at a gelsolin:actin ratio of 1:200 to 1:500. When CapG, which is a weaker capping protein, was used, it was added at a constant concentration of 120 nM in all samples, independently of actin concentration. Preliminary assays were run with each batch of CapG used in this work, to verify that the critical concentration of the pointed end was established as soon as at least 80 nm CapG was present in solution. The amount of F-actin and unassembled actin present at steady state was converted into mole concentrations by comparison of the fluorescence readings with a critical concentration curve carried out in parallel using the same actin solution, in the same concentration range as in the samples.

Initial Rate of Filament Growth or Depolymerization—The rate of filament growth off preformed F-actin seeds (either capped or uncapped) was measured as described (21). A small aliquot (~5% of total volume) of a solution of pyrenyl-labeled F-actin (10–20 μM), preassembled at steady state at least 2 h prior to the assay, was added to a solution containing pyrenyl-labeled g-actin and Tβ4. The g-actin and the F-actin (seeds) solutions were identically pyrenyl-labeled. The increase or decrease in fluorescence indicating elongation or depolymerization of the seeds was measured. The rates of fluorescence increase were monitored with a 1% pyrenyl-labeled actin, and a 5% pyrenyl-labeled actin, using a calibration curve obtained by polymerization of F-actin (seeds) solutions containing pyrenyl-labeled g-actin and Tβ4. Since only free g-actin can appreciably participate in assembly, filament growth was inhibited due to the formation of the Tβ4-actin complex (6). Gelsolin-capped filaments were used as seeds, and the value of C0 was chosen low enough (C0 ≤ 3 μM) for the free g-actin concentration dependence of the rate of growth at the pointed ends of actin filaments to vary linearly in the range (0 to C0) (22). Under these conditions, the fraction of Tβ4-bound actin, α, was directly proportional to the percent of inhibition of filament growth:

\[
\alpha = \frac{1}{1 + \frac{C_o}{K_T} \frac{(Tb_4)}{C_a}} \quad \text{(Eq. 3)}
\]

where V(0), V(Tb_4), and V(α) were the elongation rates measured in the absence or in the presence of a concentration of Tb_4, or at infinite concentration of Tb_4, respectively. (Note that V(α) theoretically equals the rate of depolymerization of filaments upon dilution, which was experimentally verified.) Data were analyzed within the following equation which describes the hyperbolic binding of Tb_4 to g-actin:

\[
\frac{1}{\alpha} = \frac{1}{K_T} \frac{C_o}{C_a} \quad \text{(Eq. 4)}
\]

The value of K_T was derived from the slope of 1/(1-α) versus [Tb_4].

Chemical Cross-linking of Tb_4 to G-actin and Tb_4 to Steady State—A solution of g-actin (15 μM) was dialyzed overnight (to remove Tris and to polymerize actin) against 5 mM phosphate, pH 7.5, containing 0.2 mM CaCl2, 2 mM MgCl2, 0.1 mM KCl, 0.2 mM diethyolothio, 0.2 mM ATP, using Spectrapore membranes. The dialyzed F-actin sample was incubated with 200 μM 14C-Tb_4 (20,000 cpm/nmol) for 16 h, then supplemented with 4 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) and 4 mM sulfo-NHS at room temperature for 1 h. The reaction was stopped by addition of 20 mM Tris. The cross-linked sample was centrifuged at 400,000 × g for 30 min in the ultracentrifuge (Beckman, TL 300). The supernatant, containing covalent and non-covalent G-actin-Tb_4 complexes, and the pellet, containing F-actin and cross-linked F-actin-Tb_4 complexes, were processed separately to identify the cross-linked Tb_4-polypeptides by SDSPolyacrylamide gel electrophoresis (23) and autoradiography. The cross-linked peptides were further characterized by cyanogen bromide degradation for 24 h at room temperature, and analyzed using a 100 times molar excess of CNBr over methionine. The peptide profiles were analyzed on Tricine-Polyacrylamide gels (24).

