Articles

Interaction of Thymosin β_4 with Muscle and Platelet Actin: Implications for Actin Sequestration in Resting Platelets†

A. Weber,*,‡ V. T. Nachmias,§ C. R. Pennise,‡ M. Pring,∥ and D. Safer§

Department of Biochemistry and Biophysics, Department of Anatomy, and Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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ABSTRACT: Quantitative measurements of the interactions of $T\beta_4$ with muscle actin suggest that its only physiological role is monomer sequestration. $T\beta_4$ forms a 1:1 complex with monomeric actin under physiological salt conditions. Its K_d for actin is not affected by calcium. $T\beta_4$ binds only to actin monomers and not to filament ends or alongside the filament. $T\beta_4$–actin complexes do not elongate actin filaments at either the barbed or the pointed end, and, unlike actobindin, $T\beta_4$ does not specifically suppress the nucleation of polymerization. We assessed the fraction of monomeric actin that can be sequestered by $T\beta_4$ in resting platelets. This was done on the basis of (a) its K_d of 0.4–0.7 μM for platelet actin, which had been prepared by a newly devised simpler method, and (b) the values for the concentrations of monomeric actin and of $T\beta_4$ which we measured as 280 and 560 μM, respectively. Using the higher K_d value of 0.7 μM, the $T\beta_4$–complexed actin is calculated to be between 70 and 240 μM, depending on the steady-state free G-actin concentration. This may vary from 0.1 to 0.5 μM, the critical concentrations for uncapped and for fully barbed-end-capped actin filaments. If the K_d in the platelet is the same as in vitro, most of the sequestered actin would be bound to $T\beta_4$ if more than 95% of the actin filaments are capped at their barbed ends in resting platelets.

We reported recently (Safer et al., 1990) that on native gels of supernatants from permeabilized human blood platelets, most of the unpolymerized actin was complexed with a 5-kDa actin-sequestering peptide, which we later showed (Safer et al., 1991) to be identical with thymosin β_4 ($T\beta_4$). The distribution of $T\beta_4$ has been studied intensively, and it is found in many vertebrate tissues and cells and is especially concentrated in macrophages, fibroblasts, neutrophils, and platelets (Hannappel et al., 1982; Goodall et al., 1983; Hannappel & Van Kampen, 1987), cells which have large pools of G-actin (Bray & Thomas, 1976; Southwick & Young, 1990; Markey et al., 1978; Chaponnier et al., 1987).

To understand the biological role of $T\beta_4$ more fully, one needs to know whether monomer sequestration is the only effect of $T\beta_4$ on actin and what proportion of a given cell's G-actin might be bound to $T\beta_4$ under physiological salt conditions. These two questions require both in vitro studies and data from specific cells. We have focused on an analysis of the blood platelet because it is rich in actin, is readily obtained, and is devoid of a nucleus and because good data on cell volume are available.

Our in vitro studies suggest that the only function of $T\beta_4$ is monomer sequestration and that it does not interact with polymerizing actin like profilin (Pollard & Cooper, 1986; Kaiser et al, 1986; Pring et al., 1992) or with actin nuclei like actobindin, the *Acanthamoeba* monomer binding protein (Lambooy & Korn, 1986; Bubb et al., 1991). We estimated the upper and lower limits for the fraction of monomeric actin sequestered by $T\beta_4$ in resting platelets and provide evidence that the upper limit for the concentration of $T\beta_4$–actin complexes in the resting platelet, expected if more than 95% of the actin filaments are capped, is close to the entire platelet pool of G-actin.

Experimental Procedures

Preparation of Proteins. (*A*) *Muscle actin* was prepared from an acetone powder of rabbit muscle as previously described (Murray et al., 1981) with some modifications in the chromatography step (Young et al., 1990). Human *platelet actin* was extracted from a platelet acetone powder prepared as described previously for brain (Pardee & Bamberg, 1979). Washed (with a buffer containing 1 mM phenylmethanesulfonyl fluoride, 126 mM NaCl, 5 mM KCl, 0.3 mM EDTA, and 10 mM Na_2HPO_4, pH 7.4) packed platelets from 8 units of Red Cross platelets were stirred with 30 mL of chilled acetone (ACS) for 15 min in ice, followed by three washings with 10 mL of chilled acetone each, dried under vacuum overnight, and ground to a fine powder in a mortar the next day. To extract actin, 0.3–0.6 g of powder (the yield from 8 units) was stirred on ice for 0.5 h in 10 mL of a buffer containing 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.5 mM $CaCl_2$, and 0.5 mM Tris, pH 8.0. The supernatant after 2-h centrifugation at 100000*g* at 0 °C was passed over a 35 cm × 3 cm Sephadex G-150 column, with a flow rate of 0.25–0.6 mL/min. Mini gel analysis (10% acrylamide) showed only the actin band across the peak region even though it was not possible to polymerize the actin by the addition of 0.1 M KCl and 2 mM $MgCl_2$. Immunoblotting of the supernatant indicated the presence of $T\beta_4$ and profilin. The peak fractions were combined and concentrated to 2 mL under vacuum in the cold, using a Millipore-immersible CX filter unit with a

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* Author to whom correspondence should be addressed.
‡ Department of Biochemistry and Biophysics.
§ Department of Anatomy.
∥ Department of Physiology.

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nominal cutoff of 10 000 daltons. Actin was dissociated from the monomer binding proteins according to Malm et al. (1983): the concentrated actin was precipitated with an equal volume of phosphate buffer, containing 4 mM ATP, 2 mM Mg, 2 mM DTT, and 2 M potassium phosphate, pH 7.6, incubated at room temperature for 1–2 h, and then centrifuged for 30 min at 20000*g* at 15 °C. The supernatant was discarded and the whitish loose precipitate homogenized with 1–2 mL of a polymerizing solution containing 0.5 mM ATP, 2 mM $MgCl_2$, 10 μM EDTA, 0.5 mM DTT, 0.2 mM $CaCl_2$, and 5 mM Tris, pH 7.6. Centrifugation at 100000*g* for 2 h at 15 °C produced a translucent actin pellet which was depolymerized by dialysis against 0.2 mM $CaCl_2$, 0.5 mM ATP, pH 8.0, 50 μM Ca-EDTA, 10 mM Tris, pH 8.0, and 1 mM sodium azide. The yield was 5 and 7 mg of pure actin/8 units of packed platelets in two preparations.

