Thymosin β_4 Sequesters the Majority of G-actin in Resting Human Polymorphonuclear Leukocytes

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Abstract. Thymosin β_4 (Tβ_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 Tβ_4 is an abundant peptide in these cells. By reverse phase HPLC of perchloric acid supernatants, human PMNs contain ~169 fg/cell ± 90 fg/cell (SD), corresponding to a cytoplasmic concentration of ~149 ± 80.5 μM. 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/Tβ_4 complex. Chemoattractant stimulation of PMNs results in a decrease in this G-actin/Tβ_4 complex. To determine whether chemoattractant induced actin polymerization results from an inactivation of Tβ_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 Tβ_4 being released as G-actin polymerizes, with no chemoattractant-induced change in its affinity for G-actin. The kinetics of bovine spleen Tβ_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)[1] 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; Sklar et al., 1985; Cassimeris et al., 1990).

Resting PMNs contain a large pool of G-actin, ~120 μM (White et al., 1983; Fechheimer and Zigmond, 1983), well above the critical concentration for polymerization of ~0.1–0.2 μM measured in vitro (reviewed in Pollard and Cooper, 1986). Thus, G-actin–binding factors are required to sequester ~120 μM 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 ~40 μM (Southwick and Young, 1990) and given its affinity for G-actin measured in vitro (K_d = 1 μM; Southwick and Young, 1990), profilin could maximally sequester <40 μM of the G-actin in a resting PMN, leaving ~80 μM G-actin to be sequestered by other factors.

Recent studies by Safer et al. (1990) suggest that thymosin β4 (Tβ_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 (Tβ_4 concentration in platelets ~560 μM; Weber et al., 1992). In this paper we have investigated: (*a*) the role of Tβ_4 in sequestering G-actin in resting human PMNs, (*b*) the possible regulation of Tβ_4 by chemoattractant, and (*c*) the kinetics of Tβ_4 binding to G-actin in vitro.

Materials and Methods

Reagents

Unless specified otherwise, reagents are from Sigma Chemical Co. (St. Louis, MO).

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1. *Abbreviations used in this paper*: F-actin, filamentous actin; FNLLP, formylnorleucylleucylphenylalanine; HPLC, high performance liquid chromatography; PMN, polymorphonuclear leukocytes; Tβ_4, thymosin β_4.

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 (Northrop et al., 1986) and stored at −80°C until used. Pyrenyl-labeled actin was prepared from acetone powder (~50–80% 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 gelsolin and a peroxidase-labeled 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; Coué 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.

Tβ_4 was isolated from a perchloric 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 Tβ_4. Bovine spleens (Rockland, Inc., Gilbertsville, PA) were chilled in liquid nitrogen, broken up with a mallet, and then pulverized to a coarse powder using an ice crusher. Batches of frozen, pulverized spleen were homogenized with 4 vol of cold 0.5 M PCA in a blender. The filtered homogenate was clarified by centrifugation (15,000 *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 *g* for 10 min, at −10°C); the supernatant, containing virtually all the Tβ_4, 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 Tβ_4 were pooled and further purified by reverse-phase HPLC. For large preps (100–1,000 g spleen) a Vydac (Hesperia, CA) 218TP510 octadecyl silica column was used; A = 20 mM ammonium acetate, pH 6.5; B = acetonitrile, and elution was performed with a gradient from 13 to 15% B in 120 min at a flow rate of 2 ml/min. Up to 20 mg (the yield from 200–250 g spleen) was injected per run. Acetonitrile was removed by rotary evaporation; the material was then lyophilized in aliquots and stored at −80°C.

Cells and Supernatants

Human PMNs were obtained by venipuncture from healthy volunteers, and the blood was immediately mixed with heparin (10 U/ml final concentration) and EDTA (5 mM final concentration) to prevent coagulation. For most experiments, PMNs were isolated by density gradient centrifugation on Polymorphprep (Accurate Chemical and Scientific Corp., Westbury, NY) according to the manufacturer's instructions (yield is >95% PMNs). We had difficulty stimulating cells isolated by Polymorphprep, so for experiments comparing resting and formylnorleucylleucylphenylalanine (FNLLP) stimulated PMNs, cells were isolated either by dextran sedimentation alone to yield a mixed population of white cells (Boyum, 1968; >70% PMNs), or by dextran sedimentation followed by Ficoll-Hypaque (Sterling Drug Co., New York, NY) density gradient centrifugation (45 min at 450 *g*; >95% PMNs). After two to three saline washes and removal of red blood cells by hypotonic lysis, the cells were resuspended in cell buffer (HBSS [Gibco Laboratories, Grand Island, NY], without phenol red, sodium bicarbonate, calcium, and magnesium, but supplemented with 10 mM Hepes, pH 7.2). The platelet contamination contributed <1% of the total cell volume (accounting for <5% of the total Tβ_4).