Incorporation of Tb_4 into F-actin—The binding of Tb_4 to F-actin was measured using a sedimentation assay. Samples of F-actin (20 μM, 0.25 ml), containing different amounts of Tb_4 in the range 0–250 μM, were sedimented at 400,000 × g for 30 min, following 16 h incubation at room temperature. Pellets of F-actin were resuspended in a 2.5-fold smaller volume of G-buffer and assayed for Tb_4. Two methods were used to quantify the amount of F-actin-bound Tb_4. In the first method, 14C-Tb_4 was used and the amount of bound Tb_4 was derived from radiactivity measurements. To estimate the amount of Tb_4 present in the interstitial volume of the pellets and not specifically bound to F-actin, pellets were run in all assays containing an amount of F-actin in the range 5–20 μM, and 5 μM 14C-Tb_4-ox prepared by HClO4 oxidation of Met6 in the 14C-Tb_4 material used in the experiments. Since Tb_4-ox is known not to bind significantly to actin at this concentration (12), it is present in the pellet as a marker of the interstitial volume. The percent of total oxidized Tb_4 present as a contaminant in each pellet was measured at each concentration of F-actin, providing an estimation of the contamination per unit volume of the pellet. The amounts of F-actin and unassembled actin in the samples run at different concentrations of Tb_4 are known (Fig. 2A). Therefore the amount of 14C-Tb_4 actually bound to F-actin could be obtained after subtraction of the appropriate percent of the total 14C-Tb_4 as a contaminant. ATP, used in these experiments, was not oxidized. In the second method, 14C-Tb_4 was used, since Tb_4 present in the pellets of F-actin was assayed by HPLC of the perchloric extract of the resuspended pellets, and comparison of the peak areas of the absorbance elution diagram at 220 nm with a calibration curve using standards in the range 0–2 nmol of Tb_4. The proportion of contaminant Tb_4 trapped in the pellet was estimated as in the first method.

Electron Microscopy—The structure of actin filaments at steady state in the presence of Tb_4 at different concentrations was examined in the electron microscope. Samples of 3–10 μM F-actin, 14–50 μM gelsolin, and Tb_4 in the range 0–250 μM were prepared 16 h in advance to make sure that steady state was established. Ten microliters of each sample were deposited on a carbon coated, air-dried glass micrograph. Following 10–20 s drying, the excess solution was blotted and the sample was negatively stained with several drops of a 2% uranyl acetate solution. Unidirectional shadowing of freeze-dried specimens was performed as
described (25). Samples were prepared and adsorbed on the grid as for negative staining, rapidly rinsed with distilled water and immediately (within 2 s) frozen by immersion into liquid nitrogen. The grid was transferred into a Cryofract (Reichert-Jung) unit and maintained at −85 °C for at least 2 h. The specimens were then shadowed with a 2-nm thick layer of carbon-platinum, evaporated at an angle of 45°, and coated with a 3–4-nm carbon film evaporated at 90°. Specimens were observed in a CM12 Philips electron microscope operated at 80 kV. Micrographs were recorded at a nominal magnification of 35,000.

RESULTS

Tβ4 Binds G-actin Selectively at Low Concentration—In living cells, the physiological ionic conditions are such that F-actin is assembled at steady state, i.e., filaments coexist with G-actin at the critical concentration. Hence in vitro measurements of F-actin at steady state lead to the closest description of the in vivo situation. The actin-sequestering activity of Tβ4 was monitored by the linear decrease in the concentration of F-actin at steady state versus total concentration of Tβ4 as described by Equation 2 (see “Materials and Methods”):

$$[TA] = [T_o] + \frac{[A_o]}{K_T}$$

(Eq 5)

When barbed ends are capped, A_o is the critical concentration at the pointed ends of filaments, which is higher than at the barbed ends, hence Tβ4 sequesters G-actin more efficiently. This situation is the most favorable, for economy of material, to measure the affinity of Tβ4 for G-actin. Fig. 1 shows that upon addition of increasing amounts of Tβ4 to a solution of 3 μM F-actin capped by gelsolin, the concentration of unassembled actin (A + TA) increased linearly with total β-thymosin until all actin was depolymerized. A value of 2 ± 0.2 μM was derived for K_T from the slope of the plot, in good agreement with previous data (5–7) obtained in the same range of actin and Tβ4 concentrations.

Tβ4 Does Not Behave as a Simple G-actin Sequestering Protein at High Concentration—The same experiment as above was repeated in a range of higher concentrations of F-actin. The data, displayed in Fig. 2A, show that above a total concentration of Tβ4 = 20 μM, Tβ4 sequestered actin less efficiently than expected, and a deviation from the linear dependence of [TA] versus [T_o] (Equation 1) was observed. Filaments remained stable in solution at concentrations of Tβ4 at which total depolymerization should have occurred. At concentrations of F-actin higher than 10 μM, an invariant curve [A + TA] = f(T_T) was obtained (E and C in Fig. 2A). The fact that the dependence of [A + TA] on [T_T] becomes independent of the amounts of F-actin means that the measured amount of unassembled actin ([A + TA]) at each [T_T] represents the concentration of monomers, A and TA, at equilibrium with filaments, that is the critical concentration.

