Tropomodulin Caps the Pointed Ends of Actin Filaments

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Abstract. Many proteins have been shown to cap the fast growing (barbed) ends of actin filaments, but none have been shown to block elongation and depolymerization at the slow growing (pointed) filament ends. Tropomodulin is a tropomyosin-binding protein originally isolated from red blood cells that has been localized by immunofluorescence staining to a site at or near the pointed ends of skeletal muscle thin filaments (Fowler, V. M., M. A., Sussman, P. G. Miller, B. E. Flucher, and M. P. Daniels. 1993. *J. Cell Biol.* 120: 411–420). Our experiments demonstrate that tropomodulin in conjunction with tropomyosin is a pointed end capping protein: it completely blocks both elongation and depolymerization at the pointed ends of tropomyosin-containing actin filaments in concentrations stoichiometric to the concentration of filament ends ($K_d \leqslant 1$ nM). In the absence of tropomyosin, tropomodulin acts as a "leaky" cap, partially inhibiting elongation and depolymerization at the pointed filament ends (K_d for inhibition of elongation = 0.1–0.4 μM). Thus, tropomodulin can bind directly to actin at the pointed filament end. Tropomodulin also doubles the critical concentration at the pointed ends of pure actin filaments without affecting either the rate or extent of polymerization at the barbed filament ends, indicating that tropomodulin does not sequester actin monomers. Our experiments provide direct biochemical evidence that tropomodulin binds to both the terminal tropomyosin and actin molecules at the pointed filament end, and is the long sought-after pointed end capping protein. We propose that tropomodulin plays a role in maintaining the narrow length distributions of the stable, tropomyosin-containing actin filaments in striated muscle and in red blood cells.

ACTIN assembly in muscle and nonmuscle cells is regulated in part by proteins that cap the fast growing (barbed) ends of actin filaments. All of the well-known capping proteins, gelsolin, villin, and capZ, block elongation and depolymerization at the barbed filament end, and they are also capable of nucleating actin polymerization (for reviews see Pollard and Cooper, 1986; Weeds and Maciver, 1993). These proteins play various roles in different cells at different times. For instance, capZ might provide nucleation sites in the Z band for thin filament formation during myofibril assembly in the development of muscle cells (Schafer et al., 1993). On the other hand, in some nonmuscle cells, capping proteins may play an important role in the sudden changes in the state of actin polymerization associated with stimulation of these cells (for a recent review, see Zigmond, 1993).

Considerably less is known about the molecules responsible for regulating actin filament assembly at the slow growing (pointed) end. Evidence for the existence of pointed end capping proteins in muscle and nonmuscle cells comes from several observations. First, there is no elongation at the pointed ends of actin filaments when G-actin is added to isolated myofibrils (Sanger et al., 1984; Peng and Fischman, 1991); furthermore, the obstacle to elongation can be removed by extraction with high salt (Ishiwata and Funatsu, 1985). Second, under some conditions, elongation at the pointed filament end of the short actin filaments in the red blood cell membrane skeleton is also blocked (Pinder et al., 1986). Finally, Tilney and colleagues (1992) have reported that there is no growth from the pointed ends of the actin filaments in the comet-like tail of *Listeria monocytogenes* in macrophages or from the pointed ends of the actin filaments in the mature hair cells of chicken cochlea (Tilney and DeRosier, 1986; Tilney et al., 1992). Depolymerization from the pointed ends of actin filaments is also blocked in muscle myofibrils since removal of monomeric actin by repeated washes of the myofibrils has no effect on the actin filaments (Perry, 1952; Weber, 1959). By itself, this observation does not necessarily indicate the presence of a capping protein since depolymerization from the pointed end can be blocked under some conditions by tropomyosin, which binds along the length of actin filaments (Broschat et al., 1989, 1990; Weigt et al., 1990). The only known pointed-end–capping protein that blocks elongation, but not depolymerization, is DNase I, an extracellular protein whose physiological interactions with actin have not yet been clearly defined (Podolski and Steck, 1988; Weber et al., 1994).