Cell supernatants were prepared by lysing cells by addition of 1/4 volume of cold 4× lysis buffer (final concentrations: 10 mM Hepes, 2 mM potassium phosphate buffer, 0.15 M KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1% NP-40, pH 7.2, supplemented with the following protease inhibitors: 1 mM 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/Tβ_4 complex and G-actin sequestering capacity, supernatants were prepared from cells incubated with and without chemoattractant. For stimulation, cells were incubated with 10^{-7} M FNLLP for 30 s at 23°C. In some experiments cytochalasin B (2–10 μM final concentration) was added with the FNLLP. A portion of each of the resting and stimulated cell suspensions were fixed by addition of glutaraldehyde (Polysciences Inc., Warrington, 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/Tβ_4 complex and G-actin sequestering capacity as described below.

Quantitation of Tβ_4

The level of Tβ_4 in human PMNs was determined by reverse phase HPLC (Hannappel and Van Kampen, 1987; Safer et al., 1991) based on the solubility of Tβ_4 after precipitation of proteins with PCA. This method recovers >90% of the Tβ_4 (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 × 10^8 cells/ml) or cell supernatants prepared as described above (from cells at 1.25 × 10^8 cells/ml). When whole cells were used, the protease inhibitors were added to the cell buffer prior to addition of PCA, and the samples were sonicated 2 × 10 s with a sonicator (model 150; Dynatech Laboratories, Chantilly, Virginia) and cooled for 30 s on ice between each sonication pulse. To look for Tβ_4 in the cytoskeleton fraction, the detergent insoluble fraction of the lysate was resuspended in 1× cold lysis buffer, PCA was added to 0.4 M and the sample sonicated as described above. For each sample, the PCA precipitate was pelleted for 5 min at 4°C in a microfuge (Eppendorf; Brinkman Instruments) and the PCA supernatant neutralized with cold potassium phosphate (0.4 M final concentration). The insoluble potassium perchlorate was pelleted as above and the resulting supernatant was stored at −80°C. 50–100-μl samples were analyzed by reverse phase HPLC using an Applied Biosystems Inc. (Santa Clara, CA) OD-300 octadecyl silica column (4.6 × 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 10 to 35% B in 12 min at a flow rate of 2 ml/min, and monitored at 220 nm. The peak corresponding to Tβ_4 was identified by spiking PMN samples with Tβ_4 purified from bovine spleen. The concentration of Tβ_4 was determined from the integrated area of the peak, calibrated against pure Tβ_4 at a known concentration (calibrated as described by Safer et al., 1991).

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 × 10^5–1 × 10^6 cells/lane. Proteins were identified either by staining gels with Coomassie blue or by immunoblotting.

A complex of actin and Tβ_4 was prepared from purified proteins to serve as a gel standard. Equimolar concentrations of muscle G-actin and spleen Tβ_4 were incubated on ice for ~5 min before adding glycerol and loading on the gel. G-actin and G-actin/Tβ_4 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 Tβ_4 rapidly diffuses from gels so its position relative to actin and actin/Tβ_4 was determined for an overloaded sample (~20 μg, compared to the typical load for the actin/Tβ_4 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 immobilon 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%

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-Tβ_4 antibodies were "lightly" fixed before transfer. This step was necessary because Tβ_4 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 × 2 min 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 Tβ_4 was raised in rabbits (V. T. Nachmias, L. Cassimeris, R. Golla, and D. Safer, manuscript submitted for publication). Bovine spleen and human Tβ_4 have an identical amino acid sequence (Gondo et al., 1987; Low et al., 1981). This antiserum binds Tβ_4 in ELISA assays (Nachmias, 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 Tβ_4 is not routinely detected on immunoblots (occasional weak staining has been observed) most likely because free Tβ_4 is not retained on the Immobilon membrane.

To determine changes in G-actin/Tβ_4 complex after chemoattractant stimulation, the anti-actin and anti-Tβ_4 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 I to average 16 frames and subtract a background image. The intensities of the G-actin/Tβ_4 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 ~10^5–~10^4 cells per lane for the Tβ_4 antibody.