It was found appropriate to verify that the ATP had not been extensively hydrolyzed during the 16-h incubation period, due to the steady-state ATPase of F-actin, and that even the most concentrated F-actin samples could be truly considered as being at steady state in the presence of ATP. G-actin (20 μM) was equilibrated in G-buffer in which the 200 μM ATP were traced by [γ-32P]ATP, and polymerized by addition of 2 mM MgCl₂ and 0.1 M KCl as described under “Materials and Methods.” The solution was then split into 3 samples containing 0, 50, and 200 μM Tβ4. After 16 h incubation, the amounts of hydrolyzed ATP were 29, 28.8, and 31.8 μM, respectively, in the three samples. Hence free nucleotide in the medium then consisted of about 10 μM ADP and 190 μM ATP. These numbers testify that the measurements made after 16 h truly reflect a situation in which F-actin is at steady-state in ATP, hence free G-actin is ATP-G-actin at the critical concentration, and Tβ4 binds ATP-G-actin as described by Equation 2.

Thus far the conclusion that Tβ4 fails to totally depolymerize F-actin at high concentration relies on fluorescence measurements of pyrenyl actin. To verify that the measurements truly reflect equilibrium values of F-actin, G-actin, and Tβ4-G-actin, the following controls were carried out. 1) Identical fluorescence readings were obtained 8 h later, indicating that measurements reflected a stable situation; 2) identical results were obtained with actin solutions containing different fractions of pyrene-labeled actin; 3) sedimentation of the samples of F-actin containing different concentrations of Tβ4 and SDS-gel electrophoresis of the pellet and supernatant (Fig. 2A, inset) established the validity of the interpretation of fluorescence measurements in terms of F-actin and unassembled actin; 4) sedimentation velocity of Tβ4 at different concentrations up to 250 μM showed that Tβ4 was a monomeric 5-kDa protein in the whole range of concentrations investigated. Finally quantitatively identical results showing incomplete depolymerization of F-actin were obtained with chemically synthesized Tβ4, which eliminates the possibility that a minor contaminant present in the preparation of Tβ4 from spleen would be responsible for the incomplete depolymerization of F-actin at high Tβ4.

The fact that Tβ4 fails to totally depolymerize actin in a range of high concentrations is a first indication that it can bind to F-actin as well as to G-actin, albeit with a lower affinity. Because other actin-binding proteins like ADF/cofilin (26, 27) have been shown to bind preferentially either F- or G-actin depending on pH, the depolymerization of F-actin (20 μM) by increasing amounts of Tβ4 was measured at pH 6.5 and 7.8 in parallel. Practically identical curves [TA] = f(T_T) showing incomplete depolymerization were obtained at the two pH values (data not shown). On the other hand, the failure of Tβ4 to totally depolymerize F-actin at high concentration was only observed at physiological ionic strength (0.1 M KCl). In a polymerization buffer containing only 2 mM MgCl₂, addition of increasing amounts of Tβ4 to 20 μM F-actin led to eventual complete depolymerization, and a linear curve [TA] = f(T_T) was obtained (like in Fig. 1), consistent with a value of K_T of 0.8 μM, in agreement with previous determinations at low ionic strength (12).

The incomplete depolymerization of F-actin at high concentration of Tβ4 was also observed when barbed ends were uncapped. Fig. 2B shows the amount of F-actin observed at steady-state upon addition of increasing amounts of Tβ4 to 12 μM F-actin, with barbed ends either capped by CapG, or un-
Tβ₄ Is Not a Simple G-actin Sequestering Protein

The concentration of F-actin at steady state was derived from pyrene fluorescence. The emissions shutter was periodically opened for 5 s to measure steady-state fluorescence. The time courses of actin desequotation/repolymerization upon uncapping by EGTA, displayed in Fig. 2C, show that the repolymerization process gets slower at higher concentrations of Tβ₄. This result is very surprising for the following reason. Upon uncapping of barbed ends due to EGTA-induced dissociation of CapG, the initial rate of repolymerization is expected to be the following:

\[
V = k_b [F]C_p^f - C_p^f
\]  
(Eq. 6)

where \(k_b\) is the rate constant for addition of G-actin to the uncapped ends at concentration [F], \(C_p^f\) is the concentration of free G-actin at time of uncapping, which is equal to the critical concentration of the pointed ends, and \(C_p^f\) is the critical concentration for actin assembly at the barbed ends, which is reached upon completion of the uncapping-linked repolymerization process. According to Equation 2, the initial rate of repolymerization should be the same at all concentrations of Tβ₄. Only the extent of repolymerization should vary in proportion with Tβ₄, reflecting the difference in the amount of Tβ₄-G-actin complex (TA), when barbed ends are capped or uncapped, as follows.

