β-Thymosins Are Not Simple Actin Monomer Buffering Proteins

INSIGHTS FROM OVEREXPRESSION STUDIES*

Hui-Qiao Sun, Katarzyna Kwiatkowska, and Helen Lu Yin‡

From the Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9040

The actin cytoskeleton of nonmuscle cells responds to extracellular stimuli through a spatially and temporally regulated series of polymerization and depolymerization reactions. Monomer binding proteins, such as β-thymosin, ADF/cofilin, and profilin, buffer actin monomers (G-actin) to prevent them from polymerizing spontaneously (reviewed in Refs. 1 and 2). During cell activation, polymerization is initiated by filament uncapping at the cell cortex, which lowers the critical concentration for actin polymerization (1, 3–6). Actin monomer binding proteins amplify the effect of filament uncapping because they create a reservoir of G-actin that can be desquestered to supply actin to filament ends. Furthermore, because profilin-actin complexes facilitate barbed end filament growth (7) and profilin competes with β-thymosin for actin, profilin can tap into the β-thromosin-actin pool (7–9) to fuel massive polymerization. After polymerization, actin filaments are transported centripetally (10, 11) and subsequently depolymerize (12, 13). The newly released actin subunits are captured by monomer-binding proteins and recycled toward the cell front.

Among the monomer-binding proteins identified thus far, β-thymosins appear to best fulfill the criteria for a simple monomer-buffering protein because no interaction with other proteins and no regulation of their activity has been described (14–16). Up until now, much of the focus has been on β-thymosin in neutrophils and platelets that contain sufficient β-thymosins to sequester the bulk of G-actin (15, 16). The contributions of other monomer-binding proteins are therefore ignored. However, in many other types of cells, β-thymosin does not predominate, and its contribution has to be considered in the context of the other G-actin-binding proteins.

In this paper, we examined the fundamental assumption that β-thymosins behave like a simple actin monomer-buffering protein in the intracellular milieu. β-Thymosin was overexpressed to a moderate level by cDNA mediated transfection, and the consequences on the polymer/monomer equilibrium were examined. Previous overexpression studies (17–19) demonstrated that swamping the cell with a large excess of β-thymosin causes extensive actin disassembly, but the cortical filaments are surprisingly resistant and the relation between β-thymosin dose and depolymerization response is not analyzed quantitatively. We find that a 3-fold increase in the predominant β-thymosin isoform (Tβ10) in NIH3T3 cells enhanced cell spreading, chemotaxis, and wound healing. It decreased the G-actin pool, contrary to the expectation for an exclusively buffering function. The paradoxical result may be explained by our recent in vitro studies, which show that thymosin β4 (Tβ4), an isoform that is functionally identical to Tβ10 (14), binds actin filaments with low affinity and decreases the Cc (47). This reduces the impact of β-thymosin overexpres-

Received for publication, October 17, 1995, and in revised form, January 8, 1996.

The actin cytoskeleton of nonmuscle cells responds to extracellular stimuli through a spatially and temporally regulated series of polymerization and depolymerization reactions. Monomer binding proteins, such as β-thymosin, ADF/cofilin, and profilin, buffer actin monomers (G-actin) to prevent them from polymerizing spontaneously (reviewed in Refs. 1 and 2). During cell activation, polymerization is initiated by filament uncapping at the cell cortex, which lowers the critical concentration for actin polymerization (1, 3–6). Actin monomer binding proteins amplify the effect of filament uncapping because they create a reservoir of G-actin that can be desquestered to supply actin to filament ends. Furthermore, because profilin-actin complexes facilitate barbed end filament growth (7) and profilin competes with β-thymosin for actin, profilin can tap into the β-thromosin-actin pool (7–9) to fuel massive polymerization. After polymerization, actin filaments are transported centripetally (10, 11) and subsequently depolymerize (12, 13). The newly released actin subunits are captured by monomer-binding proteins and recycled toward the cell front.

Among the monomer-binding proteins identified thus far, β-thymosins appear to best fulfill the criteria for a simple monomer-buffering protein because no interaction with other proteins and no regulation of their activity has been described (14–16). Up until now, much of the focus has been on β-thymosin in neutrophils and platelets that contain sufficient β-thymosins to sequester the bulk of G-actin (15, 16). The contributions of other monomer-binding proteins are therefore ignored. However, in many other types of cells, β-thymosin does not predominate, and its contribution has to be considered in the context of the other G-actin-binding proteins.