Pyrenyl labeling of muscle and platelet actin was carried out according to Kouyama and Mihashi (1981) with the modifications described previously (Northrop et al., 1986). Actin was stored in liquid nitrogen and defrosted as previously described (Young et al., 1990). The critical concentration of various actin preparations varied between 0.07 and 0.12 μM for uncapped filaments and between 0.4 and 0.6 μM for filaments capped at the barbed ends with gelsolin or villin.

(*B*) $T\beta_4$ for in vitro studies was prepared as previously described (Safer et al., 1991) and the concentration determined by the bicinchoninic acid procedure (Smith et al., 1985) calibrated by amino acid analysis (Safer et al., 1991).

(*C*) *Villin*, a generous gift from Dr. Mark Mooseker, was prepared according to Coleman and Mooseker (1985) and stored for weeks at 4 °C.

(*D*) *Gelsolin*, a generous gift from Dr. Joseph Bryan, was prepared as previously described (Bryan, 1988) and stored in liquid nitrogen.

Protein concentrations were calculated for actin using E_{290} = 24.9 mM^{-1} cm^{-1} and for gelsolin and villin using E_{280} = 155 and 123.4 mM^{-1} cm^{-1}, respectively.

Determination of Total G-Actin in the Platelet Supernatant by the DNase Assay. This was performed essentially by the method of Fox et al. (1981) with minor modifications for microcuvettes (0.45 μL). Six KIU DNase was added to an appropriate volume of extract or buffer and rapidly mixed for a few seconds; then $^5/_6$ of the volume was added with rapid mixing to the DNA in a Gilford spectrophotometer. The rate of absorbance increase was measured within 30 s of addition of extract, and the linear region was used for analysis. Inhibition was determined from rates which fell between 30 and 70% of the control rate, obtained by varying the amount of supernatant used. At least three measurements were taken of each preparation; agreement was within 10%. The amount of actin in the supernatants was estimated by comparison with freshly prepared pure muscle G-actin after we had established that the inhibition of DNase activity by muscle and platelet actin was identical. Platelets were counted in a hemocytometer as previously described (Nachmias et al., 1987); four estimates were made for each preparation; the agreement was within 10%. Platelets were used at concentrations close to 2 × 10^9/mL.

To prepare the lysate, an appropriate volume of platelet suspension at a known platelet concentration was prepared in modified Tyrode's buffer as in Safer et al. (1990), and lysed as previously described (Gonnella & Nachmias, 1981). Lysates were stored on ice for 10 min and then centrifuged in an Eppendorf microfuge (12000*g*) at 4 °C for 10 min. The clear supernatant was kept on ice and assayed within 1.5 h.

Measurement of $T\beta_4$ Concentration in Platelet Extracts. The concentration of $T\beta_4$ in platelet extracts was determined by reverse-phase HPLC, using the method of Hannappel (1986) modified as follows. To prepare samples, aliquots of platelet extracts prepared as above at 4 °C were mixed with 0.25 volume of cold 2 M perchloric acid; the precipitate was removed by centrifugation at 30000*g* for 5 min, and an aliquot of each supernatant was mixed with 0.25 volume of 2 M K_2HPO_4. The potassium perchlorate precipitate was removed by centrifugation, and the supernatant was used directly for HPLC. Recovery of $T\beta_4$ by perchloric acid extraction has been shown to be essentially quantitative (Hannappel & Van Kampen, 1987), and this was verified in our laboratory by measuring the recovery of a known quantity of pure $T\beta_4$ added to the platelet extract before addition of perchloric acid.

HPLC was performed using an Isco chromatograph, with data acquisition and analysis on an IBM-compatible computer using the ChemResearch interface and software. Separations were made on a C-18 column (ABI Aquapore OD-300, 4.6 × 250 mm); solvent A = 0.1% trifluoroacetic acid, solvent B = 0.08% trifluoroacetic acid in acetonitrile, and elution was performed with a gradient from 10 to 35% B in 12 min at a flow rate of 2 mL/min. Elution was monitored at 220 nm, and $T\beta_4$ was estimated by the integrated area of the peak, calibrated against pure $T\beta_4$ at a known concentration.

Fluorescence and Kinetic Measurements. Changes in actin polymerization were calculated from fluorescence measurements as previously described (Weber et al., 1987a) using a PTI photon-counting fluorometer. Measurements were standardized against a Raman peak. Readings of different experiments are not comparable because varying slit widths were used.

All experiments were carried out at 20 °C with Mg–actin, the physiological form of actin (Weber et al., 1969; Kitasawa et al., 1982). In a KCl–Mg medium, the fluorescence increase of pyrenylactin starts at the maximal rate with Mg–actin but not with Ca–actin as first observed by Selden et al. (1983). This is due to the shorter persistence of low-fluorescing ATP–pyrenylactin in polymerized Mg–actin as compared to Ca–actin (Carlier et al., 1986). The conversion to Mg–actin was carried out as previously described (Young et al., 1990).

Measurements of elongation rates at either filament end were carried out as previously described (Walsh et al., 1984; Northrop et al., 1986; Young et al., 1990) in a medium containing 10 mM imidazole buffer, pH 7.4, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM azide, 1 mM dithiothreitol, 0.5 mM ATP, and either 0.2 mM $CaCl_2$ or 5 mM EGTA. For elongation measurements of gelsolin-capped filaments in the presence of 5 mM EGTA, 10–20 nM gelsolin–actin monomer complexes were added in order to saturate the barbed ends with gelsolin–actin complexes [K_d about 0.1 nM according to Selve and Wegner (1986)]. Known number concentrations of pointed filament ends in the presence and absence of Ca^{2+} and of barbed filament ends in the absence of Ca^{2+} were obtained by copolymerizing actin with a strong nucleating protein, either villin or gelsolin in the presence of calcium, which results in the formation of one filament per nucleating protein molecule (Walsh et al., 1984; Coleman & Mooseker, 1985; Northrop et al., 1986).