\[
\Delta[TA] = [TA]_0(C_p^f C_p^c + K_p) - C_p^f[C_p^c + K_p]
\]  
(Eq. 7)

The Critical Concentration for Actin Assembly Decreases in the Presence of Tβ₄: Evidence for the Interaction of Tβ₄ with F-actin—The data shown in Fig. 2 suggest that the Tβ₄-G-actin complex could be a weakly polymerizing actin species able to copolymerize with actin. If the Tβ₄-G-actin complex can undergo the monomer-polymer exchange reactions which maintain filament stability at steady-state, the global critical concentration for actin assembly is the sum of the partial critical concentrations of G-actin and Tβ₄-G-actin, respectively, and the contribution of Tβ₄-G-actin to monomer-polymer exchange is expected to decrease the critical concentration of G-actin. The following simple experiment was designed to challenge this possibility.

We have checked that despite the fact that profilin does not bind pyrenyl-actin, the change in fluorescence of pyrenyl-F-actin capped by gelsolin upon increasing profilin is a valid measurement of the mass amount of F-actin at steady state over periods of several hours, as long as profilin is added to preassembled F-actin. This is no longer the case when actin has been assembled in the presence of profilin. A full study and justification of this assessment is provided elsewhere (I. Perelroizen, D. Didry, H. Christensen, N. H. Chua, M.-F. Carlier, submitted for publication).

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F-actin (1% pyrenyllabeled) was assembled at 10 μM in the presence of 120 mM Cap6 and either in the absence (●) or presence (○) of 50 μM Tβ4. Profilin was added to the samples at the indicated concentration. Samples were incubated overnight before fluorescence was measured. The linear decrease in fluorescence represents the decrease in F-actin due to the formation of profilin-actin complex. The less steep slope observed in the presence of 50 μM Tβ4 implies a 3.8-fold lower value of A, in the presence of 50 μM Tβ4 B, the concentration of free G-actin at steady state, or partial critical concentration of G-actin, [A], was derived from the measurements of unassembled actin, [A] (Eq. 7), shown in Fig. 2a (○, ▽) using Equation 9.

decrease in F-actin, [A], was 2.3-fold lower, consistent with a 3.8-fold lower value of [A]. Hence the partial critical concentration for actin assembly at the pointed ends is decreased by Tβ4. Evidence for the Tβ4-dependent decrease in the partial critical concentration of G-actin can also simply be derived from the analysis of the amounts of unassembled actin at different concentrations of Tβ4 ([T3]) shown in Fig. 2a (○, ▽), as follows. At each value of [T3], the measured amount of unassembled actin [A] can be written:

$$[A] = [A] + [TA] = [A] + [T3] \frac{[A]}{[A] + K_T} \quad (\text{Eq. } 8)$$

which leads to a quadratic equation in [A], the solution which is:

$$[A] = \frac{[A] - K_T - [T3] \pm \sqrt{[A] - K_T - [T3]^2 + 4[A] \cdot K_T}}{2} \quad (\text{Eq. } 9)$$

The change in [A] versus [T3] can therefore be derived from the measurements of [A] at different values of [T3]. Fig 3B shows that [A] calculated according to Equation 9 decreases cooperatively upon increasing [T3]. It is interesting to observe that 50 μM Tβ4, a value of [A] of 0.15 μM was derived from the data shown in Fig. 2a, that is a 3.3-fold decrease in critical concentration, in good agreement with the 3.8-fold decrease found in the experiment shown (Fig. 3A) carried out also at 50 μM Tβ4, and in which profilin was used to derive the value of [A]. Hence two independent methods agree quantitatively to demonstrate that the partial critical concentration of G-actin decreases as larger amounts of TA complex are formed at steady-state. This result accounts for: 1) the limited extent of repolymerization upon uncapping and 2) the slowing down in the repolymerization process upon uncapping observed in Fig. 2C. Indeed, because C0 decreases from 0.5 μM to less than 0.1 μM (Fig. 3B), while Ck cannot decrease by more than 0.1 μM, the value of Δ[T3] is lower than expected (Equation 7) and the value of V (Equation 6) decreases upon increasing Δ[T3]/Tβ4.

