Thymosin $\beta_4$ Sequesters the Majority of G-actin in Resting Human Polymorphonuclear Leukocytes

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Abstract. Thymosin $\beta_4$ (T$\beta_4$), a 5-kD peptide which binds G-actin and inhibits its polymerization (Safer, D., M. Elzinga, and V. T. Nachmias. 1991. J. Biol. Chem. 266:4029–4032), appears to be the major G-actin sequestering protein in human PMNs. In support of a previous study by Hannappel, E., and M. Van Kampen (1987. J. Chromatography. 397:279–285), we find that T$\beta_4$ is an abundant peptide in these cells. By reverse phase HPLC of perchloric acid supernatants, human PMNs contain $\sim$169 fg/cell $\pm$ 90 fg/cell (SD), corresponding to a cytoplasmic concentration of $\sim$149 $\pm$ 80.5 $\mu$M. On non-denaturing polyacrylamide gels, a large fraction of G-actin in supernatants prepared from resting PMNs has a mobility similar to the G-actin/T$\beta_4$ complex. Chemoattractant stimulation of PMNs results in a decrease in this G-actin/T$\beta_4$ complex. To determine whether chemoattractant induced actin polymerization results from an inactivation of T$\beta_4$, the G-actin sequestering activity of supernatants prepared from resting and chemoattractant stimulated cells was measured by comparing the rates of pyrenyl-actin polymerization from filament pointed ends. Pyrenyl actin polymerization was inhibited to a greater extent in supernatants from stimulated cells and these results are qualitatively consistent with T$\beta_4$ being released as G-actin polymerizes, with no chemoattractant-induced change in its affinity for G-actin. The kinetics of bovine spleen T$\beta_4$ binding to muscle pyrenyl G-actin are sufficiently rapid to accommodate the rapid changes in actin polymerization and depolymerization observed in vivo in response to chemoattractant addition and removal.

Within nonmuscle cells, actin subunits transit rapidly between monomer (G-actin) and polymer (F-actin) pools. This dynamic equilibrium allows rapid reorganization of the cytoskeleton in response to stimuli (reviewed in Mitchison and Kirschner, 1988; Stossel, 1989). For example, polymorphonuclear leukocytes (PMNs) respond to chemoattractant stimulation by becoming motile and assembling a dynamic array of actin filaments in the newly formed lamellipodia (Fechheimer and Zigmond, 1983; White et al., 1983; Skafr et al., 1985; Cassimeris et al., 1990).

Resting PMNs contain a large pool of G-actin, $\sim$120 $\mu$M (White et al., 1983; Fechheimer and Zigmond, 1983), well above the critical concentration for polymerization of $\sim$0.1–0.2 $\mu$M measured in vitro (reviewed in Pollard and Cooper, 1986). Thus, G-actin–binding factors are required to sequester $\sim$120 $\mu$M G-actin in PMNs. In response to chemoattractant stimulation, a fraction of this sequestered pool polymerizes into F-actin. It is not known whether the availability of free barbed ends and/or the release of G-actin from sequestering factors initiates polymerization.

Understanding how G-actin is sequestered and subsequently made available for polymerization requires characterization of the factors which bind G-actin. Profilin is present in PMNs at $\sim$40 $\mu$M (Southwick and Young, 1990) and given its affinity for G-actin measured in vitro (K$\text{d} = 1$ $\mu$M; Southwick and Young, 1990), profilin could maximally sequester $\sim$40 $\mu$M of the G-actin in a resting PMN, leaving $\sim$80 $\mu$M G-actin to be sequestered by other factors.

Recent studies by Safer et al. (1990) suggest that thymosin $\beta_4$ (T$\beta_4$), a 5-kD peptide which binds G-actin at a 1:1 molar ratio, is likely to be the major actin sequestering protein in platelets (T$\beta_4$ concentration in platelets $\sim$560 $\mu$M; Weber et al., 1992). In this paper we have investigated: (a) the role of T$\beta_4$ in sequestering G-actin in resting human PMNs, (b) the possible regulation of T$\beta_4$ by chemoattractant, and (c) the kinetics of T$\beta_4$ binding to G-actin in vitro.

Materials and Methods

Reagents

Unless specified otherwise, reagents are from Sigma Chemical Co. (St. Louis, MO).
Proteins
Rabbit skeletal muscle actin was isolated from acetone powder (Spudich and Watt, 1971) and further purified by gel filtration chromatography on a Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) column (MacLean-Fletcher and Pollard, 1980). The actin containing fractions were pooled, 100-µl aliquots were frozen in liquid nitrogen (Northrup et al., 1986) and stored at −80°C until used. Pyrenyl-labeled actin was prepared from acetone powder (30-80% labeled in different preparations) as described previously (Northrup et al., 1986; Cano et al., 1991) and was stored as G-actin at 4°C until used.

Gelsolin was isolated from rabbit serum by a slight modification of the simplified chromatographic method of Cooper et al. (1987). The changes from their method were: (a) the first DEAE-Sephacel step (in the presence of calcium) was done as a “batch” step rather than in a column; and (b) the fractions eluted from the DEAE-Sephacel column (in the presence of EGTA) with 0-0.5 M NaCl were analyzed for gelsolin by dot blots using a mAb to a porin/desialylated secondary antibody. The activity of the gelsolin was assayed by the change in fluorescence of NBD-actin upon binding gelsolin under non-polymerizing conditions (Bryan and Kurth, 1984; Couëf and Korn, 1985; calibration performed by Dr. A. Weber, University of Pennsylvania, Philadelphia, PA). The gelsolin was stored at −80°C or diluted 1:1 with ethylene glycol and stored at −20°C.