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Recently, tropomodulin has been localized to a site at or near the pointed ends of the thin filaments in rat psoas skeletal muscle by immunofluorescence staining of isolated myofibrils at resting and stretched lengths (Fowler et al., 1993). Tropomodulin is a 40-kD tropomyosin-binding protein that binds to one end of tropomyosin and was originally isolated from the red blood cell membrane skeleton (Fowler, 1987, 1990; Sung et al., 1992). The amino acid sequence of human red blood cell tropomodulin (Sung et al., 1992) is 86% identical to chicken skeletal muscle tropomodulin (Babcock and Fowler, 1994), indicating that tropomodulin is a highly conserved protein. In red blood cells, tropomodulin is associated with the short tropomyosin-containing actin filaments that form the "hubs" of the spectrin-actin lattice (Ursitti and Fowler, 1994). Estimations of relative stoichiometries in skeletal muscle and red cells suggest that there are between one and two tropomodulin molecules for each actin filament (Fowler et al., 1993). Tropomodulin is also found in the vicinity of the pointed ends of the thin filaments in cardiac muscle (Gregorio, C. C., and V. M. Fowler, unpublished observations) and is a component of the membrane skeleton in lens fiber cells (Woo and Fowler, 1994).

Here, we present evidence that tropomodulin in nanomolar concentrations completely blocks elongation and depolymerization at the pointed ends of tropomyosin-actin filaments. At higher concentrations, tropomodulin partially inhibits elongation and depolymerization at the pointed ends of pure actin filaments, demonstrating that tropomodulin can also bind directly to actin at the pointed filament end. We propose that pointed-end capping by tropomodulin helps to maintain the constant lengths of the tropomyosin-containing actin filaments in skeletal muscle and in the red cell membrane skeleton.

Materials and Methods

Proteins

Actin. Rabbit skeletal muscle actin was prepared from an acetone powder of rabbit muscle as previously described (Murray et al., 1981) with some modifications in the chromatography step (Young et al., 1990). Pyrenyl labeling of muscle actin was carried out according to Kouyama and Mihashi (1981) with the modifications described previously (Northrop et al., 1986). Actin was stored in liquid nitrogen and defrosted as previously described (Young et al., 1990). The critical concentration of various actin preparations varied between 0.08 and 0.15 μM for uncapped filaments and between 0.4 and 0.7 μM for filaments capped at their barbed ends with gelsolin.

Tropomodulin. Recombinant chicken skeletal muscle tropomodulin was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (GST)[1] and purified to homogeneity by adsorption to glutathione-Sepharose followed by thrombin cleavage to release the GST moiety and a final step of ion exchange chromatography, as described elsewhere (Babcock and Fowler, 1994). The recombinant tropomodulin obtained by this procedure contained a fusion peptide of 15 amino acids at the NH_2-terminus that was derived from the GST linker region (Guan and Dixon, 1991). In most experiments, unless otherwise indicated, the recombinant tropomodulin was also missing the first five amino acids at the NH_2-terminus. Comparisons with native tropomodulin purified from red blood cells (Fowler, 1990) show that tropomodulin and not the fusion peptide is responsible for the pointed-end capping effects. In the absence of tropomyosin, native tropomodulin inhibited the initial rate of elongation to a similar extent, as did recombinant short tropomodulin, and it also lowered the final extent of polymerization. In combination with tropomyosin, native tropomodulin was effective in very low concentrations comparable to the recombinant tropomodulin (data not shown). Furthermore, a comparison of the short- with the full-length recombinant tropomodulin preparations indicates that the first five amino terminal residues are not of major importance for tropomodulin function: both the short and long protein inhibit elongation of pure actin with a similar K_d, and both increase the critical concentration to about twice the control value (data not shown).