End points of polymerization were measured after overnight incubation of actin in the presence of a nucleating substance, since without a nucleating agent polymerization may not be complete even after an overnight incubation. We used either actin–gelsolin nuclei (actin:gelsolin ratio = 25) or a small amount of polymerized actin pushed through a 10-μL Ham-

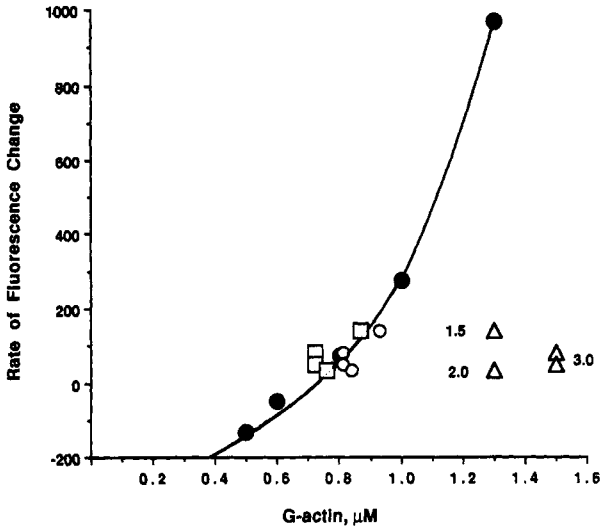

FIGURE 1: $T\beta_4$ inhibits the initial elongation rate of gelsolin-capped actin filaments. Filled circles, calibration curve in the absence of $T\beta_4$, showing the initial rate of the pyrenyl fluorescence change, taken from the initial linear part of the elongation trace, as a function of increasing concentrations of added G-actin (10% pyrenyl-labeled). The line through the control points is drawn by eye since in this concentration range the rate does not increase as a linear function of the G-actin concentration (Weber et al. 1987b). Open triangles, rates in the presence of $T\beta_4$ (concentrations in micromolar, indicated by the numbers next to the triangles) at added G-actin concentrations as indicated by the abscissa; open squares and open circles, rates in the presence of $T\beta_4$ plotted against the concentration of nonsequestered G-actin calculated with a K_d of 2.1 and 2.8 μM, respectively. Filament elongation was started by simultaneous addition of salt (final concentrations of 0.1 M KCl and 2 mM $MgCl_2$, 5 mM EGTA), gelsolin-capped actin filaments (100 nM 10% pyrenyl F-actin, 4 nM gelsolin), and gelsolin–actin dimers (4 nM) to G-actin. Gelsolin–actin dimers were added to maintain the saturation of the barbed ends with gelsolin in the presence of EGTA which lowers the gelsolin capping constant to about 0.1 nM (Selve & Wegner, 1986). When present, $T\beta_4$ was preincubated with G-actin for 5–10 min.

ilton syringe at maximal speed.

RESULTS

We measured the interaction of $T\beta_4$ and actin using pyrenyl-labeled muscle actin after first establishing that the results were independent of the extent of pyrenyl labeling, indicating that the label does not interfere with $T\beta_4$ binding to actin (data not shown). We determined the binding constant of $T\beta_4$ for actin monomers assuming a 1:1 complex (Safer et al., 1991) under physiological salt conditions, by measuring both the inhibition of the initial rate of filament elongation by $T\beta_4$ and the extent to which $T\beta_4$ lowers the end point of polymerization. $T\beta_4$ inhibits the initial rate of elongation of actin filaments capped at the barbed end with gelsolin (Figure 1, open triangles). Assuming that the inhibition is entirely caused by sequestration of actin, the concentration of free G-actin that determines the elongation rate measured in the presence of $T\beta_4$ can be read from the calibration curve, and the concentration of sequestered actin, equal to [total G-actin] − [free G-actin], and of free $T\beta_4$, equal to [total $T\beta_4$] − [$T\beta_4$–actin], can be calculated. How well a K_d value obtained according to K_d = [free G-actin][free $T\beta_4$]/[$T\beta_4$–actin] fits all of the data points can be visualized by plotting the measured rate against the calculated concentration of free G-actin using this K_d value. If the fit is perfect, all of the points fall on the calibration curve. The open squares and the open circles show the fit for two K_d values, 2.1 and 2.8 μM. The fit is slightly better for a K_d value of 2.1 μM.

Since one cannot rule out that capping of pointed filament ends by $T\beta_4$ may have contributed to the inhibition of the