Tfα4 was isolated from a perichiotic acid extract of bovine spleens, with final purification by reverse-phase HPLC. Preliminary purification has been achieved by several different methods in different preparations; in all cases, analytical HPLC was used to identify the fractions containing Tfα4. Bovine spleens (Rockland, Inc., Gilbertsville, PA) were chilled in liquid nitrogen, broken up with a meat tenderizer, and then centrifuged to a coarse powder using an ice crusher. Batches of frozen, pulverized spleen were homogenized with 4 volumes of 0.5 M NaCl; an ice bath in a blender. The filtered homogenate was clarified by centrifugation (15,000 x g for 10 min) and neutralized with cold KOH. The precipitated potassium perchlorate was removed by centrifugation. The supernatant was lyophilized, then redissolved in water to about 1/10 of its original volume, and an equal volume of acetone at −20°C was added. The precipitate was removed by centrifugation (15,000 x g for 10 min, at −10°C); the supernatant, containing virtually all the Tfα4, was then mixed with an equal volume of cold acetone and recentrifuged. The pellet obtained at 75% acetone was redissolved in water and dialyzed (in small-pore tubing) against 25 mM ammonium bicarbonate. The dialyzed material was applied to a column of DEAE-Sephacel equilibrated with 25 mM ammonium bicarbonate (0.5-mL column bed per gram of spleen), washed with 0.66 column volume of the same buffer, and eluted with 0.25 M ammonium bicarbonate.

Fractions containing Tfα4 were pooled and further purified by reverse-phase HPLC. For large preparations (100-1,000 g spleen) a Vydac (Hesperia, CA) OD-300 octadecyl silica column (4.6 x 250 mm) and an Isco chromatograph. Solvent A + 0.1% trifluoroacetic acid in water, solvent B = 0.08% trifluoroacetic acid in acetonitrile; elution was performed with a gradient from 0 to 35% B in 12 min at a flow rate of 2 ml/min, and monitored at 220 nm. The peak corresponding to Tfα4 was identified by spiking PMN samples with Tfα4 purified from bovine spleen. The concentration of Tfα4 was determined from the integrated area of the peak, calibrated against pure Tfα4 at a known concentration (calibrated as described by Safer et al., 1991).

Quantitation of Tβ4
The level of Tβ4 in human PMNs was determined by reverse phase HPLC (Hannapel and Van Kampen, 1987; Safer et al., 1991) based on the solubility of Tβ4 after precipitation of proteins with PCA. This method recovered >90% of the Tβ4 (Hannapel and Van Kampen, 1987; D. Safer, unpublished results). Cold PCA (final concentration 0.4 M) was added to either whole cells (1-1.25 x 10⁸ cells/ml) or cell supernatants prepared as described above (from cells at 1.25 x 10⁸ cells/ml). When whole cells were used, the cell pellets were homogenized by passing through an ice-cold 0.5 M NaCl; an ice bath in a blender. The filtered homogenate was clarified by centrifugation (15,000 x g for 10 min) and neutralized with cold KOH. The precipitated potassium perchlorate was removed by centrifugation. The supernatant was lyophilized, then redissolved in water to about 1/10 of its original volume, and an equal volume of acetone at −20°C was added. The precipitate was removed by centrifugation (15,000 x g for 10 min, at −10°C); the supernatant, containing virtually all the Tβ4, was then mixed with an equal volume of cold acetone and recentrifuged. The pellet obtained at 75% acetone was redissolved in water and dialyzed (in small-pore tubing) against 25 mM ammonium bicarbonate. The dialyzed material was applied to a column of DEAE-Sephacel equilibrated with 25 mM ammonium bicarbonate (0.5-mL column bed per gram of spleen), washed with 0.66 column volume of the same buffer, and eluted with 0.25 M ammonium bicarbonate.

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Native PAGE and Immunoblotting
7.5% nondenaturing polyacrylamide gels were run at 4°C as described by Safer et al. (1990). Supernatants, prepared as described above, were loaded onto gels after addition of glycerol to 10%. For most experiments, supernatant samples were prepared immediately before loading on the gel. Gels were typically loaded with supernatant equivalents of 2 x 10⁵-1 x 10⁶ cells/lane. Proteins were identified either by staining gels with Coomassie blue or by immunoblotting.

A complex of actin and Tβ4 was prepared from purified proteins to serve as a gel standard. Equimolar concentrations of muscle G-actin and spleen Tβ4 were incubated on ice for ~5 min before adding glycerol and loading on the gel. G-actin and G-actin/Tβ4 complex were loaded onto gels at about the same concentration as the G-actin concentration in the supernatants (~3-5 µM in most experiments). Free Tβ4 rapidly diffuses from gels so its position relative to actin and actin/Tβ4 was determined for an overexposed sample (~20 µg, compared to the typical load for the actin/Tβ4 complex of 0.15 µg) by examining the gel within 20-30 min of staining with Coomassie blue.

For immunoblotting, gels were transferred to Immobilon-P (Millipore Continental Water Systems, Bedford, MA) using a Hoefer Mini Transphor apparatus (San Francisco, CA) according to the method of Towbin (Towbin et al., 1979). After transfer the immunoblot was blocked and probed with antibodies as described previously (Cano et al., 1992) except that TBS containing Tween-20 (TBS-T; 50 mM Tris-HCl, pH 7.4, 154 mM NaCl, 0.05% PMSF, 1 µg/ml leupeptin, 1 µg/ml benzamidine, 10 µg/ml aprotinin, and 10 µg/ml TAME-HCl). The lysate was spun for 5 min at 4°C in a microfuge (Eppendorf; Brinkman Instruments Inc., Westbury, NY) and the supernatant removed. This low speed centrifugation would pellet at least 80% of the cytoskeletal F-actin (Cano et al., 1992), but some filaments may remain in the supernatant.