Incorporation of Tβ4 in Actin Filaments—The binding of Tβ4 to F-actin was quantitated using both the sedimentation assay and chemical cross-linking described under “Materials and Methods.” The sedimentation assays (both using chemically synthesized 14C-Tβ4 and unlabeled spleen Tβ4) showed evidence for a very weak, substoichiometric binding of Tβ4 to F-actin. The binding also appeared cooperative, as can be seen on Fig. 4. Less than 0.01 Tβ4 per F-actin subunit was bound at 100 μM Tβ4, while about 0.04 Tβ4 per F-actin was measured at 250 μM Tβ4. Although these figures are very low and indicate that the binding constant lies in the 5–10 mM range, they were significantly above the level corresponding to simple trapping of Tβ4 in the pellets. Typically, at 100 μM Tβ4, the amount of Tβ4 measured in the pellets was twice as high as the amount trapped in the interstitial volume.

Chemical cross-linking of Tβ4 to F-actin displayed in Fig. 4, inset, confirmed that at high concentration Tβ4 bound to F-actin. Approximately 5–10% of F-actin could be covalently cross-linked to Tβ4 at 200 μM 14C-Tβ4, leading to a 47-kDa 14C-labeled polypeptide migrating at the same position as the covalent Tβ4-G-actin complex. The mass amount of Tβ4-actin cross-linked polypeptide was only about 3–4-fold lower than the mass amount of the Tβ4-G-actin cross-linked polypeptide found in the supernatant of the sedimented cross-linked mixture, which rules out the possibility of a significant contamination of covalent Tβ4-actin by covalent Tβ4-G-actin trapped in the pellet. On the other hand, an artifact might arise if the covalently cross-linked Tβ4-G-actin complex aggregated and

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**Fig. 3.** Tβ4 causes the lowering of the critical concentration. A, F-actin (1% pyrenyllabeled) was assembled at 10 μM in the presence of 120 mM Cap6 and either in the absence (●) or presence (○) of 50 μM Tβ4. Profilin was added to the samples at the indicated concentration. Samples were incubated overnight before fluorescence was measured. The linear decrease in fluorescence represents the decrease in F-actin due to the formation of profilin-actin complex. The less steep slope observed in the presence of 50 μM Tβ4 implies a 3.8-fold lower value of A, in the presence of 50 μM Tβ4 B, the concentration of free G-actin at steady state, or partial critical concentration of G-actin, [A], was derived from the measurements of unassembled actin, [A] (Eq. 7), shown in Fig. 2a (○, ▽) using Equation 9.

**Fig. 4.** Evidence for low affinity binding of Tβ4 to F-actin. Samples of F-actin (20 μM) capped by gelsolin (0.05 μM) containing the indicated amounts of Tβ4 were sedimented at 400,000 × g and the amount of bound Tβ4 per F-actin subunit was determined by HPLC after correction for Tβ4 trapped in the pellet (see “Materials and Methods”). Inset, EDC cross-linking of Tβ4 to F-actin and G-actin. Samples of F-actin (15 μM) capped by gelsolin and containing either 200 μM (+) or 0 (−) 14C-Tβ4 were cross-linked for 1 h by EDC-sulfo-NHS as described under “Materials and Methods.” The samples were centrifuged and the pellets were resuspended in a volume of buffer 2.5-fold smaller than the original volume. 25 μl of supernatants (sup.) and 15 μl of the resuspended pellets (pel.) were submitted to SDS-polyacrylamide gel electrophoresis (top panel) followed by autoradiography of the gel (bottom panel).
sedimented together with F-actin. To test this possibility, G-actin (15 μM) was supplemented with 200 μM Tβ4, followed by 1 mM MgCl2 and 0.1 M KCl, and the mixture was immediately submitted to cross-linking. No polymerization of G-actin could occur during cross-linking due to the high amount of Tβ4. The sample was centrifuged at 400,000 × g. Although no pellet could be seen by the eye, any putative sedimented material was carefully resuspended. No covalent actin-Tβ4 adduct was observed in gel electrophoresis of the resuspended material. Therefore the cross-linking experiments also demonstrate weak binding of Tβ4 to F-actin. The Tricine-SDS gel patterns of the cyanogen bromide digests of the covalent 14C-Tβ4-G-actin and 14C-Tβ4-F-actin complexes were identical, which provided an indication that the contact points between Tβ4 and either G- or F-actin were identical.