Measurement of Changes in Cell Supernatant Capacity to Bind Exogenous G-actin before and after Chemoattractant 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 × 10^8 cells/ml) were prepared from resting and stimulated cells (30-s stimulation with 10^{-7} M FNLLP at 23°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 $MgCl_2$, 1 μM ATP, 0.1 mM $CaCl_2$ 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 aprotinin, and 10 μg/ml TAME-HCl. 95 μl of cell supernatant was warmed at room temperature for 1 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 $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 0.2% NP-40), and pyrene fluorescence read in a spectrofluorimeter (model LS-5; Ex 370/Em 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.

Tβ_4 Binding to Muscle G-actin

The binding affinity was determined based on the inhibition of the initial rate of elongation in the presence of Tβ_4, assuming a 1:1 complex between actin and Tβ_4 (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 through 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 Tβ_4 were preincubated with pyrenyl G-actin for 3–5 min before addition of assay buffer and F-actin nuclei. For samples containing Tβ_4, the concentration of G-actin was varied between 0.7–2 μM and the concentration of Tβ_4 was varied between 3–10 μM. Tβ_4 binds equally well to pyrene and unlabeled muscle actin (Weber et al., 1992).

Binding kinetics were estimated by spiking samples with Tβ_4 after polymerization had been initiated. We assumed that once the slope in the sample spiked with Tβ_4 had attained the same slope as the sample pre-incubated with Tβ_4, that the complex concentration equaled >95% of its equilibrium concentration. This time was used to estimate the k_{on} from the integrated second order rate equation (Weiland and Molinoff, 1981):

$$\ln\left[\frac{C_e\left[B_T - \frac{(C)(C_e)}{G_T}\right]}{B_T[C_e - C]}\right] = k_{on}t\left[\frac{(B_T)(G_T)}{C_e} - C_e\right] \quad (1)$$

Where C_e is the concentration of the actin/Tβ_4 complex at equilibrium, C is the concentration of the actin/Tβ_4 complex equal to 95% of the equilibrium complex concentration, G_T is the total G-actin concentration, B_T is the total concentration of Tβ_4, and t is the time required for the actin/Tβ_4 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_{on} ($K_d = k_{off}/k_{on}$).

Results

Concentration of Tβ_4 in Human PMNs

The concentration of Tβ_4 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. 1 *A*, the peak denoted by the asterisk has the retention time of Tβ_4 and coelutes with purified bovine spleen Tβ_4 (Fig. 1 *B*), suggesting that this peak is PMN Tβ_4. Tβ_4 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 Tβ_4 is due to PMSF.

By measuring the Tβ_4 peak, we find 169 fg ± 90 (SD) Tβ_4 per PMN (range = 51–357 fg/cell; n = 7 samples analyzed in duplicate or triplicate). Based on a cell cytoplasmic volume of 2.27 × 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