In this paper, we examined the fundamental assumption that β-thymosins behave like a simple actin monomer-buffering protein in the intracellular milieu. β-Thymosin was overexpressed to a moderate level by cDNA mediated transfection, and the consequences on the polymer/monomer equilibrium were examined. Previous overexpression studies (17–19) demonstrated that swamping the cell with a large excess of β-thymosin causes extensive actin disassembly, but the cortical filaments are surprisingly resistant and the relation between β-thymosin dose and depolymerization response is not analyzed quantitatively. We find that a 3-fold increase in the predominant β-thymosin isoform (Tβ10) in NIH3T3 cells enhanced cell spreading, chemotaxis, and wound healing. It decreased the G-actin pool, contrary to the expectation for an exclusively buffering function. The paradoxical result may be explained by our recent in vitro studies, which show that thymosin β4 (Tβ4), an isoform that is functionally identical to Tβ10 (14), binds actin filaments with low affinity and decreases the Cc (47). This reduces the impact of β-thymosin overexpres-

*This work was supported by National Institutes of Health Grants GM51112 and NS31430. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Physiology, U.T. Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9040. Tel.: 214-648-7967; Fax: 214-648-8685; E-mail: hyin@mednet.swmed.edu.

1The abbreviations used are: ADF, actin depolymerizing factor; Tβ10, thymosin β10; Tβ4, thymosin β4; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; Ctrl, control; HPLC, high pressure liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid.
sion and has repercussions for all the other monomer-binding proteins in the intracellular milieu as well.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**

The rat Tβ10 cDNA (17) was inserted through the HindIII and BamHI sites into LK588, a β-actin promoter driven expression vector that contains a neomycin resistance gene (21). NIH3T3 cells maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) were transfected by the Lipofectin protocol (Life Technologies, Inc.) and selected with G418 as described previously (22). Control clones (Ctrl) were transfected with vector containing no Tβ10 insert.

For some experiments, NIH3T3 cells were starved in DMEM/0.2% serum for 24 h and in a completely defined 1:1 DMEM/Ham’s F-12 medium supplemented with 20 mM Hepes, pH 7.4, 0.5 mg/ml bovine serum albumin, 1 μg/ml insulin, and 1 μg/ml transferrin (Sigma) (Q-medium) for an additional 18 h.

**Quantitative Immunoblotting**

Rabbit anti-Tβ10 and anti-Tβ4 antibodies were generated by immunizing against synthetic peptides corresponding to the 11 carboxyl-terminal amino acids of each protein as described (23). Their isoform specificity was established previously (14, 17, 23). Monoclonal anti-actin (clone C4) was obtained from Boehringer Mannheim, and rabbit anti-bovine spleen profilin (24) and anti-mouse gelsolin (25) were generated in this laboratory. Rabbit anti-chicken ADF (26) and monoclonal anti-chicken cofilin (MAB22) (27) were gifts from J. Bamburg (Colorado State University) and T. Obinata (Chiba University, Japan), respectively.

For β-thymosin detection, cells were trypsinized from the monolayers and washed in phosphate-buffered saline. They were resuspended in a buffer containing 100 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 1.8 mM CaCl₂, 50 mM KCl, 2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin and sonicated with a microtip probe for 30 s at 4°C. An aliquot was removed for protein quantitation by the micro-BCA method (Pierce), and the remainder was centrifuged at 15,000 rpm for 10 min in an Eppendorf microfuge. The supernatant was boiled in SDS gel sample buffer and electrophoresed in 5–20% gradient SDS-polyacrylamide gels. Western blotting was performed as described previously (17), using glutaraldehyde to cross-link β-thymosin in the acrylamide gel prior to electrophoretic transfer to enhance retention on the nitrocellulose membrane. Greater than 99% of β-thymosin was recovered in the supernatant.

For detection of proteins other than β-thymosin, cells were lysed in cold RIPA buffer (50 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 50 mM Hepes, pH 7.4) containing the protease inhibitor mixture. Polypeptides in SDS gels were transferred to nitrocellulose without glutaraldehyde fixation (28).

Semi-quantitative bands were visualized with the ECL detection system (Amersham Corp.). The intensity of the stained bands was determined by scanning with a Molecular Dynamics 300A computing densitometer. Serial dilutions of the cell lysates were analyzed, and samples within the linear range were compared with actin or actin-binding protein standards to estimate their content. Purified recombinant rat Tβ4 and Tβ10 concentrations were determined by amino acid analyses. Bovine spleen profilin, rabbit muscle actin, human plasma gelsolin, recombinant chicken ADF, and chicken cofilin (gifts of J. Bamburg) were prepared by standard methods, and their protein concentrations were determined by the micro-BCA method.

**Analytical HPLC**

The amount of β-thymosins in cells was determined by reverse phase HPLC of the pericholic acid-soluble cell extract (16). 25–67 μl samples were analyzed using a 5-μm C18 column (2.1 × 150 mm) and an ISCO chromatograph. Solvent A was 10 mM ammonium acetate, pH 6.0, solvent B was acetonitrile, and the resolving gradient was from 12–20% B in 5 min. (19) The β-thymosin concentrations determined from the integrated area of the peaks, calibrated against a known amount of pure Tβ4.