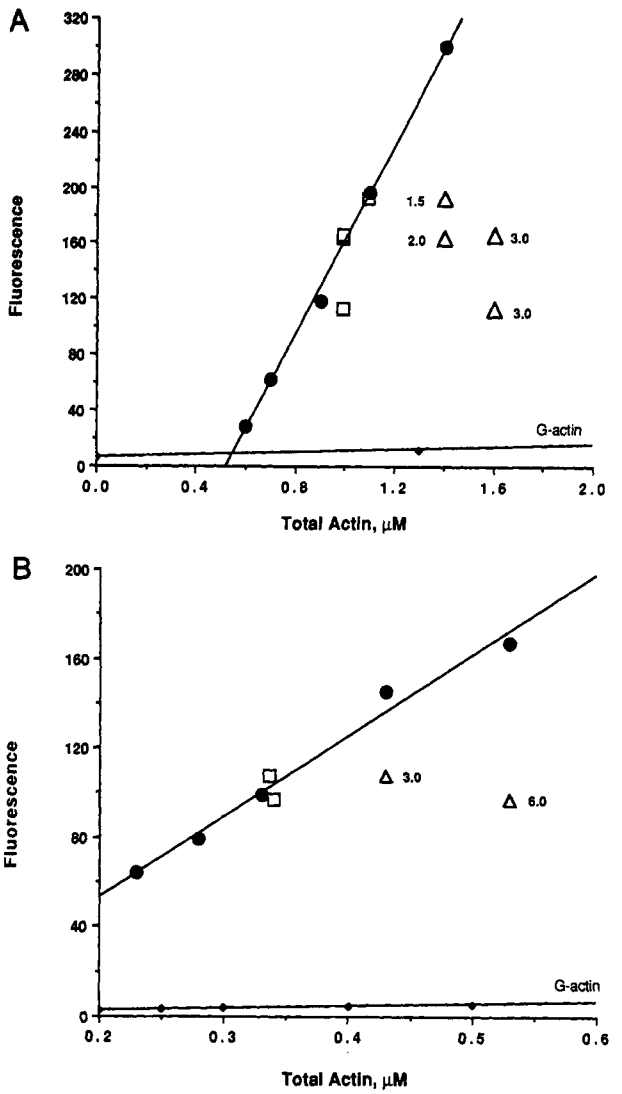

FIGURE 2: Effect of $T\beta_4$ on the end point of polymerization of skeletal muscle actin. (A) In the presence of gelsolin, 24-h end points of the experiment of Figure 1. Filled circles, controls in the absence of $T\beta_4$; triangles, end points in the presence of $T\beta_4$ (concentrations in micromolar indicated by the numbers next to the triangles); open squares, in the presence of $T\beta_4$, nonsequestered total actin calculated with a K_d of 2.1 μM. (B) In the absence of gelsolin, 24-h end points; different preparations of $T\beta_4$ and actin (20% pyrenyl-labeled). Polymerization was initiated as in Figure 1 except that villin-capped actin filaments (30 nM 20% pyrenyl F-actin, 1 nM villin) were substituted for gelsolin-capped filaments + gelsolin–actin dimers. In the presence of 5 mM EGTA, barbed end capping by villin is inactivated, and the free barbed ends act as nuclei for polymerization. Filled circles, controls in the absence of $T\beta_4$; triangles, end points in the presence of $T\beta_4$ (concentrations in micromolar indicated by the numbers next to the triangles); open squares, in the presence of $T\beta_4$, nonsequestered total actin calculated with a K_d of 1.7 μM. The lines marked G-actin in (A) and (B) represent the G-actin fluorescence, which intercepts with the control curve at 0.05 μM total actin (B).

elongation rate, we determined the K_d also from a steady-state measurement, the extent by which $T\beta_4$ decreases the end point of polymerization after an overnight incubation, using gelsolin-capped (Figure 2A) and uncapped actin filaments (Figure 2B). In this case, free G-actin equals the critical concentration indicated by the intersection of the calibration curve with the fluorescence curve for G-actin. Sequestered actin is obtained by subtracting the concentration of polymerized actin, indicated by the measured fluorescence (open triangles), from the concentration of added actin. The fit of the K_d value of 2.1 μM (open squares in Figure 2A) is visualized as described for Figure 1. That the reduction of the elongation rate and of the

Table I: Dependence of K_d on either [Free Tβ_4] or [Free G-Actin] for 1:2 and 2:1, but Not for 1:1, Actin–Tβ_4 Complexes

$[G]_{free}$ (μM)	$[T\beta_4]_{free}$ (μM) for A–Tβ_4			K_d for A–Tβ_4		
	1:2	1:1	2:1	1:2[a]	1:1[b]	2:1[a]
End Points of Polymerization of Gelsolin-Capped Actin Filaments						
0.55	1.80	2.40	2.70	2.97	2.20	2.72
0.55	1.50	2.25	2.62	1.65	1.65	2.09
0.55	1.20	1.60	1.80	1.98	2.20	2.72
0.55	0.88	1.19	1.35	1.37	2.11	2.72
End Points of Polymerization in the Absence of a Barbed End Capper						
0.056	2.83	2.92	2.96	5.34	1.95	0.22
0.056	5.57	5.78	5.89	8.04	1.50	0.17

[a] K_d in μM^2. [b] K_d in μM.

end point of polymerization both are quantitatively explained by monomer sequestration with a K_d of 2.1 μM rules out pointed end capping by Tβ_4. It also indicates that monomer binding by Tβ_4 had reached equilibrium during the 15-min preincubation before the elongation rate measurements. The K_d value of 1.7 μM derived from the data of Figure 2B, carried out with different preparations of actin and Tβ_4, is very close to the other values.

The K_d values from the three experiments agree only when calculated for a 1:1 complex. For an assumed actin–Tβ_4 complex (1:2), there is a systematic decrease of the K_d values with decreasing [free Tβ_4] and for a 2:1 complex with decreasing free G-actin concentration: the K_d values for either complex 1:2 or complex 2:1 are significantly different for the experiments in Figure 2A and Figure 2B (Table I). That the K_d value is the same indicates that a 1:1 complex is formed also under the salt conditions of these experiments, which are more physiological than those used for a native gel or for cross-linking (Safer et al., 1990, 1991).

The mean K_d value (about 25 points) of 3 different preparations of Tβ_4 and muscle actin was 2 μM, with extreme outlying values of 1.4 and 4.5 μM.

Actin binding by Tβ_4 is independent of free calcium: the K_d for muscle actin was identical in 0.2 mM calcium and in 5 mM EGTA (data not shown).

We next determined the K_d values of Tβ_4 for platelet actin purified from a platelet acetone powder as described under Experimental Procedures. The values were obtained from the effect of Tβ_4 on the end point of polymerization in the presence of gelsolin (Figure 3). The mean values of a number of determinations for each of two different actin and Tβ_4 preparations were 0.7 and 0.4 μM. A comparison with the K_d of 2 μM for muscle actin *measured at the same time* showed that platelet actin binds Tβ_4 about 3–5 times more strongly than muscle actin.