Other Proteins. Tropomyosin was purified from rabbit skeletal muscle as described by Bailey (1948). Gelsolin was a generous gift from J. Bryan (Baylor College of Medicine, Dallas, TX), and was prepared as described previously (Bryan, 1988). Spectrin-actin complexes, "crude spectrin," were prepared as described by Cohen and Branton (1979) and stored at −20°C in 50% ethylene glycol. Vitamin D–binding protein, which runs as single band on SDS gels, was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Protein concentrations were determined for actin and gelsolin by light absorption, using E_{290} = 24.9 $mM^{-1}\cdot cm^{-1}$, and E_{280} = 150 $mM^{-1}\cdot cm^{-1}$, respectively; for tropomodulin and tropomyosin by Lowry's method, using bovine serum albumin as a standard; and for vitamin D–binding protein, according to weight dissolved.

Actin Polymerization Measurements

Fluorescence and Kinetic Measurements. Changes in actin polymerization were calculated from fluorescence measurements of pyrenyl-actin (excitation = 366.5; emission = 407 nm) as indicated in the legends (usually ~10% pyrenyl-actin) (Weber et al., 1987*a*), using a PTI photon counting fluorimeter. Measurements were standardized against a Raman excitation peak (excitation = 357 nm; emission = 407 nm). Absolute readings from different experiments are not comparable because varying slit widths were used.

All experiments were carried out at 20°C with Mg-actin, the physiological form of actin (Weber et al., 1969; Kitasawa et al., 1982). The conversion to Mg-actin was carried out as previously described (Young et al., 1990).

Measurements of elongation and depolymerization rates at the pointed filament end were carried out as previously described (Walsh et al., 1984*a*,*b*; Northrop et al., 1986; Young et al., 1990) in a medium containing 10 mM imidazole buffer, pH 7.4, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM azide, 1 mM dithiothreitol, 0.5 mM ATP, 5.0 mM EGTA, and 10 nM gelsolin-actin dimers to ensure full capping of the barbed ends after dilution from the stock solution. During depolymerization measurements, 2 μM vitamin D–binding protein was also included to maintain the concentration of free G-actin at <10 nM throughout the whole time course. Vitamin D–binding protein only sequesters actin monomers and has no effect on depolymerization (Weber et al., 1994).

Endpoints of polymerization were measured after overnight incubation of actin in the presence of actin nuclei to ensure completion of polymerization. We used spectrin-actin complexes for the polymerization of uncapped actin with free barbed filament ends.

Known number concentrations of free pointed filament ends were obtained by copolymerizing actin (usually 10 μM) with gelsolin or gelsolin-actin dimers in the presence of calcium. These are strong barbed-end capping and nucleating proteins that produce one filament per gelsolin according to our measurements, as has been published previously for villin (Walsh et al., 1984*a*,*b*; Coleman and Mooseker, 1985; Northrop et al., 1986). Average sizes for filaments used in elongation and depolymerization experiments are given in the legends (*average length = number of monomers/actin filament*). For the preparation of tropomyosin-actin filaments used as nuclei for elongation or for depolymerization experiments, tropomyosin in excess over the amount necessary for filament saturation (excess of ~0.5 μM) and when present, tropomodulin (excess of 0.1–2.5 μM) were added before copolymerization was started by the addition of salt.

Known number concentrations of free barbed filament ends were obtained by using spectrin-actin complexes (Cohen and Branton, 1979). These were used after a fourfold dilution of the 50% ethylene glycol stock solution into 0.3 mM phosphate buffer, pH 7.6. The concentration of the barbed filament ends was determined by titration with gelsolin. Briefly, the spectrin-actin complexes were preincubated with increasing concentrations of gelsolin-actin dimers in polymerizing solution for ~1 h, and the extent of capping was evaluated from the extent of inhibition of elongation. Capping increases with increasing gelsolin-actin (in 5 mM EGTA) nearly stoichiometrically (K_d of gelsolin-actin for barbed filament ends = 0.1 nM; Selve and Wegner, 1986). Therefore, the concentration of barbed ends = 2× the concentration of gelsolin-actin required to inhibit elongation by 50%. In some assays, spectrin-actin complexes were preelongated with G-actin overnight before the elongation assays. For example, in the experiment shown in Fig. 7 *B*, 34 nM spectrin-actin complexes were incubated

1. *Abbreviation used in this paper*: GST, glutatione *S*-transferase.

overnight with 1.8 μM G-actin in polymerizing medium (average filament length = *53 monomers/filament*) in the presence or absence of 0.5 μM tropomyosin and 0.5 μM tropomodulin, if also present.