**Northern Blotting**

Total mouse cell RNA prepared as described in Ref. 29 was electrophoresed, blotted onto GeneScreen Plus (DuPont NEN), and hybridized with Tβ10 or Tβ4 probes that were labeled by random priming, in 50% formamide at 42°C. The Tβ10 probe encompassed the entire 444 base pairs of the full-length rat Tβ10 cDNA (14). The Tβ4 probe was derived from a mouse genomic clone that encompassed 331 base pairs of the Tβ4 mRNA, including 33 base pairs of translated sequence at the COOH terminus of Tβ4 and the contiguous 3′-untranslated sequence (30).

**Fluorescence Microscopy and Image Processing**

NIH3T3 cells were fixed with 3% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM MgCl₂, pH 6.9) for 30 min, permeabilized with ice-cold 0.1% Triton X-100 for 7 min. They were labeled with phalloidin conjugated with rhodamine (Molecular Probes, Eugene, OR) for 1 h in PHEM buffer containing 1% goat serum globulins. Coverslips were mounted in moldov containing 2.5% DABCO to retard photobleaching.

Fluorescence images were acquired with a cooled CCD camera (Photometrics, Tuscon, AZ) on an Axiosvert 135 microscope (Carl Zeiss Instruments, Thornwood, NY) using 63× or 100× oil immersion Plan-Neofluar objectives.

**Morphometric Analysis**

**Diameter—**Cells were trypsinized, washed, and suspended at 3 × 10⁵/ml in DMEM without serum, applied to coverslips, and mounted onto a microscope stage at 18°C. They were viewed using a 32× LD Ph1 Acrosimat lens with an Axiosvert 135 microscope within 30 min of plating to minimize cell spreading. Images were digitized using a camera (Dage-MTI, Michigan City, IN) and a Perceptics Pixel Pipeline video acquisition board (Perceptics, Knoxville, TN) in a Mac IIx. NIH-Image was used for morphometric analysis. Three separate experiments were performed, and the diameters of more than 80 cells were measured for each cell line. Cells that were not round were excluded. Mean values of the cell diameter were used to calculate the cell volume with the formula for a sphere.

**Nucleus—**The diameters of nuclei were measured from images of cells that were plated for several hours in the presence of serum and were still approximately spherical. The cells were stained with rhodamine phalloidin, which labeled the cytoplasm but not the nucleus. The nuclear volume was subtracted from the cell volume determined in suspension to give the cytoplasmic volume. A cytoplasmic volume of 48 ± 3% (n = 20) cell volume was obtained.

**Length and Width—**Cells were plated on glass coverslips at low density in the presence of 10% FCS for 24 h. Dimensions were measured in images captured in the BioRad confocal microscope.

**Quantitation of Actin Filament Content**

Actin filament content was determined using two methods. First, bulk actin filament content in cell monolayers was determined by measuring rhodamine-phalloidin binding in a fluorimeter as described in Refs. 24 and 31. Monolayers were fixed with 4% paraformaldehyde in phosphate-buffered saline containing 2 mM EGTA, and 1 μg/ml rhodamine-phalloidin was added and permeabilized with 0.1% Triton X-100. They were labeled with 0.7 μg rhodamine-phalloidin for 1 h at room temperature. Bound phalloidin was extracted with methanol, and fluorescence was measured at excitation/emission wavelengths of 540/570 nm and normalized against total protein content in parallel coverslips. Nonspecific binding was determined by adding 10-fold excess unlabelled phalloidin and was found to be less than 5% of total binding. The assay was within the linear range of the binding curve, determined by varying the concentration of cells used. Experiments were performed in duplicate. Second, rhodamine-phalloidin binding to individual cells was quantitated by image processing. Digital fluorescence images, acquired under identical non saturating optical conditions (constant gain and background correction), were analyzed with the BDS Image Program (22, 24). Following the elimination of background subtraction, contours of cells were carefully delineated, and the area was recorded. Integrated fluorescence intensities were determined. Cells were chosen randomly from different fields and TK and Ctrl cells were analyzed in a double-blind fashion.

**Cell Spreading Assay**

Cells were trypsinized and plated in 10% FCS/DMEM on coverslips that had been previously exposed to 10% FCS for 24 h. At timed intervals, coverslips were fixed, permeabilized, and stained with rhodamine-phalloidin. Adherent cells with irregular outlines were counted and expressed as the percentage of total adherent cells. TK and Ctrl samples were randomized for double-blind analysis.