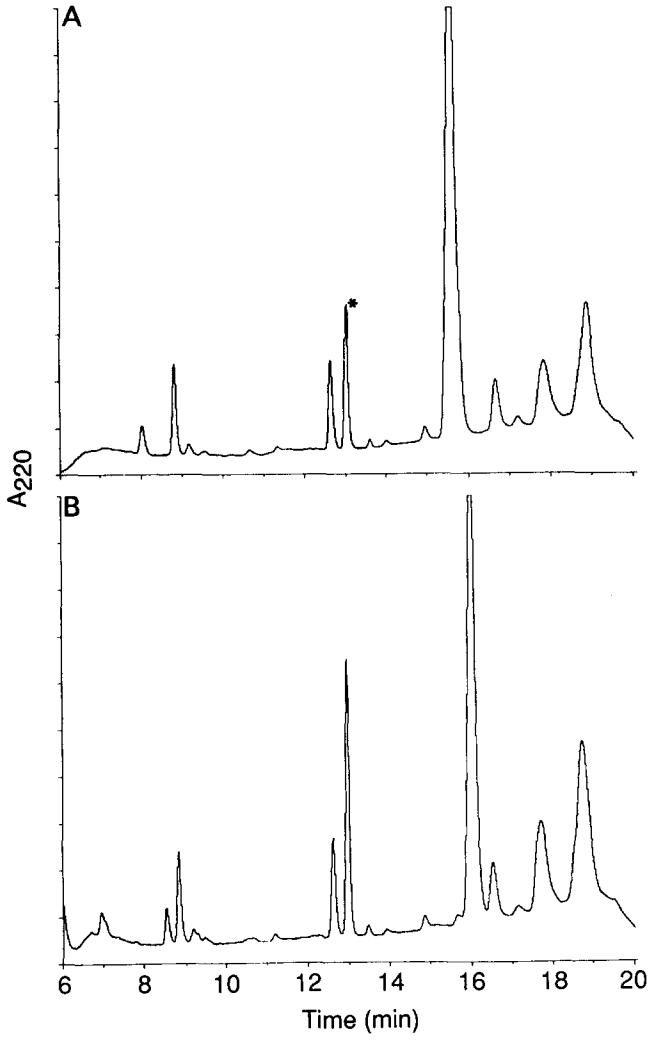

Figure 1. Quantitation of Tβ_4 in human PMNs by reverse phase HPLC. (*A*) HPLC elution profile of the PCA soluble material from human PMNs. The peak marked with the asterisk has the retention time of Tβ_4. (*B*) HPLC elution profile of a mixture of 0.5 μg bovine spleen Tβ_4 and the sample shown in *A*.

that PMNs contain ~400 fg Tβ_4 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 Tβ_4 standards, but these differences would likely be small. It is unlikely that proteolysis degraded the Tβ_4 in our samples since: (*a*) we included protease inhibitors in our buffers, while Hannappel and Van Kampen (1987) did not; and (*b*) Tβ_4 appeared stable in cell lysates since samples lysed on ice for 5 min before addition of PCA had 95% of the Tβ_4 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 Tβ_4 level by 220 fg/PMN (based on ~22 fg Tβ_4 per platelet) (Hannappel 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 Tβ_4 peak from platelet contamination).

In agreement with Hannappel and Van Kampen (1987), we find a large range of Tβ_4 concentrations (~300 fg/cell) between different individuals, while duplicate samples within a given preparation agree within ~25 fg/cell. While Tβ_4 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 Tβ_4 may be compensated for by other thymosins.

Tβ_4 Is Bound to G-actin in Human PMN Supernatants

To determine whether Tβ_4 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 Tβ_4 in platelets based on the migration of the G-actin/Tβ_4 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/Tβ_4 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 Tβ_4 complex, i.e., ahead of pure actin. The PMN band typically runs slightly slower than the muscle G-actin/spleen Tβ_4 complex. Free Tβ_4, examined on a separate gel as described in Materials and Methods, has lower mobility compared to actin or actin/Tβ_4 complex (Fig. 2, *arrowhead*).

The PMN band with similar mobility to the actin/Tβ_4 complex reacts positively with antibodies to both actin (Fig. 2, lane *4*) and Tβ_4 (Fig. 2, lane *5*). Additional lower mobility actin bands were also detected after longer exposure of

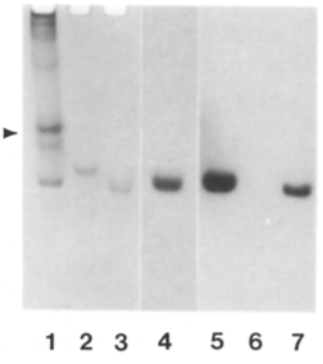

Figure 2. Identification of G-actin/Tβ_4 complex in human PMN supernatants by non-denaturing acrylamide gel electrophoresis. (Lanes *1–3*) Coomassie blue–stained gel of PMN supernatant (lane *1*, supernatant equivalent of 2 × 10^5 cells); muscle G-actin (lane *2*); and muscle G-actin/spleen Tβ_4 complex (lane *3*). In all lanes the actin concentration loaded on the gel was equal to ~3 μM. The G-actin/Tβ_4 complex runs slightly faster than G-actin (compare lanes *2* and *3*). The position of free Tβ_4, determined on a separate gel, is denoted with an arrowhead. Lanes *4–7* are anti-actin and anti-Tβ_4 immunoblots from the same gel as lanes *1–3*. Lanes *4* and *5* show anti-actin and anti-Tβ_4 immunoblots, respectively, from PMN samples equivalent to lane *1*. The band migrating with a mobility similar to the actin/Tβ_4 complex contains both actin and Tβ_4. Lane 6 is an anti-Tβ_4 immunoblot of purified actin (corresponding to lane *2*), and lane *7* is an anti-Tβ_4 immunoblot of G-actin/Tβ_4 complex (corresponding to lane *3*).

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/Tβ_4 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.