Calculations of Lowest Possible K_d for Tropomodulin Binding to Actin Monomers. If tropomodulin had a K_d of 100 μM for monomer binding, and the free monomer concentration in the presence of tropomodulin was 0.14 μM (critical concentration indicated by the control intercept with the G-actin slope in Fig. 3), then it can be calculated that 10 μM tropomodulin would sequester 14 nM actin monomer, thus raising the total G-actin concentration (free + sequestered) to 0.154 μM. However, we estimated by eye that a line through the tropomodulin data with an intercept at 0.154 μM G-actin fits the data points significantly less well than the line through the control points with an intercept at 0.14 μM actin (critical concentration).

Calculation of Steady-State G-Actin Concentrations. We used the expression

G-actin = total actin − F-actin;
F-actin = [fluorescence − a (total actin)]/[b-a];
rearranged from fluorescence = b (F-actin) + a (G-actin).

The value for *a*, which is the fluorescence value for 1 μM G-actin, was determined from the slope of fluorescence versus increasing concentrations of G-actin (e.g., Fig. 3, *crosses*). The value for *b*, which is the fluorescence value for 1 μM F-actin, was determined either from the slope of F-actin fluorescence versus increasing concentrations of total actin (e.g., Fig. 3, *closed circles*) or from the fluorescence of a high actin concentration, usually 3 μM, assuming 2.9 μM to be in the F-actin state.

Results

The Effects of Tropomodulin on Actin Polymerization in the Absence of Tropomyosin

Tropomodulin Reduces the Rate of Elongation at the Pointed End of the Actin Filament. Tropomodulin is proposed to be specifically associated with the terminal tropomyosin molecules at the pointed ends of muscle thin filaments (Fowler et al., 1993). In addition, tropomodulin might also be bound directly to domains II and IV of actin, which are fully accessible only at the pointed filament ends (Holmes et al., 1990; Kabsch et al., 1990). We investigated this possibility by checking whether tropomodulin affected the kinetics of actin assembly at the pointed end of tropomyosin-free actin filaments. Indeed, tropomodulin inhibits elongation at the pointed end, showing that it can bind directly to actin at the pointed filament end (Fig. 1). For these experiments, short gelsolin-capped actin filaments served as nuclei for elongation, and a small amount of pyrenyl-actin (usually ~10%) was added to the native actin as an indicator of polymerization. This makes it possible to estimate the extent of polymerization from the increase in fluorescence. Gelsolin was used to cap the barbed filament ends since otherwise elongation primarily occurs at the 10 times faster barbed end (for a review see Pollard and Cooper, 1986). The initial rate of polymerization decreased with increasing tropomodulin concentrations and at saturation was reduced to 20–30% of the control rate (Fig. 1 *B*). The inhibition was half maximal at a tropomodulin concentration close to 0.1 μM (abscissa intercept of the double-reciprocal plot). This value varied between 0.1 and 0.4 μM for different preparations of actin and tropomodulin.

Tropomodulin Reduces the Rate of Depolymerization at the Pointed End of the Actin Filament. Tropomodulin also reduces the rate of depolymerization from the pointed filament end (Fig. 2 *A*): the rate was ~40% of the control at saturating concentrations of tropomodulin (Fig. 2 *B*). To ensure that depolymerization went to completion in these experiments, we measured the depolymerization of gelsolin-capped actin filaments in the presence of an excess of vitamin D-binding protein. This protein sequesters virtually all of the newly released G-actin (K_d for monomeric actin = 10^{-9} M; see Leod et al., 1989) and maintains the concen-