Morphology of Actin Filaments Assembled in the Presence of Increasing Amounts of Tβ4—Images of negatively stained specimens of F-actin at steady state in the presence of increasing amounts of Tβ4 in the range 0–250 μM are displayed in Fig. 5. In the absence of Tβ4, filaments showed a distinct periodicity of the two-start long pitch helix (Fig. 5a). This periodic feature was already less apparent in filaments assembled in the presence of 22 μM Tβ4 (Fig. 5b). Unraveling of the two-start long pitch helix was more frequent in the presence than in the absence of Tβ4. The mean number of unraveled areas per half-micron length of filament was 0.7 (S.D. = 0.6, n = 20 measurements) in the absence of Tβ4, and 1.5 (S.D. = 1.0, n = 30) in the presence of 22 μM Tβ4. The length of the unraveled areas also depends on Tβ4. The average unravelled length was 150 Å (S.D. = 40 Å, n = 18) in the absence of Tβ4, and 200 Å (S.D. = 80 Å, n = 18) in the presence of 22 μM Tβ4. In the presence of 100 μM Tβ4 (Fig. 5c) a small proportion of filaments twisted around each other in a rope-like or torsadefashion. The number of filaments in each torsade is not clearly defined and the individual filament cannot be recognized in the twisted polymer. In the presence of 250 μM Tβ4 (Fig. 5d) the proportion of intertwining and twisting of filaments increased, and few isolated filaments could be seen. The intertwining of filaments in torsades was concentration-dependent, fewer torsades being observed at a lower concentration of F-actin in the presence of 250 μM Tβ4. The destabilizing effect of Tβ4 on the structure of the individual filament can best be seen in freeze-dried and shadowed specimens (Fig. 5, e and f). In the absence of Tβ4, actin filaments present clear transverse striations arising from the short pitch helix. The contrast of the transverse striations is reduced in the presence of 250 μM Tβ4, and longitudinal depressions (arrows), which arise from the long pitch (2-start) helix, are longer and more frequent than in the absence of Tβ4. These observations suggest that incorporation of very few Tβ4 molecules in the filament creates defects in the helical arrangement of subunits causing the local destabilization of the lateral actin-actin bonds in the filament. The resulting "opening" of the two strands of the long pitch helix allows lateral sidewise pairing of other filaments, which leads to the observed stiffer wider "ropes." As the number of defects is increased, the twisting of the torsades is increased. The Tβ4-induced structural changes of actin filaments were only observed at physiological ionic strength, which correlates with the thermodynamic data. In a polymerization buffer containing only 1–2 mM MgCl2, only single actin filaments were observed in the presence of high concentrations of Tβ4 under conditions where 2–3 mM F-actin remained at steady state in the presence of large amounts of Tβ4-G-actin complex. Therefore the images observed in Fig. 5 do not result from an artifact of background created by the presence of large amounts of Tβ4 and Tβ4-G-actin in the solution.

Control of the Tβ4-Actin Complex on Filament Dynamics—The fact that an invariant curve [TA] = f([Tβ4]) was obtained (Fig. 2a) suggests that the increase in concentration of Tβ4-actin complex, rather than of free Tβ4, is linked to the lowering in critical concentration of G-actin. This point was further addressed in kinetic experiments aimed at understanding how
Effect of $T\beta_4$ on filament growth was studied by U. Aebi on standard filaments (29). Therefore our results were obtained when the inhibition of filament growth by $T\beta_4$ was assayed at higher concentrations (6.5 $\mu$M, 10.5 $\mu$M, 14 $\mu$M) of G-actin. Using the value of 1.6 $\mu$M derived above for the equilibrium dissociation constant of the $T\beta_4$ complex, the values of $[T\beta_4]_0$ and $[A]$ were calculated for each pair of total concentrations of G-actin ($C_J$) and of $T\beta_4$ ($T_{J0}$) (see Eq. 1). Since the incorporation of $T\beta_4$ in filaments is extremely low, the process of filament growth was fed essentially by addition of free G-actin to filament pointed ends. Accordingly, the rate of elongation $J$ varied linearly with the concentration of free G-actin, but the plots obtained at 10.5 $\mu$M and 14 $\mu$M total G-actin did not superimpose, in the region 0–3 $\mu$M free G-actin, with the regular $J$ (c) plot obtained in the absence of $T\beta_4$ (Fig. 6B).