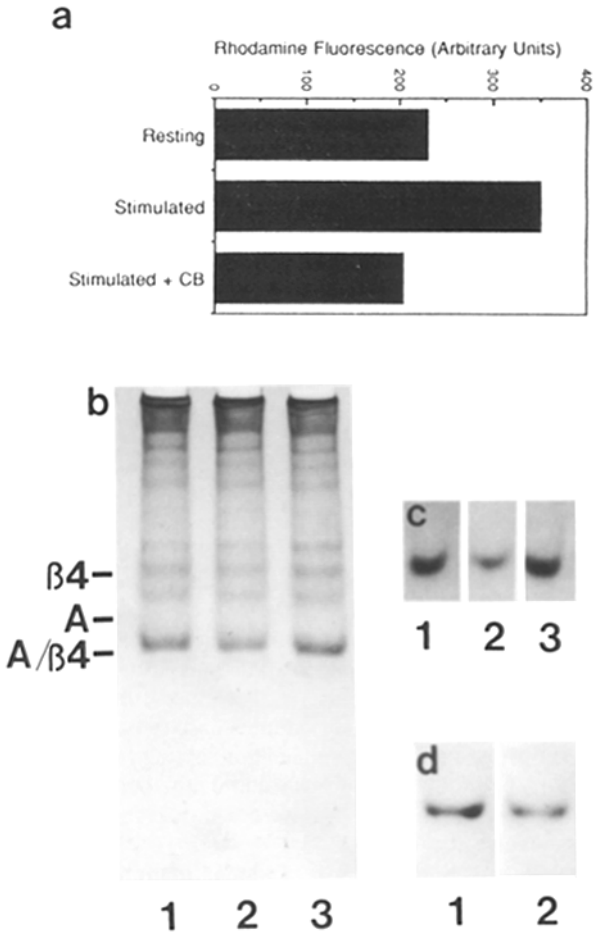

Figure 3. (*a*) F-actin levels, determined by rhodamine phalloidin binding, in resting cells, cells stimulated for 30 s with 10^{-7} M FNLLP, and cells stimulated in the presence of cytochalasin B (2 μM in this experiment). In this experiment stimulation increased the F-actin 1.5-fold. (*b*) Coomassie blue–stained non-denaturing gel of supernatants from resting cells (lane *1*), cells stimulated for 30 s with FNLLP (lane *2*), and cells stimulated in the presence of 2 μM cytochalasin B (lane *3*). (*c*) Anti-actin immunoblot of the G-actin/Tβ_4 complex in resting cells (lane *1*), 30 s after addition of FNLLP (lane *2*), and 30 s after stimulation in the presence of 2 μM cytochalasin B. (*d*) Anti-Tβ_4 immunoblot of the G-actin/Tβ_4 complex in resting cells (lane *1*) and 30 s after addition of FNLLP (lane *2*). The sample prepared by stimulation in the presence of cytochalasin B was not run on this blot. Additional experiments showed that this band also did not decrease compared to the level in resting cells. All data shown is from a single representative experiment.

Because the electrophoretic mobility of actin/Tβ_4 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 *1*) 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/Tβ_4 in a single immunoblot.

Actin-Tβ_4 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/Tβ_4 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/Tβ_4 band on Coomassie blue–stained non-denaturing gels, consistent with a decrease in the G-actin/Tβ_4 complex. This result was confirmed with anti-actin and anti-Tβ_4 immunoblots. By anti-actin immunoblots chemoattractant stimulation results in a decrease in the G-actin/Tβ_4 band to 60.2 ± 8.5% (n = 6) of the resting cell level (Fig. 3 *c*). Similar results were found with an antibody to Tβ_4: after stimulation, Tβ_4 staining of the actin/Tβ_4 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/Tβ_4 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/Tβ_4 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-Tβ_4 (not shown) immunoblots. These observations suggest that release of G-actin from Tβ_4 requires free-barbed ends and that Tβ_4 is not stably modified by chemoattractants.

Because free Tβ_4 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 Tβ_4 concomitant with the decrease in the complex observed after chemoattractant stimulation. It is unlikely that the decrease in Tβ_4 in the complex after chemoattractant stimulation is caused by proteolysis of Tβ_4

since stimulation in the presence of cytochalasin B does not reduce the $T\beta_4$ concentration in the complex and $T\beta_4$ appears stable in the lysate (above).

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 $T\beta_4$, could be caused by either an increase in free-barbed ends which have a higher affinity for G-actin than does $T\beta_4$ (Weber et al., 1992), and/or a reduced affinity of $T\beta_4$ for G-actin. If the increase in F-actin were due merely to an increased availability of free-barbed ends, with no change in $T\beta_4$-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 FNLLP 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 $\pm$ 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 $T\beta_4$ (see Discussion). In addition, boiled supernatants from resting and stimulated cells had approximately equal inhibitory activity (not shown). Since boiling does not inactivate $T\beta_4$ (Safer et al., 1990), but denatures actin, the concentration of $T\beta_4$ available to bind exogenous actin and inhibit polymerization would be the same in the two boiled supernatants.