To measure changes in the G-actin/Tβ4 complex and G-actin sequestering capacity, supernatants were prepared from cells incubated with and without chemotactrant. For stimulation, cells were incubated with 10⁻⁷ M FNLP for 30 s at 35°C. In some experiments cytochalasin B (2-10 µM final concentration) was added with the FNLP. A portion of each of the resting or stimulated cell suspensions were fixed by addition of glutaraldehyde (Polysciences Inc., Warington, PA) to 1% final concentration (for F-actin quantitation), while the remainder of each cell suspension was lysed and the supernatant fraction prepared as described above. F-actin levels were measured using the phalloidin binding assay of Howard and Oresajo (1985) as described previously (Cassimeris et al., 1990). Supernatants were assayed for the relative levels of G-actin/Tβ4 complex and G-actin sequestering capacity as described below.
TWEEN-20) was substituted for PBS. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and Kodak XRP-5 film (Eastman Kodak Co., Rochester, NY). No bands were detectable in samples incubated without antibodies, or with secondary antibodies alone.

Gels containing samples to be probed with anti-Tß4 antibodies were "lightly" fixed before transfer. This step was necessary because Tß4 does not bind well to either Immobilon or nitrocellulose transfer membranes. Gels were fixed in 0.4% glutaraldehyde (in distilled water) for 4 mins, rinsed with distilled water, and the remaining active aldehyde groups blocked with 0.1 M Tris, 0.1 M glycine (2 x 2 rain with a distilled water rinse in between). The gel was then incubated for 2 min in two changes of transfer buffer and transferred as described above. After transfer no protein bands were detectable on the gel suggesting that fixation did not hinder transfer (not shown).

The antibodies used included a rabbit polyclonal anti-nonmuscle gamma actin (Otey et al., 1986; a generous gift of Dr. J. C. Bulinski, Columbia University, New York, NY) and a mouse monoclonal anti-actin reactive with all actin isoforms (Lessard, 1988; a generous gift of Dr. J. L. Lessard, Childrens Hospital Research Foundation, Cincinnati, OH). A polyclonal antibody to bovine spleen Tß4 was raised in rabbits (V. T. Nashmias, L. Cassimeris, R. Golla, and D. Safer, manuscript submitted for publication). Bovine spleen and human Tß4 have an identical amino acid sequence (Condo et al., 1987; Low et al., 1981). This antisera binds Tß4 in ELISA assays (Nashmias, V. T., and R. Golla, unpublished observations), recognizes only one band on immunoblots of lightly fixed nature gels, and does not bind actin (shown in Fig. 2). Free Tß4 is not routinely detected on immunoblots (occasional weak staining has been observed) most likely because free Tß4 is not retained on the Immobilon membrane.

To determine changes in G-actin/Tß4 complex after chemotacticant stimulation, the anti-actin and anti-Tß4 staining of immunoblots were quantified using an Image I image processor (version 3.95; Image I, West Chester, PA). An image of the blot was acquired using a camera (Dage MTI 65) and the Image 1 to average 16 frames and subtract a background image. The intensities of the G-actin/Tß4 bands were determined by measuring the average pixel intensity within a box equal to the size of the band. For each sample, the intensity of four different gel loads was measured. The intensity measurements were used to calculate absorbance (Absorbance = log [Background Intensity/Band Intensity]), since absorbance is linearly proportional to concentration. The average intensity of the background was measured in a box directly below the band (the box size was equal to the size of the band). For each experiment the absorbance measurements for resting and stimulated cell supernatants were compared. With the antibodies (and dilutions) and the detection system used here, the supernatants loaded on the gel showed a linear change in absorbance with supernatant equivalents of ~2,000-~600 cells per lane for actin antibodies and ~10^3-~10^4 cells per lane for the Tß4 antibody.

**Measurement of Changes in Cell Supernatant Capacity to Bind Exogenous G-actin before and after Chemotacticant Stimulation**

We measured the G-actin sequestering capacity of cell supernatants by examining the ability of supernatants to inhibit the initial rate of pyrenyl G-actin polymerization from gelsolin capped filaments. Cell supernatants (final concentrations equivalent to 1 x 10^6 cells/ml) were prepared from resting and stimulated cells (30-s stimulation with 10^-7 M FNLLP at 37°C) as described above except that the lysis buffer also contained 5 mM ATP. Cell lysates were spun at either 80,000 rpm for 15 min at 4°C (TL-100 ultracentrifuge; Beckman Instruments, Palo Alto, CA) or for 15 min at 4°C in an Eppendorf microfuge (Brinkman Instruments Inc., Westbury, NY) and the supernatants removed and kept on ice. The results did not differ with the different centrifugation conditions. Since stimulation could also affect a barbed end capping activity (Hall et al., 1989), we examined the rates of pointed end polymerization using gelsolin capped filaments as nuclei for polymerization. Gelsolin capped filaments were prepared by polymerizing overnight: 10 µM unlabeled actin with 0.17 µM gelsolin in 0.15 M KCl, 2 mM MgCl2, 1 mM ATP, 0.1 mM CaCl2 in 10 mM Tris-HCl, pH 7.4, supplemented with the following protease inhibitors: 1 µg/ml leupeptin, 1 µg/ml benzamidine, 10 µg/ml aprotonin, and 10 µg/ml TAME-HCl. 95 µl of cell supernatant was warmed at room temperature for 15 min and then mixed with pyrenyl G-actin (2 µM final concentration) and 10 µl of gelsolin capped filaments. At time points between 0-3 min, samples were diluted into cuvettes containing 900 µl assay buffer (25 mM Tris HCl, pH 7.4, 0.138 M KCl, 2 mM MgCl2, 1 mM ATP, 1 mM EGTA, and 0.2% NP-40), and pyrene fluorescence read in a spectrofluorimeter (model LS-5; Ex 370/EEm 410; Perkin Elmer Corp., Norwalk, CT) 5 s after addition of the sample. The background reading from the cuvette containing the buffer was measured for each cuvette before the addition of the sample, and this background reading was subtracted from the sample reading. This method of dilution of samples at time points was required because supernatants prepared from high cell concentrations (>10^8 cells/ml) scatter enough light to interfere with the pyrene signal.