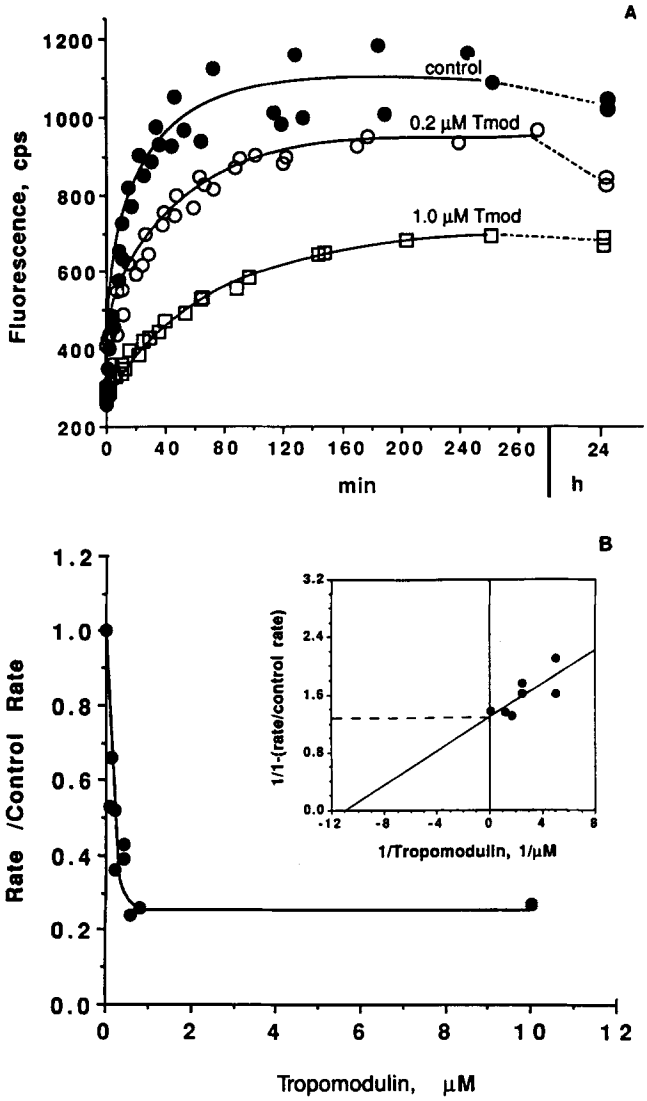

Figure 1. Effect of tropomodulin on the elongation rate of gelsolin-capped actin filaments. (*A*) Raw data: Elongation was initiated by the simultaneous addition of gelsolin-capped actin filaments and polymerizing salts to a 1.4-μM G-actin solution (10% pyrenyl-actin), containing the indicated concentrations of tropomodulin (*Tmod*) and in addition, 10-nM gelsolin-actin complexes as described in Materials and Methods. The gelsolin-capped actin filaments were prepared by copolymerizing 10 μM actin with 0.13 μM gelsolin overnight to obtain an average size of 75 actin molecules per filament, and were diluted to a final concentration of 0.2 μM F-actin and 2.7 nM gelsolin in the assay. (*B*) The normalized initial rates (rate divided by control rate) plotted against increasing tropomodulin concentrations using the full-length recombinant tropomodulin with 2.4 μM G-actin; the gelsolin-capped nuclei for elongation had an average size of 20 actin monomers per gelsolin (5 nM gelsolin). The inset is a double-reciprocal plot of the extent of saturation of the pointed ends with tropomodulin [1-(*rate/control rate*)] vs tropomodulin concentration, calculated from the data in *B*. The abscissa intercept indicates a K_d of ~0.1 μM.