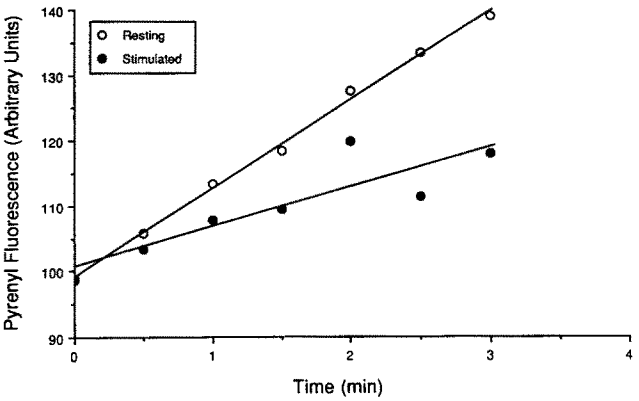

Figure 4. 2 μM pyrenyl actin polymerization from gelsolin capped filaments in supernatants prepared from resting and stimulated cells. Data shown are the means of two to three samples per time point and linear regression lines are plotted through the data. In this experiment polymerization in the stimulated supernatant was 45% of the rate in the resting supernatant (slope in resting supernatant = 13.6 fluorescent U/min, R^2 = 0.997; slope in stimulated cells = 6.1 fluorescent U/min, R^2 = 0.777). Stimulated cells contained approximately twice the F-actin of resting cells (not shown); this decrease in G-actin should increase the concentration of free $T\beta_4$ as described in the text.

$T\beta_4$ Binding to Muscle G-actin

F-actin polymerizes rapidly in PMNs stimulated with chemoattractants. If $T\beta_4$ sequesters a large fraction of the actin that polymerizes upon addition of chemoattractant, and if $T\beta_4$ is not itself modulated by chemoattractant, then release of $T\beta_4$ 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 $T\beta_4$ 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 $T\beta_4$ and pyrenyl actin were used to estimate the binding kinetics. The rate of binding of bovine spleen $T\beta_4$ to muscle G-actin was estimated by adding $T\beta_4$ 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 $T\beta_4$ 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 $T\beta_4$ 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 $T\beta_4$; 5 μM $T\beta_4$ was added after 3 min and the rate of polymerization rapidly shifted to that observed in samples of G-actin preincubated with $T\beta_4$. This is shown with greater time resolution in Fig. 5 *B*; here 5 μM $T\beta_4$ was added at 2 min (Fig. 5 *B*, curve *c*). In all (n = 7) samples receiving $T\beta_4$ after polymerization had been initiated, the slope shifted within the 6 s mixing interval to a slope equivalent to samples preincubated with $T\beta_4$. 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} ($\pm$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 $T\beta_4$ for muscle actin based on initial rates of polymerization in the presence of different concentrations of $T\beta_4$ (K_d ~2–3 μM; data not shown) confirmed the results of Weber et al. (1992) (K_d = 2 μM for muscle actin). From the K_d and the minimal value for the k_{on}, we determined that the k_{off} must be greater than 0.09 s^{-1}.

Discussion

Non-denaturing Gels

The non-denaturing gel system has been useful for identifying $T\beta_4$ as an actin binding protein (Safer et al., 1990; Safer, 1992), but the affinity of $T\beta_4$ for actin appears much higher in this system compared to the affinity measured in