**Tß4 Binding to Muscle G-actin**

The binding affinity was determined based on the inhibition of the initial rate of elongation in the presence of Tß4, assuming a 1:1 complex between actin and Tß4 (Weber et al., 1992). The initial rates of elongation of pyrenyl G-actin onto F-actin nuclei were followed in assay buffer plus 0.1% BSA. F-actin nuclei were created by rapid passage of 2 µM pyrenyl F-actin in a Hamilton syringe. The sheared F-actin (0.02 µM final concentration in the cuvette) was delivered to cuvettes containing pyrenyl G-actin at 0.5-2.0 µM in assay buffer and the rates of elongation determined by increases in pyrene fluorescence. Samples containing Tß4 were preincubated with pyrenyl G-actin for 3-5 min before addition of assay buffer and F-actin nuclei. For samples containing Tß4, the concentration of G-actin was varied between 0.7-2 µM and the concentration of Tß4 was varied between 3-10 µM. Tß4 binds equally well to pyrene and unlabeled muscle actin (Weber et al., 1992).

Binding kinetics were estimated by spiking samples with Tß4 after polymerization had been initiated. We assumed that once the slope in the sample spiked with Tß4 had attained the same slope as the sample pre-incubated with Tß4, that the complex concentration equaled >95% of its equilibrium concentration. This time was used to estimate the koff, from the integrated second order rate equation (Weiland and Molinoff, 1981):

\[
\frac{\frac{C_c}{B_r} - \frac{C(C_c)}{G_T}}{\frac{B_r}{C_t} - C_t} = k_{off} \frac{B_r}{C_t} - C_t
\]

Where \(C_c\) is the concentration of the actin/Tß4 complex at equilibrium, \(C\) is the concentration of the actin/Tß4 complex equal to 95% of the equilibrium complex concentration, \(G_T\) is the total actin concentration, \(B_r\) is the total concentration of Tß4, and \(t\) is the time required for the actin/Tß4 complex concentration to reach 95% of the equilibrium value. These experiments were complete while the amount of G-actin polymerized was small (<10% of total) and no correction for this small decrease in G-actin concentration was included. The \(k_{off}\) was then calculated from the \(K_d\) and the \(K_{off}\) (\(K_d = k_{off}/k_{on}\)).

**Results**

**Concentration of Tß4 in Human PMNs**

The concentration of Tß4 in human PMNs (>95% PMNs) was determined by reverse phase HPLC of the PCA soluble material from PMNs (Hannappel and Van Kampen, 1987; Safer et al., 1991). In the elution profile shown in Fig. 1A, the peak denoted by the asterisk has the retention time of Tß4 and coelutes with purified bovine spleen Tß4 (Fig. 1B), suggesting that this peak is PMN Tß4. Tß4 was found in PCA supernatants prepared from either whole cells or cell supernatants, but was not detectable in PCA supernatants prepared from the cytoskeletal fraction (not shown). The peak with a retention time slightly shorter than Tß4 is due to PMSF.

By measuring the Tß4 peak, we find 169 fg ± 90 (SD) Tß4 per PMN (range = 51-357 fg/cell; \(n = 7\) samples analyzed in duplicate or triplicate). Based on a cell cytoplasmic volume of 2.27 x 10^-13 liter (Roos et al., 1983), this corresponds to a cytoplasmic concentration of 149 µM ± 80.5 (SD).

Hannappel and Van Kampen (1987) previously determined...
that PMNs contain ∼400 fg Tβ4 per PMN (range = 264–564 fg/cell). The reason for the difference between their results and ours is not clear. The disparity may partially reflect the different methods used to quantify the Tβ4 standards, but these differences would likely be small. It is unlikely that proteolysis degraded the Tβ4 in our samples since: (a) we included protease inhibitors in our buffers, while Hannapel and Van Kampen (1987) did not; and (b) Tβ4 appeared stable in cell lysates since samples lysed on ice for 5 min before addition of PCA had 95% of the Tβ4 found in samples receiving PCA <30 s after lysis (not shown). Platelet contamination, a common feature of PMNs isolated from blood, could give falsely high values. 10 platelets per PMN would increase the Tβ4 level by 220 fg/PMN (based on ∼22 fg Tβ4 per platelet) (Hannapel and Van Kampen, 1987; Weber et al., 1992). We corrected our data for the small contribution from platelet contamination (platelet: PMN ratios varied between 0.02–0.3, corresponding to <5% of the PMN Tβ4 peak from platelet contamination).

In agreement with Hannapel and Van Kampen (1987), we find a large range of Tβ4 concentrations (∼300 fg/cell) between different individuals, while duplicate samples within a given preparation agree within ∼25 fg/cell. While Tβ4 is the most abundant of the thymosins, other similar polypeptides have been identified (Erikson-Viitanen et al., 1983; Hannapel et al., 1982). Since the putative actin binding site is conserved among all thymosins (Safer, 1992), it is interesting to speculate that individual variation in the concentration of Tβ4 may be compensated for by other thymosins.

**Tβ4 Is Bound to G-actin in Human PMN Supernatants**

To determine whether Tβ4 binds G-actin in human PMNs, we analyzed the actin containing complexes in PMN supernatants by nondenaturing polyacrylamide gel electrophoresis and immunoblotting. In this gel system proteins which interact with sufficient affinity run as a complex, and their mobility is altered compared to the mobility of the separate components. This system was used previously to identify Tβ4 in platelets based on the migration of the G-actin/Tβ4 complex to a position ahead of purified G-actin (Safer et al., 1990; see Fig. 2).

Fig. 2 shows a Coomassie blue stained nondenaturing polyacrylamide gel of a human PMN supernatant compared with purified muscle G-actin and muscle G-actin/Tβ4 complex (lanes 1–3). The band with the greatest mobility in the human PMN supernatant runs at a position similar to that of the muscle G-actin/spleen Tβ4 complex, i.e., ahead of pure actin. The PMN band typically runs slightly slower than the muscle G-actin/spleen Tβ4 complex. Free Tβ4, examined on a separate gel as described in Materials and Methods, has lower mobility compared to actin or actin/Tβ4 complex (Fig. 2, arrowhead).