In the context of the various possible mechanisms that might be responsible for the rapid rate of actin polymerization after platelet stimulation, it is of interest to know whether Tβ_4–G-actin complexes, like profilin–G-actin complexes (Pollard & Cooper, 1984; Kaiser et al., 1986; Pring et al., 1992), are able to contribute to the elongation of actin filaments at their barbed but not at their pointed ends. Therefore, we measured the effect of Tβ_4 on the elongation rate at the barbed end to see whether the rate was faster than could be accounted for by the concentration of nonsequestered G-actin, calculated with the K_d for monomer sequestration obtained from *contemporaneous measurements with the same proteins described* in Figure 1. The open squares (K_d 2.1 μM) and open circles (K_d 2.8 μM) of Figure 4 show that in the presence of Tβ_4 the rate was quite close to that expected for the nonsequestered G-actin

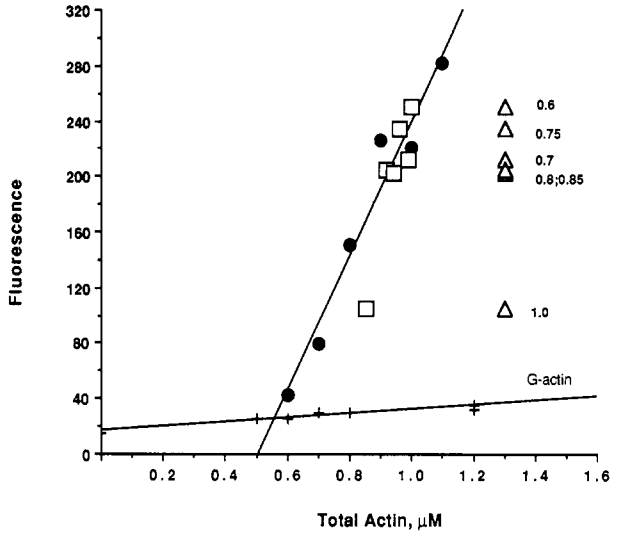

FIGURE 3: Effect of Tβ_4 on the end point of elongation of gelsolin-capped platelet actin filaments. Conditions: 4 nM gelsolin; 5% platelet pyrenylactin; 24-h end points; Tβ_4 concentrations in micromolar indicated by numbers next to data points. Procedures as in Figure 2 except that EGTA in excess over Ca^{2+} was 10 μM; filled circles, controls in the absence of Tβ_4; triangles, end points in the presence of Tβ_4 (concentrations in micromolar indicated by the numbers next to the triangles); open squares, fluorescence in the presence of Tβ_4 plotted against the concentration of nonsequestered actin calculated with a K_d of 0.7 μM.

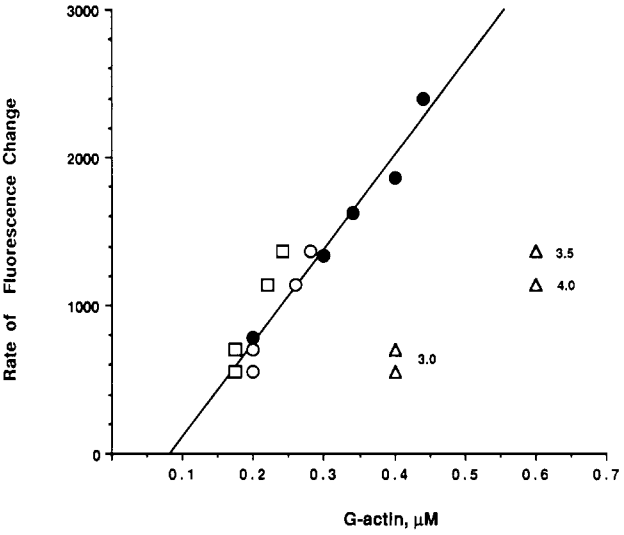

FIGURE 4: Tβ_4 inhibits the barbed end elongation rate as much as expected from the extent of monomer sequestration. The measurements were carried out under the same conditions as in Figure 1 except that the gelsolin-capped filaments were replaced by villin-capped actin filaments (2 nM villin) and the gelsolin–actin dimers were omitted. In the presence of 5 mM EGTA, villin is rapidly released from the barbed filament ends (Northrop et al., 1986), and a reproducible number of free barbed ends becomes available for elongation. Filled circles, controls in the absence of Tβ_4; triangles, rates of fluorescence change in the presence of Tβ_4 (concentrations in micromolar indicated by the numbers next to the triangles). The open squares and open circles show the rates in the presence of Tβ_4 plotted against the nonsequestered G-actin calculated with K_d values of 2.1 and 2.8 μM, respectively, obtained from the contemporaneous control experiment shown in Figure 1.

concentration. The rate of elongation was slightly, but not significantly, faster than expected for a concentration of nonsequestered G-actin calculated with a K_d of 2.1 μM, the K_d value that described best the inhibition of the pointed-end elongation rate. By contrast, in the presence of profilin, a similar experiment shows rates 2–5 times higher than predicted