**Chemoaxis**

5 × 10⁴ cells (in 50 μl) were loaded in the top wells of a modified 48-well Boyden chamber (Nuncopore, Pleasanton, CA) and 28 μl of medium containing 10% FCS was added to the bottom wells (22). The
Fig. 1. Analysis of Tβ10 overexpression. A, Western blot. Tβ10 antibody was used to probe NIH3T3 cells transfected with Ctrl or Tβ10 expression vector. 15 μg of cell extracts were analyzed. B, Western blot. Tβ4 antibody was used to probe purified Tβ4 standards, CV1 and NIH3T3 cells. Lanes 1-3, 7, 14, 28 ng of Tβ4; lanes 4 and 5, 1.8 and 3.8 μg of CV1 extract; lanes 6 and 7, 15 μg of Ctrl and TK9 extracts. The streak between lanes 5 and 6 was due to gel loading problems and should be ignored. C, analytical HPLC. Equal amount of lysate samples were analyzed. The arrow indicates the peak that is not Tβ4. The TK9 tracing was more compressed relative to the others. D, Northern blot. Right panel, Tβ10 cDNA probe was used to probe U937, TK9, and Ctrl RNA. Left panel, Tβ4 probe. 10 μg of total RNA was analyzed per lane.

Wound Healing Assay

The rate of migration of cells to cover a “wound” made by scratching an approximately 1-mm line in a confluent monolayer was measured as described (22).

RESULTS

Stable Overexpression of Tβ10—After NIH3T3 cells were transfected with a Tβ10-expression vector containing a neo gene and selected with G418, many neomycin-resistant cell lines were obtained. Western blotting with a Tβ10-specific antibody showed that there was a range of Tβ10 overexpression (Fig. 1A). Clones TK4 and TK9 had 1.8- and 2.9-fold more Tβ10 than cells transfected with the control vector (Ctrl) (Table I). In contrast, a Tβ4-specific antibody that detected purified Tβ4 and Tβ14 in CV1 cells was not positive against Ctrl or TK9 cells (Fig. 1B) even when four times more cell proteins were loaded.

Tβ10 overexpression and the predominance of endogenous Tβ10 over Tβ4 were confirmed by analytical HPLC (Fig. 1C). CV1, which contains abundant Tβ4 and Tβ10 (17), was used as a standard for identifying the thymosin isoforms in the HPLC profiles. Two major peaks that coincided with purified Tβ4 and Tβ10 standards were present at a 1.7:1 ratio. Ctrl NIH3T3 cells had a small Tβ10 peak and no peak coinciding with the Tβ4 standard. The neighboring peak (indicated by an arrow), which overlapped slightly with the tail end of the bona fide Tβ4 peak, did not shift the mobility of actin on nondenaturing gels (data not shown) and was unlikely to be Tβ4. TK4 and TK9 had progressively more Tβ10 than Ctrl cells. The extent of overexpression, based on the integrated area under the peaks, were 1.6- and 2.7-fold, respectively (Table I). These values were similar to Western blot estimates.

The abundance of Tβ10 relative to Tβ4 in Ctrl cells and the overexpression of Tβ10 in TK cells were also evident at the mRNA level. In Northern blots, the Tβ10-specific probe hybridized strongly with Ctrl cells and more strongly with the Tβ10-transfected clone TK9 (Fig. 1D). Only one positive band was detected in the overexpressing cells, because the Tβ10 overexpression construct was approximately the same size as the Tβ10 mRNA. In contrast, the Tβ4-specific probe gave minimal signal for either clone, even though it hybridized strongly to U937, a mouse macrophage cell line. These results demonstrated that the Tβ10 and Tβ4 probes were specific for each mRNA species and Tβ10 was the dominant isoform in the NIH3T3 line used in these studies.

Actin Monomer Sequestering Protein Content—Previously published data suggest that β-thymosins are passive actin sequestering proteins that bind monomers as governed by mass action (7, 15, 16, 32). If this were the case, the amount of actin bound to Tβ10 should increase with Tβ10 overexpression. The percentage of increase in the unpolymerized actin pool (A₀) depends on the Tβ10 content relative to that of other G-actin sequestering proteins and the concentration of free G-actin at steady state (critical concentration, Cₛ), which is itself regulated by filament capping (7, 9, 33). The value of A₀ is determined by the following equation:
somewhat. Thus, the upper limit for unpolymerized actin was 20.9\,\text{	extmu}M. The amount of T\beta10-G-actin complex [TA] can be calculated as follows:

\[
[A_d] = \frac{[C_i] + i[S_o]}{[C_i] + K_d} \quad \text{(Eq. 1)}
\]

where $S_{iO}$ is the concentration of G-actin-binding proteins $S_i$ and $K_d$ is the equilibrium dissociation constant for the binding of $S_i$ to G-actin.