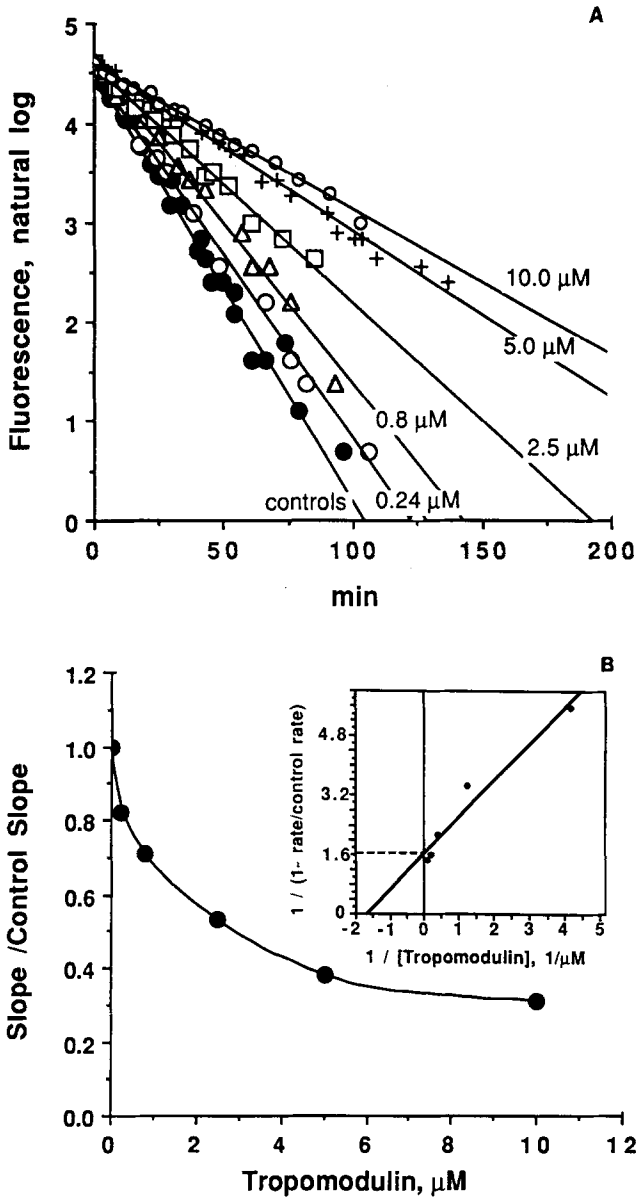

Figure 2. Effect of tropomodulin on the depolymerization rate of gelsolin-capped actin filaments. (*A*) Time course of depolymerization in the presence of increasing concentrations of tropomodulin. The ordinate gives the natural log of the fraction of the total actin that is still polymerized: ln (*fluorescence − endpoint fluorescence*)/(*initial fluorescence − endpoint fluorescence*). The slopes = k_{off} (free pointed ends) decrease with increasing saturation of the pointed ends with tropomodulin as a result of the decrease in the concentration of free pointed ends. Depolymerization was initiated by diluting 50 μl of 10 μM F-actin (10% pyrenyl-actin) copolymerized with 0.1 μM gelsolin, into 1 ml polymerizing solution containing vitamin D-binding protein (see Materials and Methods) and increasing concentrations of tropomodulin as indicated by the numbers on the lines (same preparation as in *B*). The gelsolin-capped actin filaments had an average size of 100 actin molecules per gelsolin (average length of ~250 nm). (*B*) Decrease in the concentration of free pointed ends with increasing tropomodulin concentration. The ordinate values give the fraction of the free uncapped pointed ends: *Slopes in A/control slope* (actin alone). (*Inset*) Double reciprocal plot of the fraction of tropomodulin-capped pointed ends [1 − (*slope/control slope*)] vs tropomodulin concentration, calculated from the data presented in B.

tration of free G-actin <1 nM, preventing any reassociation with the filament. Under these conditions, the time course of depolymerization (Fig. 2 *A*) is exponential either in the absence or presence of tropomodulin, as indicated by the straight slopes of the natural log of fluorescence against time (Fig. 2 *A*). The exponential time course is caused by the exponential length distribution of the actin filaments (for a review see Oosawa and Asakura, 1975), which apparently was not disturbed by tropomodulin.