The linear $J$ (c) plots obtained at about 90% saturation of G-actin by $T\beta_4$, i.e. in a range of concentrations of free G-actin of 0–2 $\mu$M (i.e. in the presence of 20–100 $\mu$M $T\beta_4$), were characterized by a higher slope than the control $J$ (c) curve carried out in the absence of $T\beta_4$, a lower critical concentration (defined as the concentration of free G-actin at which the rate of filament growth is zero), and the same value of the ordinate intercept. At the somewhat lower value of $C_J$ of 6.5 $\mu$M, data clearly showed a gradual shift from the coincidence with the standard $J$ (c) at concentrations of TA $\approx 3$ $\mu$M, toward coincidence with the plots obtained at $A_0 = 10.5$ $\mu$M and 14 $\mu$M and high $T\beta_4$ concentrations, as the saturation of G-actin by $T\beta_4$ increased. In other words, at high concentrations $T\beta_4$ is less efficient to inhibit filament growth than expected from the extent of inhibition at low concentration of $T\beta_4$, a result essentially in agreement with previous observations (6). These data demonstrate that in the presence of large concentrations of TA complex, the partial critical concentration of actin is lower, and this decrease in critical concentration is mediated by an increase in the rate constant $k^-$ for association of G-actin to filament ends, while the dissociation rate constant $k^-$ seems practically unchanged. This kinetic piece of data agrees with and further expands upon the data of incomplete depolymerization of actin filaments in the presence of high concentrations of TA reported in Fig. 2, A and B, the slow rate of repolymerization upon uncapping shown in Fig. 2C, and the steady state measurements of the decrease in critical concentration shown in Fig. 3.

**DISCUSSION**

The present results demonstrate that the function of $T\beta_4$ in the regulation of actin assembly is more complex than previously thought. We confirm that in a range of low concentrations (~20 $\mu$M), typical of the physiological concentration of $\beta$ thymosins in many cells, $T\beta_4$ acts as a simple G-actin binding protein. The 1:1 thymosin-actin complex, which may accumulate at steady state up to ~4 $\mu$M when barbed ends are capped, does not participate in actin assembly and does not affect the kinetic parameters of filament growth. On the other hand, in a range of higher concentrations (20–250 $\mu$M), $T\beta_4$ appears to also interact with F-actin with a very low affinity ($K_D \approx 5$–10 $\mu$M). The weak incorporation of $T\beta_4$ into filaments creates defects in the structure of the polymer. These defects can be described in terms of local points of destabilization of actin-actin contacts perpendicular to the filament axis, which results in local separation of the two strands of the long-pitch helix, thus enhancing the incidental “lateral slipping” feature noticed by U. Aebi on standard filaments (29). Therefore our results indicate that $T\beta_4$ binding to G-actin inhibits polymerization by interfering with the formation of lateral actin-actin bonds along the short pitch helix, rather than with the formation of longitudinal bonds. At high filament concentration, the destabilized, partially unraveled filaments tend to self-associate and twist around each other to yield thick rope-like structures. The effect of $T\beta_4$ on the F-actin structure may be compared to how...
The effect of intercalating drugs on DNA structure.

The main consequence of the incorporation of Tβ4 in F-actin is the decrease in the partial critical concentration of G-actin. The Tβ4-G-actin complex cannot be considered as a good polymerizing actin monomer since filaments containing on average 5% or less Tβ4-actin subunits exhibit a destabilized structure. These unstable filaments therefore are maintained at steady state by exchanging subunits at their ends with a pool of monomers at a high critical concentration. The total critical concentration of monomeric actin is the sum of free G-actin and Tβ4-G-actin at steady state. The partial critical concentration of Tβ4-actin monomer is 2 orders of magnitude higher than the partial critical concentration of G-actin itself under these conditions, while the copolymer consists of less than 5% Tβ4-actin subunits. These figures illustrate the fact that although Tβ4-actin is a weak polymerizing species, its contribution to filament assembly, via a high partial critical concentration, helps to diminish the partial critical concentration of G-actin. Therefore, in addition to its G-actin sequestering function, Tβ4 also possesses the power to control the steady state of actin assembly, via a high partial critical concentration, to diminish the partial critical concentration of G-actin. The decrease in partial critical concentration of G-actin occurs smoothly over a range of high concentrations of Tβ4 (only a 3-4-fold decrease was observed at 50 μM Tβ4).

The following consequences can be derived from our results: the property of Tβ4 to decrease the concentration of G-actin at steady state causes a self-limitation of the G-actin sequestering function of Tβ4, but also promotes a decrease in the amount of G-actin sequestered by other G-actin binding proteins like ADF/cofilin and, when barbed ends are capped, of profilin (as illustrated in Fig. 3A). The present in vitro results and their analysis provide an explanation of in vivo observations of actin dynamics in cells overexpressing Tβ10 (a variant of Tβ4), which show evidence for a paradoxical decrease in the amount of unassembled actin in overexpressing cells as compared to control cells (see accompanying article, Ref. 33). Examination of the measured cellular amounts of Tβ10 and other G-actin binding proteins leads to the conclusion that a putative 2-fold decrease in critical concentration linked to the 3-fold overexpression of Tβ10 is enough to account for the observed lower amount of unassembled actin. Under these conditions, the moderate increase in Tβ10-actin complex in overexpressing cells cannot fully compensate the actual decrease in the pool of actin sequestered by other G-actin binding proteins which is linked to the decrease in steady state concentration of G-actin. It should be emphasized here that, within our model, the change in the amount of unassembled actin linked to overexpression of β-thymosin can lead to very different situations depending on the concentration of other G-actin binding proteins in cells. Let us