T\beta10 and actin accounted for 0.06 ± 0.007 and 2.8 ± 0.1\% (average and range) of total cell proteins in Ctrl cells, respectively (Table I). Based on the protein content per cell and cell diameter (Table III), the cytoplasmic concentrations of T\beta10 and actin in Ctrl cells were 8.8 and 44.9\,\text{	extmu}M, respectively. The unpolymerized actin pool, estimated from the amount of actin that was Triton X-100 soluble after a 10-min centrifugation at 14,000 rpm in an Eppendorf microfuge, was 46.6 ± 5.2\% (mean ± S.E., $n = 5$) of the total actin. Because actin filaments that contain fewer than 50 monomers and are not cross-linked to the cytoskeleton will not pellet under these conditions (34), the figure for unpolymerized actin is likely to be overestimated somewhat. Thus, the upper limit for unpolymerized actin was 20.9\,\text{	extmu}M.

The amount of T\beta10 overexpression on actin filament content and cell motility

| Cell line | F-dil T\beta10 overexpression | F-actin content | Motility |
|-----------|-------------------------------|-----------------|----------|
|           | Western blotting ($n = 4$)     | HPLC* ($n = 9$, p < 0.05) | Chemotaxis | Migration |
| Ctrl      | 1                             | 1               | 45.7 ± 6.6 ($n = 13$) | 16.4 ± 1.2 ($n = 4$) |
| TK4       | 1.8 ± 0.03                    | 1.6             | 173.4 ± 34.8 ($n = 10$, p < 0.01) | 20.2 ± 0.6 ($n = 5$, p < 0.05) |
| TK9       | 2.9 ± 0.04                    | 2.7             | 268.5 ± 36.7 ($n = 10$, p < 0.001) | 23.7 ± 0.8 ($n = 4$, p < 0.01) |

* Results from one of two experiments.

\[
[T_{o}] = \frac{[C_o] + [S_o]}{[C_i] + K_{AT}} \quad \text{(Eq. 2)}
\]

where $[T_{o}]$ is the T\beta10 concentration, $K_{AT}$ is the equilibrium dissociation constant for the TA complex. Using values of 0.6\,\text{	extmu}M for $K_{AT}$ (14, 32) and 0.5\,\text{	extmu}M for $C_i$ (when barbed ends of actin filaments are capped as would be expected in "resting" cells), 8.8\,\text{	extmu}M T\beta10 would bind 4\,\text{	extmu}M actin monomers (Table II). Assuming that the other actin monomer-binding proteins were maximally active and that each bound a single actin in a mutually exclusive manner (8), the total actin sequestered maximally active and that each bound a single actin in a mutually exclusive manner (8), the total actin sequestered therefore (25), serum-deprived Ctrl fibroblasts were flatter and had thinner stress fibers than cells maintained in serum (Fig. 2B). In contrast, TK cells continued to have thick stress fibers (Fig. 2D), although there was a reduction in the number of cell processes, as would be consistent with a more quiescent state.

Cell Spreading—When fully spread, TK9 had 1.3-fold more surface area than Ctrl cells (Table III). Because they had similar diameters (and hence volumes) when in suspension and similar protein content per cell as well, the increase in TK surface area after attachment was most likely due to increased cell spreading.

This was confirmed directly from the rate of spreading after reseeding. Within 0.5 h of plating, TK cells were more spread than Ctrl cells (Fig. 3), and the cell margins were highly stained with phalloidin. Cell spreading was quantitated by tallying the number of adherent cells that did not have a round shape. TK9 had almost twice as many spread cells as Ctrl at 0.7, 1, and 1.5 h after plating (Fig. 4B). After 2 h, TK and Ctrl had comparable numbers of spread cells. TK4 spread at a rate intermediate between that of TK9 and Ctrl. Direct quantitation of phalloidin binding showed that at zero time, TK9 cells in suspension had 1.19 ± 0.05 times (n = 6; p < 0.05) more polymerized actin than Ctrl. One hour after plating, TK had 1.25 ± 0.06 times (n = 6; p < 0.05) more polymerized actin than Ctrl (Fig. 4A). Therefore, TK cells in suspension had more polymerized actin than Ctrl, and the difference was maintained during spreading.

Chemotaxis and Wound Healing—TK cells had increased chemotactic ability compared with Ctrl cells (Table I). The difference between pooled data from multiple experiments was statistically significant, with confidence intervals (p) of less than 0.01–0.001.

Likewise, motility as measured by the rate of migration of cells into monolayer wounds was also significantly higher in TK than Ctrl. The extent of increase was not as high as in chemotaxis.

**DISCUSSION**

The actin monomer pool in cells is an important component of the actin machinery (1, 9), and \beta-thymosins are the currently favored candidates for maintaining actin in the reserve pool (2,
35). In this paper, we find that Tβ10 does not behave like a simple G-actin buffering agent in vivo. Raising Tβ10 concentration to a modest extent in NIH3T3 fibroblasts unexpectedly promotes filament assembly. Consistent with an increase in F-actin, the cells are more motile in a variety of actin-based activity, including spreading, chemotaxis, and wound healing. This phenotype is observed with several overexpressing cell lines, confirming that it is a direct consequence of overexpression and not due to clonal variations or artefacts of DNA integration. Enhancement of actin polymerization is contrary to what is expected for an increase in monomer buffering capacity and suggests that additional factors are involved. A discrepancy between β-thymosin level and actin monomer pool size has also been reported by another group in a preliminary form (35, 36).