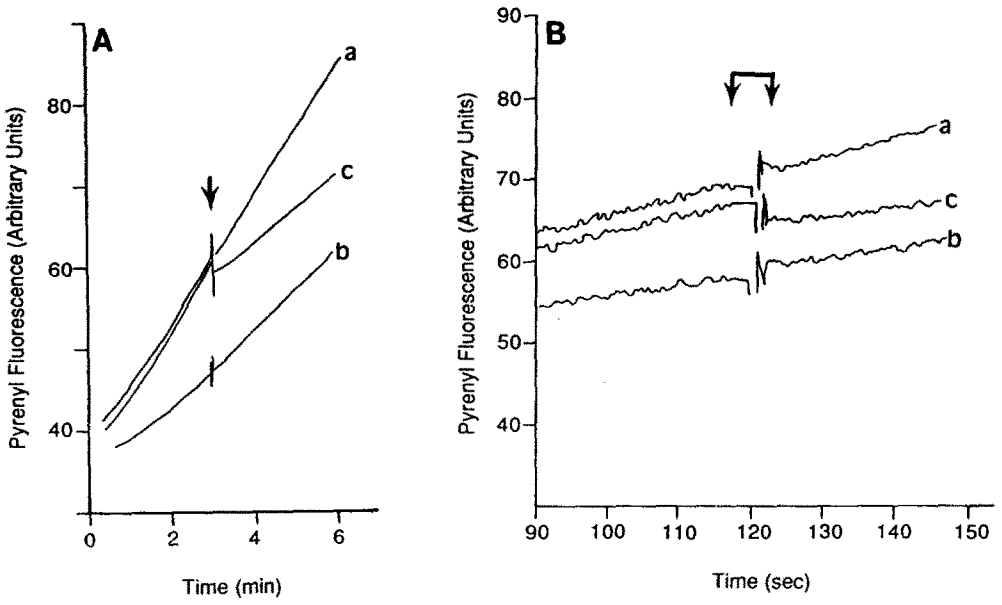

Figure 5. Kinetics of bovine spleen Tβ_4 binding to pyrenyl muscle actin. (*A*) All samples contained 2 μM G-actin and polymerization was initiated by addition of 0.02 μM F-actin as described in Materials and Methods. Curve *a* shows polymerization in the absence of Tβ_4, and curve *c* shows polymerization before and after addition of Tβ_4 (5 μM final concentration). For curve *c*, Tβ_4 was added at the arrow. All samples were mixed at the arrow. In *B* a similar experiment, but with greater time resolution is shown. *a*, *b*, and *c* are as given above. Tβ_4 was added at the left arrow; the arrows outline the mixing time when fluorescence cannot be followed. The slopes of duplicate samples typically varied by <10%.

actin polymerization buffers. For example, an equimolar complex of muscle actin and spleen Tβ_4 runs as a single band with the mobility of the complex (Fig. 2), but based on the K_d of 2 μM (determined for muscle actin; Weber et al., 1992) only about half of the 3 μM actin loaded on the gel should be complexed with Tβ_4. We do not yet know if the affinity is higher under the salt conditions of the non-denaturing gel or whether other factors are responsible for the apparently higher affinity on these gels. Therefore, it is not possible to determine the fraction of G-actin in cell supernatants bound to different monomer binders using their distribution on non-denaturing gels.

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 Tβ_4 and to profilin can be calculated based on the concentrations and affinities of these monomer binders using the following equation (Limbird, 1986):

$$[G_{bound}] = \frac{[Profilin][G_{free}]}{K_{d,Profilin} + [G_{free}]} + \frac{[T\beta4][G_{free}]}{K_{d,T\beta4} + [G_{free}]} \quad (2)$$

This equation assumes that profilin and Tβ_4 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 Tβ_4 and profilin are the only monomer sequesterers 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: [Tβ_4] = 149 μM (this report), K_d, Tβ_4 = 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 Tβ_4 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 Tβ_4. Thus, profilin and Tβ_4 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 ~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 Tβ_4 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 ~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 Tβ_4 and leaves 39 μM G-actin which must be sequestered by other factors, perhaps including other thymosin peptides. Our nondenaturing 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 Tβ_4 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-

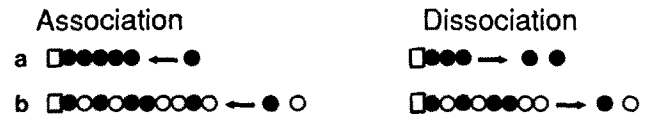

Figure 6. Diagrammatic representation of association and dissociation reactions of labeled actin in the absence and presence of unlabeled actin. Solid balls represent pyrenyl actin subunits, open balls represent unlabeled actin subunits, and the open box represents the capping of a filament end. *a* shows association and dissociation reactions in 100% labeled actin and *b* shows these same reactions at the same concentration of labeled subunits plus an equal concentration 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 detectable 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 hypothetical 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 critical 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 (K_d in the micromolar range; Larsson and Lindberg, 1988; Southwick and Young, 1990; reviewed in Pollard and Cooper, 1986). In addition, since profilin also binds PIP_2, 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 $T\beta_4$ binding to actin has yet been identified. $T\beta_4$ binding is neither calcium sensitive (Weber et al., 1992) nor affected by PIP_2 (Janmey et al., 1992), and phosphorylation of $T\beta_4$ has not been detected (V. T. Nachmias, L. Cassimeris, R. Golla, and D. Safer, submitted for publication). Our functional analysis of the pyrenyl G-actin sequestering activity of PMN supernatants also suggests that $T\beta_4$ is not stably modified by chemoattractant stimulation, as discussed below.