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assume that a living cell contains Tβ₄ and other “bona fide” G-actin binding proteins that do not affect the critical concentration. For simplicity, all these non-Tβ₄ G-actin binding proteins will be collectively considered as a single species called “GBP.” The pool of unassembled actin consists of Tβ₄-actin and GBP-actin complexes. The concentration of unpolymerized actin in cells at different total concentrations of Tβ₄ and GBP is described in a three-dimensional plot shown in Fig. 7. Iso-GBP lines outline the effect of overexpression of Tβ₄ at different concentrations of GBP. At low concentration of GBP, the increase in Tβ₄-actin concentration predominates over the decrease in GBP-actin concentration, and a net increase in unassembled actin is linked to overexpression of Tβ₄. In the intermediate range of GBP concentration, the two effects roughly compensate each other, and very little change in unassembled actin is observed upon overexpression of Tβ₄. At high concentration of GBP, the decrease in GBP-actin complex predominates over the increase in Tβ₄-actin, resulting in a net decrease in unassembled actin upon overexpression of Tβ₄. Our in vitro results therefore allow understanding of the discrepancies reported by different groups concerning the effects of Tβ₄ overexpression on the level of actin assembly, in terms of differences in concentrations of GBPs in different cell types. It will be interesting to challenge this interpretation by detailed measurements of the amounts of GBPs in different cell types.

The present in vitro data also provide an explanation for the unexplained slower rate of propulsion of Listeria in Xenopus egg extracts supplemented with F-actin together with high amounts of Tβ₄ (Fig. 6 in Ref. 30). According to the proposed model for actin-based Listeria movement, the rate of actin assembly is controlled by the difference in critical concentrations between the bulk cytoplasm (where capping proteins act to establish the high critical concentration of pointed ends) and the bacterium surface (where anchored uncapped barbed ends, characterized by a low critical concentration, are actively growing). If the critical concentration in the bulk cytoplasm is decreased by large amounts of Tβ₄, then the rate of assembly at the bacterium surface is expected to decrease (such as observed here in Fig. 2C).

Our work raises questions concerning the actual amount of actin sequestered by Tβ₄ in resting platelets and neutrophils and the physiological significance of the “in vitro physiological ionic conditions.” Clearly according to the present in vitro data, very little actin (∼10 μM) would be sequestered by Tβ₄ in resting platelets or neutrophils, while in vivo data clearly indicate that at least 100 μM actin would be unpolymerized in these cells, profilin (estimated at 50 μM in platelets) and Tβ₄ (estimated at 400 μM in platelets) being the major actin sequestering agents. Therefore some cytoplasmic component, or macromolecular crowding, has to be thought of, which would limit the effects of Tβ₄ at the high concentration that we observed in vitro. This component could either stabilize F-actin (as tropomyosin would do) and consequently prevent the incorporation of Tβ₄-actin in filaments, or it could screen the effects of ionic strength, thereby favoring the sequestering activity of Tβ₄ over its interaction with F-actin. Nonetheless, the intrinsic properties of Tβ₄ illustrated here have to be considered to some extent in the in vivo situation. It may be worth noting that complete agreement has not been reached among different groups concerning the actual value of the Tβ₄ content of motile blood cells (5, 11). In addition, the dilution of cytoplasm that takes place in the preparation of cellular extracts without fixation causes depolymerization of a part of the F-actin pool (31, 32), which may lead to a somewhat overestimated concentration of unassembled actin.

From a structural point of view, the fact that Tβ₄-actin is able, although weakly, to copolymerize with actin, accounts for the difficulties encountered in the crystallization of the Tβ₄-actin monomer in salt-containing solutions. The observation that Tβ₄-actin incorporates into filaments only in high ionic strength (0.1 M KCl) assembly buffers indicates that either electrostatic bonds in the actin-Tβ₄ interface have to be weakened, or hydrophobic bonds have to be strengthened, to allow incorporation of Tβ₄-actin in the filament. More detailed studies of the structure of Tβ₄-actin complex will challenge these expectations.

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T Is Not a Simple G-actin Sequestering Protein and Interacts with F-actin at High Concentration

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