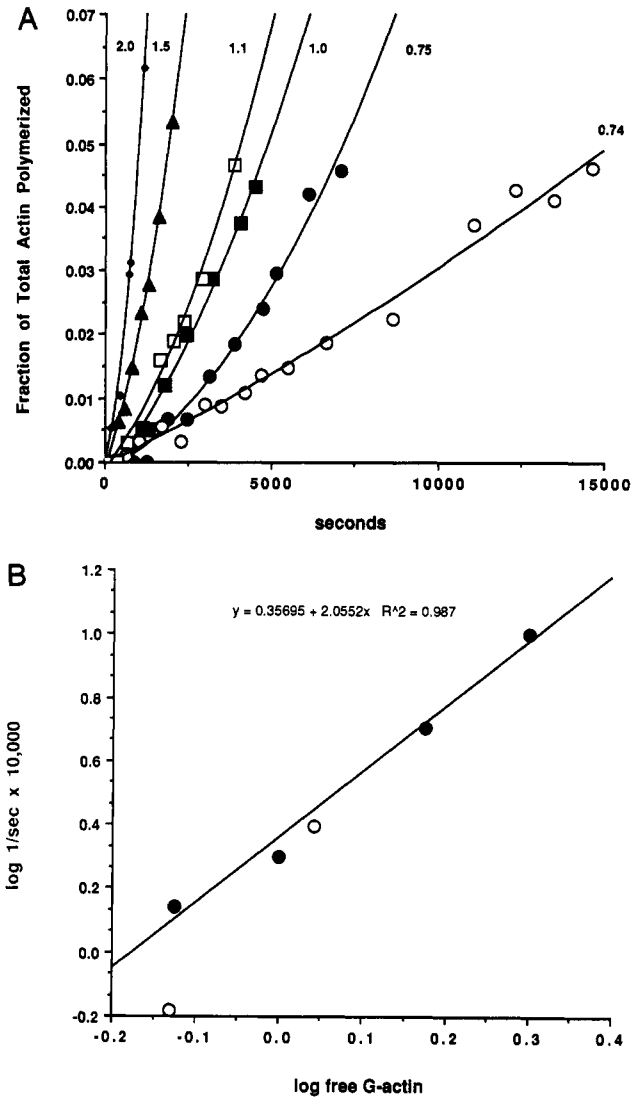

FIGURE 5: $T\beta_4$ prolongs the lag period in keeping with the extent of monomer sequestration. (A) Spontaneous polymerization with time at different concentrations of free G-actin (in micromolar, indicated by the numbers on the curves) which, in the controls, equalled the concentration of total actin. In the presence of $T\beta_4$, the free G-actin concentrations were calculated, using K_d = 2.0 μM, to give a value of 1.1 μM for 2.0 μM total actin + 2.5 μM total $T\beta_4$ and a value of 0.74 μM for 1.5 μM total actin + 2.8 μM total $T\beta_4$; 5% pyrenyl-labeled actin, freshly prepared from acetone powder in the absence (controls, closed symbols) and in the presence of $T\beta_4$ (open symbols). EGTA was 10 μM rather than 5 mM in excess over calcium in order to slow down the nucleation rate. (B) Log–log plot of the reciprocal of the lag period (time required for 5% polymerization) versus the concentration of G-actin; closed symbols, controls in the absence of $T\beta_4$; open symbols, $T\beta_4$ present.

by the free G-actin concentration (Pring et al., 1992).

We next investigated whether $T\beta_4$, like actobindin in *Acanthamoeba* (Lambooy & Korn, 1986; Bubb & Korn, 1991; Bubb et al., 1991), could serve as an inhibitor of spontaneous nucleation. Therefore, we determined whether the lag period for spontaneous polymerization was much longer in the presence of $T\beta_4$ than expected from the reduction in [free G-actin] caused by monomer sequestration by $T\beta_4$ (Figure 5A). For these experiments, we prepared fresh actin from acetone powder and checked the lag period of each column fraction to eliminate actin containing Cap Z, a frequent contaminant in muscle actin (Casella & Maack, 1987). The K_d value of 2.0 μM for monomer binding by $T\beta_4$ and by the actin fraction used for this experiment was determined on the same day. We measured the whole time course but plotted

Table II: Concentrations of Total G-Actin and Total $T\beta_4$ in Resting Platelets[a]

expt	[G-actin], μM	[total $T\beta_4$], μM
1	248	
2	311	547
3		572

[a] Both measurements were made on Triton supernatants prepared as described. Each concentration of G-actin is the mean of three and each of $T\beta_4$ the mean of several independent measurements. The inhibition of DNase activity by freshly prepared platelet and muscle actin was indistinguishable. The platelets were counted for each experiment in a hemocytometer, and the mean of four measurements was taken.

only the initial part (to 5% of maximal polymerization), where the concentrations of added free and total G-actin are close to initial values. The later time course is complicated by the fact that during polymerization the change in [free G-actin] in the presence of $T\beta_4$ is different from that in the controls since actin is continuously released from its complex with $T\beta_4$ which buffers the level of free G-actin. Although in the presence of $T\beta_4$ nucleation with 0.74 μM free G-actin was slower than in the control (Figure 5A, open circles), the difference is very small compared to the effects of actobindin (Lambooy & Korn, 1986; Bubb & Korn, 1991; Bubb et al., 1991). This can also be seen from the graph (Figure 5B) showing the log of the reciprocal of the lag period (the time to reach 5% polymerization) versus the logarithm of the concentration of free G-actin. Note that the concentration of free G-actin was equal to the added actin in the controls, while in the presence of $T\beta_4$ it was calculated from the K_d of 2 μM and the total concentrations of $T\beta_4$ and actin. While one of the data points in the presence of $T\beta_4$ is clearly below the straight line that connects the control points, the other is very close to it, indicating that $T\beta_4$ is not a significant inhibitor of nucleation. The slope of the line was 2. This is expected for a nucleus of 4 and agrees with previous determinations (Tobacman & Korn, 1983; Cooper et al., 1983), defining as nucleus the smallest polymer with the on- and off-rate constants of a filament.

Finally, we determined whether $T\beta_4$ can bind to F-actin by incubating pure $T\beta_4$ with a 10-fold excess of filamentous actin (40 μM F-actin and 4 μM $T\beta_4$) for 10 min. There was no uptake of $T\beta_4$ by the actin filaments: the $T\beta_4$ content of the supernatant was the same as that of the control without F-actin (data not shown).