Comparing the β-Thymosin Overexpression Phenotype to That of Profilin, Gelsolin, and CapG—Cells overexpressing β-thymosin are more motile in each of the three broad categories of actin-based forward movement, a classification proposed recently (37). These include: first, lamellipodial extension, which is primarily driven by the polymerization of actin monomers at the cell edge (chemotaxis and membrane ruffling are good examples); second, forward movement of the cell edge driven by actomyosin contraction, as seen in wound healing; and third, postmitotic cell spreading, which is driven by myosin transporting dorsal actin filaments forward against anchored ventral filaments. Spreading after replating is morphologically similar to postmitotic spreading and may therefore use a similar mechanism. We find that β-thymosin overexpression enhanced all three types of motility, suggesting that the phenotype is likely to be a manifestation of a fundamental change in the actin machinery.

Overexpression of profilin by cDNA-mediated transfection in tissue culture cells also increases F-actin content (38). However, unlike β-thymosin overexpression, this is the result of a preferential increase in filaments in the dynamic cell cortex accompanied by disassembly of stress fibers. We do not observe a redistribution of actin filaments between these two compartments in the β-thymosin overexpressing cells, implicating a different mechanism for raising F-actin concentration.

The β-thymosin overexpression phenotype is also superficially similar to that of CapG (22) and gelsolin (39) overexpression. These proteins are filament barbed end capping proteins that are regulated by Ca\(^{2+}\) and polyphosphoinositides (40). In addition, gelsolin also severs actin filaments and binds monomers (41). When they are overexpressed using the same vector backbone and in the same parent NIH3T3 cell line as employed in the present study, the cells are more active in chemotaxis and wound healing. However, there is no increase in actin filament content and cell spreading. Instead, increased motility is associated with enhanced polyphosphoinositide signaling. Parallel studies with TK cells did not show an increase in polyphosphoinositide metabolism,\(^2\) again suggesting that although the motile phenotypes are superficially similar, different mechanisms are involved in increasing cell motility. In the

\(^2\) H. Q. Sun and H. L. Yin, unpublished results.
case of β-thymosin overexpression, an increase in F-actin content can best explain the phenotypic changes.

How Can Overexpression of Tβ10 Increase the Amount of F-Actin in Cells?—We find that raising Tβ10 concentration 3-fold increased the F-actin content by 30% without changing the amount of actin and the other major actin monomer-binding proteins assayed. This rules out the obvious explanation of compensatory adjustments in the expression of other proteins to overcome the effect of excessive monomer sequestration by Tβ10. Previous studies have also shown that this mechanism is not generally invoked (22, 38, 39, 42).

Because the amount of unpolymerized actin (A_u) is also dictated by the K_d of the binding protein-actin complex and the C_c, these variables will be considered. The K_d of the regulatory protein for actin can be altered by the nucleotide bound state of the actin monomer as well as the activation state of the binding protein. Among the proteins examined, β-thymosin is particularly sensitive to the nucleotide content of actin (4), but β-thymosin itself is not known to be regulated directly by other effectors. Its K_d for ATP-actin is 50-fold less than ADP-actin, so a shift from ATP-actin to ADP-actin in TK cells will render Tβ10 inactive. However, there is no evidence for a large pool of actin damped in the ADP-bound form in cells (43). Furthermore, ADP-actin will increase the C_c and cannot explain the increase in F-actin in TK cells. On the other hand, inactivation of other monomer-binding proteins by increasing their K_d for actin is possible, because each of the proteins studied (except for β-thymosin) can be regulated by physiologically relevant signals or post-translational modifications. These include pH (44, 45) and phosphorylation for cofilin and ADF (26), Ca^{2+} and phospholipids for gelsolin (41, 46), and phospholipids for profilin (20). However, because each class of proteins is only present at approximately equimolar ratio to the endogenous β-thymosin and a portion of it is probably already in the inactive state (e.g. 38% of the ADF in NIH3T3 is phosphorylated and therefore inactive (26)), more than one class of actin monomer-binding proteins will have to be inactivated simultaneously. This possibility is beyond the scope of the present paper and cannot be addressed definitively. A major problem is that it is difficult to directly ascertain the intracellular activation states of these proteins because each is regulated by multiple signals and can bind both G- and F-actin. For example, although phosphorylation of ADF and cofilin can be assessed by two-dimen-
sional gel electrophoresis (26), the activity of the unphosphorylated proteins is not known, because they are further regulated by pH and can bind actin filaments as well.