The PMN band with similar mobility to the actin/Tβ4 complex reacts positively with antibodies to both actin (Fig. 2, lane 4) and Tβ4 (Fig. 2, lane 5). Additional lower mobility actin bands were also detected after longer exposure of the PMN samples.

![Figure 2](image-url)
immunoblots (data not shown). It is likely that these are actin-containing complexes, and not nonspecific antibody binding, since two actin antibodies gave similar actin patterns (data not shown). The proteins complexed with actin in these bands have not been identified. An additional band is sometimes observed at the base of the gel well. While this band may represent either polymerized or denatured actin, it is of low abundance compared to the actin/Tβ4 band and thus polymerization of actin does not occur to any great extent in the supernatant over the course of these experiments. An additional concern that polymerized actin may not enter the gel, and thus may not be detected, is unlikely since Coomassie blue staining of the major supernatant actin band is similar to the staining of the muscle actin standards, and the supernatant and standards contain equal amounts of actin.

Because the electrophoretic mobility of actin/Tβ4 is only slightly altered compared to the mobility of pure actin (Fig. 2, lanes 1–3), and because there is spreading of the actin bands during blotting (detected by India ink staining of lanes after transfer, not shown), it is not possible to say with certainty whether free G-actin is also present in the PMN supernatants. The low level of Coomassie blue staining at the position of free G-actin (Fig. 2, lane J) suggests that free G-actin is a minor component of the PMN supernatant under these conditions, but it has not been possible to resolve both free G-actin and G-actin/Tβ4 in a single immunoblot.

**Actin-Tβ4 Complex Decreases after Chemoattractant Stimulation**

Chemoattractant stimulation of PMNs results in the polymerization of ~60–80 μM G-actin, causing an approximate doubling of F-actin (Rao and Varani, 1982; Fechheimer and Zigmond, 1983; Howard and Meyer, 1984; Fig. 3 a). The concomitant ~7 μM decrease in the G-actin isolated in a high affinity complex with profilin (Southwick and Young, 1990) is insufficient to account for the increase in F-actin after chemoattractant stimulation. Therefore, we examined the change in the relative intensity of the G-actin/Tβ4 band after chemoattractant stimulation. As shown in Fig. 3 b, 30-s stimulation with 10^{-7} M FNLLP reduces the intensity of the G-actin/Tβ4 band on Coomassie blue-stained non-denaturing gels, consistent with a decrease in the G-actin/Tβ4 complex. This result was confirmed with anti-actin and anti-Tβ4 immunoblots. By anti-actin immunoblots chemoattractant stimulation results in a decrease in the G-actin/Tβ4 band to 60.2 ± 8.5% (n = 6) of the resting cell level (Fig. 3 c). Similar results were found with an antibody to Tβ4; after stimulation, Tβ4 staining of the actin/Tβ4 band decreased to 53 ± 9% (n = 6) of the resting cell level (Fig. 3 d). In these experiments F-actin increased upon stimulation to 200 ± 42% (n = 6) of the amount found in resting cells. If F-actin increased from 60 to 120 μM then the total G-actin would have decreased from 140 to 80 μM (57% of its initial value). This change is compatible with the change in the G-actin/Tβ4 band occurring after stimulation.

Cytochalasin B blocks the chemoattractant induced increase in F-actin (Fig. 3 a), but not signal transduction induced secretion (Norgauer et al., 1988). When cells were stimulated in the presence of cytochalasin B (2–10 μM), the intensity of the G-actin/Tβ4 band on Coomassie blue-stained non-denaturing gels was similar to that in resting cells (Fig. 3 b). This result was confirmed on anti-actin (Fig. 3 c) and anti-Tβ4 (not shown) immunoblots. These observations suggest that release of G-actin from Tβ4 is not stably modified by chemoattractants. Because free Tβ4 is not consistently detectable on immunoblots, presumably because it is not retained on the Immobilon membrane, it has not been possible to document an increase in free Tβ4 concomitant with the decrease in the complex observed after chemoattractant stimulation. It is unlikely that the decrease in Tβ4 in the complex after chemoattractant stimulation is caused by proteolysis of Tβ4.
Supernatants from Stimulated Cells Inhibit Pyrenyl Actin Pointed End Polymerization to a Greater Extent than Supernatants Prepared from Resting Cells

A chemoattractant-induced increase in F-actin, and thus a decrease in G-actin complexed with Tβ4, could be caused by either an increase in free-barbed ends which have a higher affinity for G-actin than does Tβ4 (Weber et al., 1992), and/or a reduced affinity of Tβ4 for G-actin. If the increase in F-actin were due merely to an increased availability of free-barbed ends, with no change in Tβ4-binding affinity, then supernatants from stimulated cells should contain an increased concentration of free monomer binders (because the concentration of cell G-actin is less) that would be capable of binding and sequestering exogenous G-actin. Conversely, if the increase in F-actin were due to inactivation of the monomer binders, then supernatants from stimulated cells should not have an increased ability to sequester exogenous G-actin. To address this question, we examined the G-actin sequestering capacity of cell supernatants prepared from resting cells and cells stimulated for 30 s with 10^{-7} M FcLLP by comparing the rate of 2 μM pyrenyl actin polymerization from a constant number of filament pointed ends.

As shown in Fig. 4, stimulated cell supernatants inhibit the initial rate of pointed end polymerization to a greater extent than supernatants prepared from resting cells. In three experiments, polymerization in supernatants from stimulated cells was 0.57 ± 0.1 (SD) of the rate in supernatants from resting cells. These results suggest that stimulated cell supernatants contain a higher concentration of free monomer sequestering factors compared to that in resting cell supernatants and these results are consistent with no chemoattractant-induced modification of Tβ4 (see Discussion). In addition, boiled supernatants from resting and stimulated cells had approximately equal inhibitory activity (not shown). Since boiling does not inactivate Tβ4 (Safer et al., 1990), but denatures actin, the concentration of Tβ4 available to bind exogenous actin and inhibit polymerization would be the same in the two boiled supernatants.