Having thus established that $T\beta_4$ appears to function only as a monomer-sequestering protein, we wanted to estimate what fraction of the monomeric actin could be sequestered by $T\beta_4$ within the platelet. Therefore, we determined the total concentration of $T\beta_4$ in resting platelets by quantitative extraction from a known platelet volume using a modification of Hannappel's method (1986) as described under Experimental Procedures. Complete extraction was verified by measuring the recovery of a known quantity of $T\beta_4$ added to the platelets. We obtained a mean value for [total $T\beta_4$] of 560 μM (Table II).

According to K_d = [free G-actin][free $T\beta_4$]/[$T\beta_4$–actin], using a K_d value of 0.7 μM for platelet actin and 560 μM for the concentration of total $T\beta_4$, the concentration of $T\beta_4$–actin may vary from 70 to 233 μM depending on the concentration of nonsequestered free G-actin, i.e., the critical G-actin concentration in resting platelets. It has a value of about 0.1 μM for uncapped and of 0.5 μM for fully barbed end capped filaments [cf. Pollard and Cooper (1986)].

We then determined the content of monomeric actin in two platelet preparations, using the DNase assay. The total platelet

volume was obtained prior to lysis on the basis of the platelet count and the average volume of individual platelets of 7 fL (Nachmias et al., 1987). We also used freshly prepared actin for the calibration of the DNase assay since we found that storage of G-actin for even a few days at 4 °C decreased its ability to inhibit DNase which would result in an overestimation of the G-actin content. We obtained a mean value of 280 μM total G-actin in resting platelets (Table II).

Discussion

We draw two major conclusions from our study. First, we have found no evidence for an interaction of $T\beta_4$ with actin other than monomer sequestration. Second, assuming that the K_d value of $T\beta_4$ for platelet actin is valid under in vivo conditions, the fraction of G-actin sequestered by $T\beta_4$ in vivo may vary between 25 and 83% depending on the critical G-actin concentration in resting platelets.

Our value for the G-actin concentration in resting platelets is at the low end of estimates that have been given in the reviews cited in the literature (from 150 to 950 μM; Blikstad et al., 1978; Fox & Phillips, 1983). However, detailed perusal of the basis of the literature estimates showed that either a careful comparison with pure actin standards or a precise determination of the total cell volume based on cell count and individual cell size was missing in previous work. We based our value for platelet cell volume on cell count and the previously established value for intracellular volume.

The concentration of total $T\beta_4$ in resting platelets of 560 μM measured by us agrees well with the values reported by Hannappel and Van Kampen (1987); they found a mean value of 22.3 fg/cell, which, using our value of 7 fL/cell, yields 638 μM. According to the expression

$$[G\text{-}T\beta_4] = ([\text{free G-actin}]/K_d)[T\beta_4]_{free}$$

the concentration of sequestered monomer for any given concentration of total $T\beta_4$ depends on the ratio of free G-actin to the K_d.

The concentration of steady-state free G-actin in platelets is not known; it should be somewhere between 0.1 μM, if the barbed ends of the actin filaments were uncapped, and 0.5 μM [Bonder et al., 1983; cf. Pollard and Cooper (1986) and Figures 3 and 4 of the paper], if all of the filaments were capped. Partial capping of the actin filaments should increase the steady-state G-actin concentration relatively little until more than 90% of the filaments are capped because the rate constants are 10 times faster at the barbed than at the pointed ends (Walsh et al., 1984; Selve & Wegner, 1985). We showed by direct measurements that the critical concentration remains relatively low when the fraction of capped filaments increases from 0 to 90% but then rises sharply between 90 and 99% capping (Northrop et al., 1986; Young et al., 1990). This means that $T\beta_4$ alone can be responsible for most of the actin monomer pool in resting platelets only if more than 95% of the actin filaments are capped.

Other known mammalian monomer binding proteins are profilin, discovered by Lindberg (Carlsson et al., 1977) and extensively studied in the laboratories of Korn (Reichstein & Korn, 1979; Mockrin & Korn, 1980; Tobacman & Korn, 1982; Tobacman et al., 1983; Lal & Korn, 1985) and of Pollard (Tseng & Pollard, 1982; Tseng et al., 1984; Pollard & Cooper, 1984, 1986; Kaiser et al., 1986), and destrin (Moriyama et al., 1990), the mammalian form of ADF previously discovered by Bamberg et al. (1980). It is not clear how much profilin is bound to actin in resting platelets since it is possible that most of it is bound to PIP_2 (Markey et al., 1978; Goldschmidt-Clermont et al., 1990, 1991). Even if all of the 50 μM profilin (Goldschmidt-Clermont et al., 1991) were bound to actin, it could sequester only about 20% of the monomeric actin. The presence of destrin in mammalian platelets has not yet been established. In conclusion, if the steady-state G-actin concentration is close to that for free barbed ends because 20% or more of the actin filaments are not capped, either a monomer binding protein other than profilin or $T\beta_4$ must be present or the K_d of $T\beta_4$ in resting platelets must be lower than the value measured with the purified protein.

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References

Bamberg, J. R., Harris, H. E., & Weeds, A. G. (1980) *FEBS Lett. 121*, 178–181.

Blikstad, I., Markey, F., Carlsson, L., Persson, T., & Lindberg, U. (1978) *Cell 15*, 935–943.

Bonder, E. M., Fishkind, D. J., & Mooseker, M. S. (1983) *Cell 34*, 491–501.

Bray, D., & Thomas, C. (1976) *J. Mol. Biol. 105*, 527–544.

Bryan, J. (1988) *J. Cell Biol. 106*, 1553–1562.

Bubb, M. R., & Korn, E. D. (1991) *Methods Enzymol. 196*, 119–125.

Bubb, M. R., Lewis, M. S., & Korn, E. D. (1991) *J. Biol. Chem. 266*, 3820–3826.

Carlier, M. F., Pantaloni, D., & Korn, E. D. (1986) *J. Biol. Chem. 261*, 10785–10792.

Carlsson, L., Nystrom, L. E., Sundkvist, I., Markey, F., & Lindberg, U. (1977) *J. Mol. Biol. 115*, 465–483.