The possibility that β-thymosin overexpression reduces the C_c is supported by our recent in vitro data with Tβ4 (47). We find that although Tβ4 behaves like a simple actin sequestrating protein at low concentration (<20 μM), it fails to depolymerize actin as efficiently as expected at higher concentrations. Sedimentation and chemical cross-linking show that Tβ4 binds actin filaments with low affinity and substoichiometric binding induces a dose-dependent increase in C_c. Electron microscopy shows that the filaments are twisted around each other into bundles. This activity of β-thymosin was not noticed previously because relatively low concentrations of β-thymosin were used.

Lowering the C_c mitigates the impact of Tβ10 overexpression and reduces the effectiveness of the other monomer-binding proteins as well. Assuming from the in vitro data (47) that 26 μM Tβ10 decreases the C_c from 0.5 to 0.2 μM, total sequestered actin will drop from 23.2 to 14.3 μM (Table I). The unpolymerized actin pool in TK9 will be 9.9% smaller than Ctrl, in spite of a 3-fold increase in Tβ10. Thus, our in vitro observation that raising intracellular β-thymosin decreases C_c can, in theory, account for our in vivo results quantitatively. Likewise, Tβ4 overexpression in another NIH3T3 cell line also does not increase the actin monomer pool (35, 36). However, unlike our results, the actin monomer pool is not decreased, possibly because the cell line used has a different monomer binding profile compared with our cells. Nonetheless, the important conclusion is that an increase in β-thymosin does not necessarily result in a proportionate increase in actin monomer content in a complex environment containing different types of actin monomer-binding proteins.

The actin stress fibers in TK cells appeared thicker, suggesting that they may be bundled. The finding that even TK cells in suspension had more F-actin than Ctrl is consistent with the possibility that the C_c is decreased. Cells in suspension do not have extrinsic cues generated by substrate attachment, favoring a mechanism that alters an intrinsic property of the actin machinery itself. Although our preliminary immunofluorescence studies did not show colocalization of Tβ10 with phal-
loidin-stained actin filaments, low affinity binding of thymosin to actin filaments is a viable mechanism because β-thymosin binds F-actin substoichiometrically and therefore may not be present in sufficient quantity to be detected.

The Physiological Implications of Our Findings—Our results show that a small increase in β-thymosin produces a distinct motile phenotype in NIH 3T3 cells that have moderate levels of endogenous β-thymosin. The concentrations of β-thymosin attained in these cells after cDNA-mediated overexpression are comparable with and in some cases considerably lower than what is observed in a number of cell types. Furthermore, many cells are able to regulate β-thymosin expression, which fluctuates according to their developmental and/or activation state (reviewed in Ref. 35). It was assumed that an increase in β-thymosin level results in an expansion of the monomer pool to meet the demands for altered polymerization dynamics. Our current result would suggest that β-thymosin can self-regulate its ability to sequester G-actin, and its biphasic action must be considered in models for the regulation of the actin cycle. Further experiments will be required to determine whether additional mechanisms such as compensatory inactivation of other actin monomer sequestering proteins may also be involved.

Acknowledgment—We thank Daniel Safer (University of Pennsylvania) for performing the analytical HPLC measurements.