Tβ4 Binding to Muscle G-actin

F-actin polymerizes rapidly in PMNs stimulated with chemoattractants. If Tβ4 sequesters a large fraction of the actin that polymerizes upon addition of chemoattractant, and if Tβ4 is not itself modulated by chemoattractant, then release of Tβ4 from actin would need to be fast enough to keep up with the rate of polymerization. Thus, it is important to determine if the kinetics of Tβ4 binding to, and release from, G-actin are fast enough to allow the rate of polymerization observed in vivo. In vitro experiments with purified bovine spleen Tβ4 and pyrenyl actin were used to estimate the binding kinetics. The rate of binding of bovine spleen Tβ4 to muscle G-actin was estimated by adding Tβ4 to a sample of pyrenyl G-actin 2-3 min after polymerization onto F-actin nuclei had begun. The time required for the slope to shift to that observed in samples preincubated with Tβ4 was used to estimate the k_on as described in Materials and Methods.

Fig. 5 A, curve a shows the initial rate of 2 μM pyrenyl G-actin polymerization from F-actin nuclei. Preincubation of 2 μM pyrenyl G-actin with 5 μM Tβ4 results in a slower rate of polymerization, consistent with a reduction in free G-actin (Fig. 5 A, curve b). Fig. 5 A, curve c shows a sample of G-actin initially polymerizing from F-actin nuclei without Tβ4. 5 μM Tβ4 was added after 3 min and the rate of polymerization rapidly shifted to that observed in samples of G-actin preincubated with Tβ4. This is shown with greater time resolution in Fig. 5 B; here 5 μM Tβ4 was added at 2 min (Fig. 5 B, curve c). In all (n = 7) samples receiving Tβ4 after polymerization had been initiated, the slope shifted within the 6 s mixing interval to a slope equivalent to samples preincubated with Tβ4. No change in slope was observed after merely mixing samples (curves a and b). Because it was not possible to reduce this mixing time interval with our spectrofluorimeter, we could not determine the value of the rate constants, but we could determine that the rate constant had to be >0.045 μM^{-1} s^{-1} (±0.003 SD, n = 7; calculated using Equation 1). The maximum rate of binding could be up to ~44 times faster (~2 μM^{-1} s^{-1} for diffusion limited protein–protein associations; Northrup and Erickson, 1992). Our measurement of the affinity of Tβ4 for muscle actin based on initial rates of polymerization in the presence of different concentrations of Tβ4 (K_a = 2 μM; data not shown) confirmed the results of Weber et al. (1992) (K_a = 2 μM for muscle actin). From the K_a and the minimal value for the k_on, we determined that the k_on must be greater than 0.09 s^{-1}.

Discussion

Non-denaturing Gels

The non-denaturing gel system has been useful for identifying Tβ4 as an actin binding protein (Safer et al., 1990; Safer, 1992), but the affinity of Tβ4 for actin appears much higher in this system compared to the affinity measured in
Cytoplasmic G-actin Sequestering Capacity

With the assumption that the G-actin concentration is 120 µM in the resting cell and 40 µM in the stimulated cell, the fraction of G-actin bound to TB4 and to profilin can be calculated based on the concentrations and affinities of these monomer binders using the following equation (Limbird, 1986):

\[
\frac{[\text{G}_{\text{bound}}]}{[\text{G}_{\text{free}}]} = \frac{[\text{Profilin}][\text{G}_{\text{free}}]}{K_{d,\text{Profilin}}} + \frac{[\text{TB4}][\text{G}_{\text{free}}]}{K_{d,\text{TB4}}} + [\text{G}_{\text{free}}] + [\text{G}_{\text{free}}] + [\text{G}_{\text{free}}]
\] (2)

This equation assumes that profilin and TB4 compete for G-actin as demonstrated (Goldschmidt-Clermont, P. J., M. I. Furman, and T. D. Pollard. 1991. J. Cell Biol. 115:3a).

Equation 2 was solved by successive iteration for two different conditions: first, assuming TB4 and profilin are the only monomer sequesters and that there are no free filament ends; and second, after setting the free G-actin to 0.5 µM, approximately the critical concentration of the pointed end. For both cases we used the following concentrations and affinities: [TB4] = 149 µM (this report), \( K_d, TB4 = 0.6 \) µM (determined using platelet actin; Weber et al., 1992), and [Profilin] = 40 µM, \( K_d, Profilin = 1 \) µM (Southwick and Young, 1990). The total G-actin concentration was set at 120 µM for resting PMNs and 40 µM for stimulated PMNs.

If we assume TB4 and profilin provide all the cytoplasmic sequestering of G-actin, and that there are no free filament ends, in a resting cell (total G-actin = 120 µM), 21.3 µM actin would be bound to profilin, and 97.6 µM bound to TB4. Thus, profilin and TB4 could reduce the concentration of unsequestered G-actin to 1.1 µM, close to the critical concentration for the pointed end of actin filaments measured in vitro (by most measurements the pointed end critical concentration is \( \approx 0.5 \) - 1 µM; reviewed in Pollard and Cooper, 1986).

In a stimulated cell (total G-actin = 40 µM) the concentration of unsequestered G-actin will decrease to 0.18 µM, close to the critical concentration of the barbed end. Interestingly, the calculations predict that the combined G-actin "buffering" capacity of profilin and TB4 would lead to a free G-actin concentration which falls in a range approximately between the critical concentrations for the barbed and pointed filament ends. Based on this analysis, it appears that the G-actin buffer is sufficient to allow polymerization of \( \approx 80 \) µM G-actin before the concentration of unsequestered G-actin decreases to the critical concentration of the barbed end, thus limiting further polymerization.

If we assume that the pointed ends of filaments are free in the cytoplasm, the concentration of unsequestered G-actin is unlikely to exceed the critical concentration of the pointed end. Resolving equation 2 after setting the free G-actin to 0.5 µM indicates that 13 µM G-actin is bound to profilin and 68 µM bound to TB4, and leaves 39 µM G-actin which must be sequestered by other factors, perhaps including other thymosin peptides. Our non-denaturing gels showed three actin-containing complexes, suggesting that additional monomer binding factors are present in PMNs.