Casella, J. F., & Maack, D. J. (1987) *Biochem. Biophys. Res. Commun. 145*, 625–630.

Chaponnier, C., Yin, H. L., & Stossel, T. P. (1987) *J. Exp. Med. 165*, 97–106.

Coleman, T. R., & Mooseker, M. S. (1985) *J. Cell Biol. 101*, 1850–1857.

Cooper, J. A., Buhle, E. L, Walker, S. B., Tseng, T. Y., & Pollard, T. D. (1983) *Biochemistry 22*, 2193–2202.

Cooper, J. A., Blum, J. D., Williams, R. C., Jr., & Pollard, T. D. (1986) *J. Biol. Chem. 261*, 477–485.

Fox, J. E. B., Dockter, M. E., & Phillips, D. R. (1981) *Anal. Biochem. 117*, 170–177.

Fox, J. E. B., & Phillips, D. R. (1985) *Semin. Hematol. 20*, 243–260.

Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J., & Pollard, T. D. (1990) *Science 247*, 1575–1578.

Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G., & Pollard, T. D. (1991) *Science 251*, 1231–1233.

Gonnella, P., & Nachmias, V. T. (1981) *J. Cell Biol. 89*, 746–751.

Goodall, G. J., Morgan, J. I., & Horecker, B. L. (1983) *Arch. Biochem. Biophys. 221*, 598–601.

Hannappel, E. (1986) *Anal. Biochem. 156*, 390–396.

Hannappel, E., & Van Kampen, M. (1987) *J. Chromatogr. 397*, 279–285.

Hannappel, E., Xu, G.-J., Morgan, J., Hempstead, J., Horecker, B. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2172–2175.

Kaiser, D. A., Sato, M., Ebert, R. F., & Pollard, T. D. (1986) *J. Cell Biol. 102*, 221–226.
Kitasawa ., Shuman, H., & Somlyo, A. P. (1982) *J. Muscle Res. Cell Motil. 3*, 437–454.
Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38.
Lal, A. A., & Korn, E. D. (1985) *J. Biol. Chem. 260*, 10132–10138.
Lambooy, P. K., & Korn, E. D. (1986) *J. Biol. Chem. 261*, 17150–17155.
Lind, S. E., Janmey, P. A., Chaponnier, C., Herbert, T. J., & Stossel, T. P. (1987) *J. Cell Biol. 105*, 833–842.
Malm, B., Larsson, H., & Lindberg, U. (1983) *J. Muscle Res. Cell Motil. 4*, 569–588.
Markey, F., Lindberg, U., & Eriksson, L. (1978) *FEBS Lett. 88*, 75–79.
Mockrin, S. C., & Korn, E. D. (1980) *Biochemistry 19*, 5359–5362.
Moriyama, K., Nishida, E., Yonezawa, N., Sakai, H., Matsumoto, S., Iida, K., & Yahara, I. (1990) *J. Biol. Chem. 265*, 5768–5773.
Murray, J. M., Weber, A., & Knox, M. K. (1981) *Biochemsitry 20*, 641–649.
Nachmias, V. T., Yoshida, K., & Glennon, M. C. (1987) *J. Cell Biol. 105*, 1761–1769.
Northrop, J., Weber, A., Mooseker, M. S., Franzini-Armstrong, C., Bishop, M. F., Dubyak, G. R., Tucker, M., & Walsh, T. P. (1986) *J. Biol. Chem. 261*, 9274–9281.
Pardee, J. D., & Bamberg, J. R. (1979) *Biochemistry 18*, 2245–2252.
Pollard, T. D., & Cooper, J. A. (1984) *Biochemistry 23*, 6631–6641.
Pollard, T. D., & Weeds, A. G. (1984) *FEBS Lett. 170*, 94–96.
Pollard, T. D., Cooper, J. A. (1986) *Annu. Rev. Biochem. 55*, 987–1035.
Pring, M., & Weber, A. (1992) *Biochemistry* (in press).
Reichstein, E., & Korn, E. D. (1979) *J. Biol. Chem. 254*, 6174–6179.
Safer, D., Golla, R., & Nachmias, V. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2536–2540.
Safer, D., Elzinga, M., & Nachmias, V. T. (1991) *J. Biol. Chem. 266*, 4029–4032.
Selden, L. A., Estes, J. E., & Gershman, L. C. (1983) *Biochem. Biophys. Res. Commun. 116*, 478–485.
Selve, N., & Wegner, A. (1986) *Eur. J. Biochem. 160*, 379–387.
Smith, P. K., Korn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Southwick, F. S., & Young, C. L. (1990) *J. Cell Biol. 110*, 1965–1973.
Tobacman, L. S., & Korn, E. D. (1982) *J. Biol. Chem. 257*, 4166–4170.
Tobacman, L. S., & Korn, E. D. (1983) *J. Biol. Chem. 258*, 3207–3214.
Tobacman, L. S., Brenner, S. L., & Korn, E. D. (1983) *J. Biol. Chem. 258*, 8806–8812.
Tseng, P. C., & Pollard, T. D. (1982) *J. Cell Biol. 94*, 213–218.
Tseng, P. C., Runge, M. S., Cooper, J. A., Williams, R. C., Jr., & Pollard, T. D. (1984) *J. Cell Biol. 98*, 214–221.
Walsh, T. P., Weber, A., Higgins, J., Bonder, E. M., & Mooseker, M. S. (1984) *Biochemistry 23*, 2613–2621.
Weber, A., Herz, R., & Reiss, J. (1969) *Biochemistry 8*, 2266–2271.
Weber, A., Northrop, J., Bishop, M. F., Ferrone, F. A., & Mooseker, M. S. (1987a) *Biochemistry 26*, 2528–2536.
Weber, A., Northrop, J., Bishop, M. F., Ferrone, F. A., & Mooseker, M. S. (1987b) *Biochemistry 26*, 2537–2544.
Young, C. L., Southwick, F. S., & Weber, A. (1990) *Biochemistry 29*, 2232–2240.