REFERENCES
1. Fedchheimer, M., and Zigmond, S. H. (1993) J. Cell Biol. 123, 1–5
2. Sun, H.-Q., Kwiatkowska, K., and Yin, H. L. (1995) Curr. Opin. Cell Biol. 7, 102–110
3. Stossel, T. P. (1993) Science 260, 1086–1094
4. Carlier, M.-F., J. ean, C., Rieger, K. J., and Lenfant, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5034–5038
5. Schafer, A. D. A., and Cooper, J. A. (1995) Annu. Rev. Cell Dev. Biol. 11, 497–518
6. Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janney, P. A., Taylor, L. A., Toker, A., and Stossel, T. P. (1995) Cell 82, 643–653
7. Pantaloni, D., and Carlier, M.-F. (1993) Cell 75, 1007–1014
8. Goldschmidt-Clermont, P. J., Furman, M. I., Wachsstock, D., Safer, D., Nachmis, V. T., and Pollard, T. D. (1992) Mol. Biol. Cell 3, 1015–1024
9. Carlier, M.-F., and Pantaloni, D. (1994) Semin. Cell Biol. 5, 183–191
10. Fisher, G. W., Conrad, P. A., DeBiasio, R. L., and Taylor, D. L. (1988) Cell Motil. Cytoskeleton 11, 235–247
11. Heath, J. P., and Hollier, B. F. (1991) Cell Motil. Cytoskeleton 18, 245–257
12. Theriot, J. A., and Mitchison, T. J. (1991) Nature 352, 126–131
13. Zigmond, S. H. (1993) Cell Motil. Cytoskeleton 25, 309–316
14. Yu, F.-X., Lin, S.-C., Morrison-Bogorad, M., Atkinson, M. A. L., and Yin, H. L. (1993) J. Biol. Chem. 268, 502–509
15. Nachmis, V. T., Cassimeris, L., Golli, R., and Safer, D. (1993) Eur. J. Cell Biol. 61, 314–320
16. Cassimeris, L., Safer, D., Nachmis, V. T., and Zigmond, S. H. (1992) J. Cell Biol. 119, 1261–1270
17. Yu, F., Lin, S., Morrison-Bogorad, M., and Yin, H. L. (1994) Cell Motil. Cytoskeleton 27, 13–25
18. Sanders, M. C., Goldstein, A. L., and Wang, Y.-L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4678–4682
19. Sanger, J. M., Golla, R., Safer, D., Cho, J. K., Yu, K., Sanger, J. W., and Nachmis, V. T. (1995) Cell Motil. Cytoskeleton 31, 307–322
20. Lassing, I., and Lindberg, U. (1985) Nature 314, 472–474
21. Gunning, P., Leavitt, J., Mucsi, G., Ng, S. Y., and Kedes, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4831–4835
22. Sun, H.-Q., Kwiatkowska, K., Wooten, D. C., and Yin, H. L. (1995) Cell Biol. 129, 147–156
23. Lin, S. C., and Morrison-Bogorad, M. (1991) J. Biol. Chem. 266, 23347–23353
24. Muilem, S., Kwiatkowska, K., Xu, X., and Yin, H. L. (1995) J. Cell Biol. 128, 589–598
25. Onoda, K., Yu, F.-X., and Yin, H. L. (1993) Cell Motil. Cytoskeleton 26, 227–238
26. Morgan, T. E., Lockerbie, R. O., Minamide, L. S., Browning, M. D., and Bamberg, J. R. (1993) Cell Biol. 122, 623–633
27. Abe, H., and Obinata, T. (1989) J. Cell Biol. 128, 172–180
28. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4355
29. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
30. Gunning, P., Leavitt, J., Mucsi, G., Ng, S. Y., and Kedes, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4831–4835
31. Marchand, J.-B., Moreau, P., Paulet, C., Cossart, P., Carlier, M.-F., and

3. K. Kwiatkowska and H. L. Yin, unpublished results.
Actin Monomer Polymer Equilibrium/ β-Thymosin Overexpression

Pantaloni, D. (1995) J. Cell Biol. 130, 331–343
34. Cano, M. L., Cassimeris, L., Joyce, M., and Zigmond, S. H. (1992) Cell Motil. Cytoskeleton 21, 147–158
35. Safer, D., and Nachmias, V. T. (1994) Bioessays 16, 473–479
36. Golla, R., Safer, D., Sanger, J. M., Sanger, J. W., Choi, J., Yu, K., Holtzer, H., Holtzer, S., Philip, N., and Nachmias, V. T. (1994) Mol. Biol. Cell 4, 383 (abstr.)
37. Cramer, L. P., and Mitchison, T. J. (1995) J. Cell Biol. 131, 179–189
38. Finkel, T., Theriot, J. A., Dise, K. R., Tomaselli, G. F., and Goldschmidt-Clermont, P. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1510–1514
39. Cunningham, C. C., Stossel, T. P., and Kwiatkowski, D. J. (1995) Science 259, 1233–1236
40. Yu, F.-X., Johnston, P. A., Sudhof, T. C., and Yin, H. L. (1990) Science 250, 1413–1415
41. Yin, H. L., and Stossel, T. P. (1980) J. Biol. Chem. 255, 9490–9493
42. Babcock, G., and Rubenstein, P. A. (1993) Cell Motil. Cytoskeleton 24, 179–188
43. Rosenblatt, J., Peluso, P., and Mitchison, T. J. (1995) Mol. Biol. Cell 6, 227–236
44. Hayden, S. M., Miller, P. S., Brauweiler, A., and Bamberg, J. R. (1993) Biochemistry 32, 9994–10004
45. Hawkins, M., Pope, B., Maciver, S. K., and Weeds, A. G. (1993) Biochemistry 32, 9985–9993
46. Janney, P. A., and Stossel, T. P. (1987) Nature 325, 362–364
47. Carlier, M.-F., Didry, D., Erk, I., Lepault, J., Van Troys, M. L., Vanderkooi, J.-P., Pérez-Roths, I., Yin, H., Doi, Y., and Pantaloni, D. (1996) J. Biol. Chem. 271, 9231–9239
-Thymosins Are Not Simple Actin Monomer Buffering Proteins: INSIGHTS FROM OVEREXPRESSION STUDIES
Hui-Qiao Sun, Katarzyna Kwiatkowska and Helen Lu Yin

J. Biol. Chem. 1996, 271:9223-9230.
doi: 10.1074/jbc.271.16.9223

Access the most updated version of this article at http://www.jbc.org/content/271/16/9223

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 22 of which can be accessed free at http://www.jbc.org/content/271/16/9223.full.html#ref-list-1