The calculations of the G-actin sequestering capacity of TB4 and profilin in PMNs are based on binding affinities measured using purified components and as such are likely only an estimate of the situation in the cell. Additional fac-

Cassimeris et al. Thymosin \( \beta_4 \) Binds G-actin in PMNs
The same concentration of labeled subunits plus an equal concentra-
tional concentration. Having a labeled subunit at the filament end is 0.5. The association
of unlabeled subunits in a and as one labeled and one unlabeled subunit
would add onto each filament in both cases a and b. Although subunit association would be twice as fast in b, the de-
tectable association in each case is five labeled subunits. Over this
same time interval, dissociation of two subunits would occur as two
labeled subunits in a and as one labeled and one unlabeled subunit
in b. In this example, the presence of unlabeled subunits reduces
the apparent dissociation rate by one-half, since the probability of
having a labeled subunit at the filament end is 0.5. The association
of five to ten subunits and dissociation of two subunits is purely hy-
thetical and is not meant to represent the situation at a particular
actin concentration. It is interesting to note that the lowering of the
apparent dissociation rate by the presence of unlabeled subunits
will be less significant at G-actin concentrations well above the crit-
ical concentration.

The Journal of Cell Biology, Volume 119, 1992 1268

Figure 6. Diagramatic representation of association and dissocia-
tion reactions of labeled actin in the absence and presence of unla-
beled actin. Solid balls represent pyrenyl actin subunits, open balls
represent unlabeled actin subunits, and the open box represents the
capping of a filament end. a shows association and dissociation re-
actions in 100% labeled actin and b shows these same reactions at
the same concentration of labeled subunits plus an equal concentra-
tion of unlabeled subunits. During a hypothetical time interval, five
labeled subunits would add onto each filament in both cases a and
b. Although subunit association would be twice as fast in b, the de-
tectable association in each case is five labeled subunits. Over this
same time interval, dissociation of two subunits would occur as two
labeled subunits in a and as one labeled and one unlabeled subunit
in b. In this example, the presence of unlabeled subunits reduces
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ical concentration.

tors may affect the binding affinity of each monomer binding
protein for G-actin, or may compete with actin for binding. For
example, a high affinity profilin–actin complex has been
isolated (Carlsson et al., 1977), but reconstituted profilin/actin
complex has a lower affinity (Ki in the micromolar
range; Larsson and Lindberg, 1988; Southwick and Young,
1990; reviewed in Pollard and Cooper, 1986). In addition,
since profilin also binds PIP2, some of the profilin in the
cell may be bound to phospholipids and not available to bind
G-actin (Goldschmidt-Clermont et al., 1990; Lassing and
Lindberg, 1988). No regulation of Tβ4 binding to actin has
yet been identified. Tβ4 binding is neither calcium sensitive
(Weber et al., 1992) nor affected by PIP2 (Janmey et al.,
1992), and phosphorylation of Tβ4 has not been detected
(V. T. Nachmias, L. Cassimeris, R. Golla, and D. Safer, sub-
mitted for publication). Our functional analysis of the pyrenyl
G-actin sequestering activity of PMN supernatants also sug-
gests that Tβ4 is not stably modified by chemoattractant
stimulation, as discussed below.

**Tβ4 Does Not Appear Modified in Supernatants from Chemoattractant-stimulated Cells**

30-s stimulation of cells with chemoattractant decreased the
amount of G-actin migrating in a complex with Tβ4 (Fig.
3). This result is not surprising since in resting PMNs the
majority of G-actin appears complexed with Tβ4 (Fig. 2)
and after stimulation the total G-actin has decreased by ~80
µM. However, the mechanism responsible for this decrease
was not clear. Chemoattractant-induced polymerization could result either from a modification of monomer seque-
resters to reduce their affinity for G-actin and increase
the pool of free G-actin, or from an increase in free barbed
ends, without modification of the sequesters, provided
barbed ends have a higher affinity for G-actin than do the se-
questers (as is the case with Tβ4; Weber et al., 1992). To
differentiate between these possible mechanisms, we exam-
ined the exogenous G-actin binding capacity of supernatants
from resting and stimulated cells. We find that stimulated

cell supernatants had an increased capacity to bind exoge-

ous pyrenyl actin, suggesting that stimulation does not sta-

bly inactivate the G-actin sequestering capacity of the
cytoplasm (see Discussion below).

While this functional assay cannot differentiate between
individually monomeric binding factors, the observed inhibi-
tion of polymerization is consistent with sequestering due to
Tβ4, for several reasons. First, boiling did not destroy the
activity (not shown) and Tβ4 remains active after boiling
(Safer et al., 1990). Second, the extent to which the rate of
polymerization was slowed is similar to that predicted based
on the concentration of Tβ4 in the supernatants and its mea-
sured affinity for muscle actin (below). Third, the low con-
centration of profilin and its low affinity for pyrenyl actin
(Lai and Korn, 1985; Kaiser et al., 1986) make it unlikely
that profilin contributes significantly to the inhibitory activ-
ity. In contrast, Tβ4 is present at high concentration and its
affinity for muscle actin is unaffected by the presence of the
pyrene probe (Weber et al., 1992).