$T\beta_4$ 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 $T\beta_4$ (Fig. 3). This result is not surprising since in resting PMNs the majority of G-actin appears complexed with $T\beta_4$ (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 sequesterers 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 sequesterers, provided barbed ends have a higher affinity for G-actin than do the sequesterers (as is the case with $T\beta_4$; Weber et al., 1992). To differentiate between these possible mechanisms, we examined 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 exogenous pyrenyl actin, suggesting that stimulation does not stably inactivate the G-actin sequestering capacity of the cytoplasm (see Discussion below).

While this functional assay cannot differentiate between individual monomer binding factors, the observed inhibition of polymerization is consistent with sequestering due to $T\beta_4$ for several reasons. First, boiling did not destroy the activity (not shown) and $T\beta_4$ 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 $T\beta_4$ in the supernatants and its measured affinity for muscle actin (below). Third, the low concentration of profilin and its low affinity for pyrenyl actin (Lal and Korn, 1985; Kaiser et al., 1986) make it unlikely that profilin contributes significantly to the inhibitory activity. In contrast, $T\beta_4$ 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 resting and stimulated cell supernatants, and the lower affinity of $T\beta_4$ for muscle actin compared to cell actin (K_d = 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 $T\beta_4$ 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 $T\beta_4$ were not altered by stimulation; and (*b*) supernatants from stimulated cells assuming enough $T\beta_4$ 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):

$$[T\beta4_{bound}] = \frac{[\text{Cell Actin}_{total}][T\beta4_{free}]}{K_{d,\ \text{cell actin}} + T\beta4_{free}} + \frac{[\text{Pyrenyl Actin}_{total}][T\beta4_{free}]}{K_{d,\ \text{pyrenyl actin}} + T\beta4_{free}} \quad (3)$$

The concentration of free pyrenyl actin was determined by successive iteration using the following values (based on supernatants equal to a 20-fold dilution of the cytoplasm): [Cell actin_{total}] = 6 μM (resting supernatant) and 2 μM (stimulated supernatant), [Pyrenyl actin_{total}] = 2 μM in all cases, [$T\beta_{4Total}$] = 7.5 μM (resting cells and case a), and 2.3 μM (case *b*), and K_d's = 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 supernatants from resting cells, 0.7 μM for case (*a*) (stimulated cells with no modification of $T\beta_4$) and 1.5 μM for case (*b*) (stimulated cells with modification of $T\beta_4$). 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 supernatants 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 $[G_{labeled} + G_{unlabeled}]$. 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 $T\beta_4$) would equal 30% of the rate in supernatants from resting cells. In contrast, the rate of pyrenyl actin polymerization in case *b* (inactivation of $T\beta_4$) 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 $T\beta_4$ is not modified by chemoattractant 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 $T\beta_4$. In the two boiled supernatants the concentrations of available $T\beta_4$ 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 chemoattractant 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 $T\beta_4$ plays a passive role in regulating actin assembly, or that modification of $T\beta_4$ affinity for G-actin is transient.

$T\beta_4$ 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 chemoattractants. If $T\beta_4$ 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 $T\beta_4$ from G-actin in the cell must be $\geq$0.06–0.08 s^{-1} (calculated from 60–80 μM G-actin released from 97.6 μM G-actin/$T\beta_4$ complex in 10 s). The minimal k_{off} estimated using muscle actin is 0.09 s^{-1}. While the binding affinity of $T\beta_4$ for nonmuscle actin is approximately threefold higher than for muscle actin (Weber et al., 1992) and the k_{off} 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 k_{off} of 0.75 s^{-1}. Thus the rate of release of G-actin from $T\beta_4$ is sufficient (without requiring modification of $T\beta_4$) for the rate of polymerization observed in vivo.

Upon chemoattractant 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 k_{on} of 0.045 μM^{-1} s^{-1} estimated for $T\beta_4$ binding to muscle actin, and the concentrations of $T\beta_4$ and G-actin in vivo, binding of 80 μM actin by $T\beta_4$ 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, $T\beta_4$ 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 $T\beta_4$ and profilin is sufficient to allow polymerization of ~80 μM G-actin with less than a 1 μM change in free G-actin. These results, combined with the apparent lack of chemoattractant modification of $T\beta_4$, are consistent with models where polymerization is regulated by the availability of free barbed ends.

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