Interpretation of the experiment shown in Fig. 4 must take
into account the different concentrations of cell actin in rest-
ing and stimulated cell supernatants, and the lower affinity
of Tβ4 for muscle actin compared to cell actin (Kd = 2 µM
for muscle actin and 0.6 µM for cell actin; Weber et al.,
1992). To model the experimental results, the rate of 2 µM
pyrenyl actin polymerization was calculated with unlabeled
G-actin and Tβ4 concentrations equivalent to those present
in supernatants from resting cells, and this rate compared to
the rate calculated for: (a) supernatants from stimulated cells
assuming the total concentration and affinity of Tβ4 were
not altered by stimulation; and (b) supernatants from stimu-
lated cells assuming enough Tβ4 was inactivated to release
the G-actin which polymerized. The concentration of free
pyrenyl actin was calculated for these three cases using the
following equation (Limbird, 1986):

\[
[Tβ4_{bound}] = \frac{[Cell Actin_{total}][Tβ4_{free}]}{K_{4, cell actin} + Tβ4_{free}} + \frac{[Pyrenyl Actin_{total}][Tβ4_{free}]}{K_{4, pyrenyl actin} + Tβ4_{free}}
\]

The concentration of free pyrenyl actin was determined by
successive iteration using the following values (based on super-
натants equal to a 20-fold dilution of the cytoplasm):

\[
[Cell actin_{total}] = 6 \, \mu M \text{ (resting supernatant) and } 2 \, \mu M \text{ (stimulated supernatant),}
\]

Pyrenyl actin concentration was set at 2 µM in all cases,

\[
[Tβ4_{free}] = 7.5 \, \mu M \text{ (resting supernatants and case a), and } 2.3
\]

µM for case (b), and Ki = 2 µM (pyrenyl actin) and 0.6 µM
(cell actin) (Weber et al., 1992). This analysis suggests that

the concentration of free pyrenyl actin is 1 µM in super-

natants from resting cells, 0.7 µM for case (a) (stimulated cells
with no modification of Tβ4) and 1.5 µM for case (b)
(stimulated cells with modification of Tβ4). The calculation
is an estimate since the contribution from profilin binding to
cell actin was not included.

It is not valid to compare directly the rates of pyrenyl
fluorescence increase in supernatants with the rates observed
with 1, 0.7, and 1.5 µM pyrenyl actin in the absence of super-
натants because the presence of unlabeled actin subunits
causes a reduction in the apparent dissociation rate of the
pyrenyl actin (since dissociation of the unlabeled subunits is
not detected). In contrast, the association rate for pyrenyl ac-
tin is not affected by the presence of unlabeled G-actin, while the total association rate for each filament will be equal to the association rate constant times the \([G_{\text{bound}} + G_{\text{unbound}}]\). Since the detection system only "sees" labeled subunits the observed rate would only reflect the concentration of labeled subunits (Fig. 6).

Taking into account the percent labeled actin in each case (the pyrenyl actin stock was 75% labeled in these experiments), the rate of pyrenyl actin polymerization predicted for case a (no modification of Tβa) would equal 30% of the rate in supernatants from resting cells. In contrast, the rate of pyrenyl actin polymerization in case b (inactivation of Tβb) would have been 200% of the rate in supernatants from resting cells. Our results show that the rate of polymerization in supernatants from stimulated cells is 57% of the rate in supernatants from resting cells, and this is in good agreement with a model (case a) where Tβa is not modified by chemotactrant stimulation. After boiling, resting and stimulated cell supernatants had approximately equal inhibitory activity. This result is expected since boiling should denature the cell actin, but not the Tβ. In the two boiled supernatants the concentrations of available Tβa would be equal, and thus the concentrations of free pyrenyl actin would also be equal in the two supernatants.

Activation of a pointed-end capping protein by chemotactrant stimulation might generate the greater inhibition of pyrenyl actin polymerization in supernatants from stimulated cells. However, this seems unlikely since F-actin rapidly depolymerizes in stimulated PMNs when the barbed ends are capped (by cytochalasin B), suggesting that the pointed ends are free to depolymerize (Cassimeris et al., 1990). Our results suggest that either Tβa plays a passive role in regulating actin assembly, or that modification of Tβa affinity for G-actin is transient.

**Tβ, Binding to G-actin with Rapid Kinetics Is Necessary for Regulation of Polymerization In Vivo**

In PMNs, F-actin increases by 60–80 μM within 10 s after addition of chemotactants. If Tβ, plays a passive role in regulating actin assembly, it would need to dissociate rapidly from G-actin to supply the G-actin for polymerization. For 60–80 μM G-actin to be available in 10 s, the off-rate of Tβa from G-actin in the cell must be >0.06–0.08 s⁻¹ (calculated from 60–80 μM G-actin released from 976 μM G-actin/Tβ complex in 10 s). The minimal koff estimated using muscle actin is 0.09 s⁻¹. While the binding affinity of Tβa for nonmuscle actin is approximately threefold higher than for muscle actin (Weber et al., 1992) and the koff may be up to threefold slower, our rate constants are minimal estimates and the true rate constants may be 10–100 times faster. Recent results by Goldschmidt-Clermont et al. (1992) calculate a koff of 0.75 s⁻¹. Thus the rate of release of G-actin from Tβa is sufficient (without requiring modification of Tβb) for the rate of polymerization observed in vivo.

Upon chemotactrant dilution or cytochalasin B addition, 60–80 μM F-actin depolymerizes in 10 s (at 37°C) (Cassimeris et al., 1990). In this situation, G-actin must be bound by monomer binding proteins at a rate of 6–8 μM/s (60–80 μM in 10 s) to maintain a low free G-actin concentration and to prevent readdition of released subunits back onto the pointed ends. Based on the minimal koff of 0.045 μM⁻¹ s⁻¹ estimated for Tβa, binding to muscle actin, and the concentrations of Tβa and G-actin in vivo, binding of 80 μM actin by Tβa would reach 95% of the equilibrium complex concentration in 0.7 s (calculated using equation 1) which is equivalent to a rate of binding of >100 μM/s. Thus, Tβa binds actin fast enough to bind rapidly the free subunits generated by what is likely the maximal rate of G-actin concentration increase found in vivo.

**Summary**

The combined G-actin buffering capacity of Tβa and profilin is sufficient to allow polymerization of ~80 μM G-actin with less than 1 μM change in free G-actin. These results, combined with the apparent lack of chemotactrant modification of Tβa, are consistent with models where polymerization is regulated by the availability of free barbed ends.

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Cassimeris et al. Thymosin β1, Binder G-actin in PMNs