Tropomodulin Does not Sequester Actin Monomers, but It Increases the Critical Concentration for the Pointed Filament End. Tropomodulin decreased the extent of polymerization when the barbed filament ends were capped by gelsolin (Fig. 1 *A*). This would be expected if tropomodulin, as DNase I, sequestered actin monomers. Sequestration of actin monomers decreases the concentration of polymerizable actin and, therefore, would be expected to reduce the extent of polymerization whether or not barbed ends are capped. However, in the absence of gelsolin, neither the initial time course (see below, Fig. 7 *A*) nor the final extent of polymerization (Fig. 3) were affected by high concentrations of tropomodulin (10 μM). From the data in Fig. 3 we estimated (see Materials and Methods) that the K_d of tropomodulin for actin monomers would have to be ≥100 μM, assuming that it binds monomers at all.

Therefore, since tropomodulin lowers the extent of polymerization without sequestering monomers, it must be raising the concentration of free monomeric actin at steady state with the gelsolin-capped actin filaments, i.e., the critical concentration for the pointed filament end must be increased. We measured the final extent of polymerization at the pointed filament end with increasing actin concentrations in the presence and absence of 4 μM tropomodulin (Fig. 4). In this experiment, tropomodulin increased the critical con-

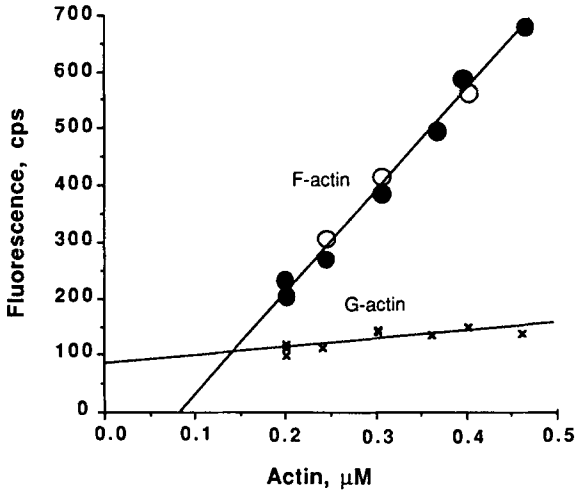

Figure 3. Tropomodulin does not increase the steady-state actin concentration when the barbed ends are not capped. Increasing concentrations of actin (10% pyrenyl-actin) were polymerized (nucleated by 0.8 nM spectrin-actin complexes) in the absence (*closed circles*; control) and in the presence of 10 μM tropomodulin (*open circles*). The endpoints of fluorescence were read after 24 h. The critical concentration is given by the intercept with the line of G-actin fluorescence (*crosses*). Note that the open circles are superimposed on the control curve.

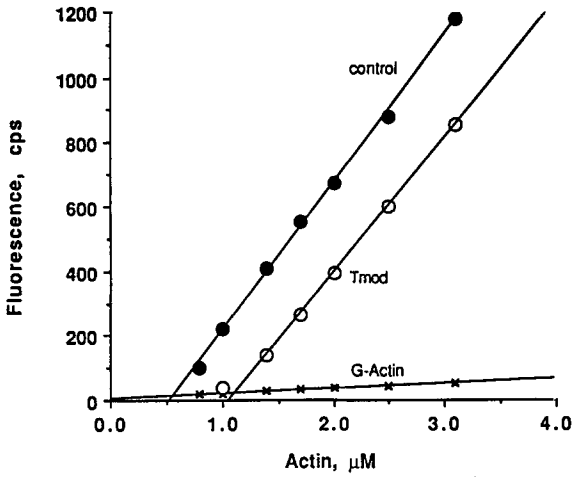

Figure 4. Increase in the critical concentration for the pointed filament end by 4 μM tropomodulin. Increasing concentrations of actin were polymerized overnight in the absence (*closed circles*; control) and in the presence (*open circles*) of 4 μM full-length tropomodulin. The concentration of nuclei in the assay (i.e., pointed filament ends) was 4 nm with an average size of 20 actins per gelsolin. The critical concentration is indicated by the intercept between the F-actin line (*circles*) and the G-actin line (*crosses*).

centration at the pointed filament end from 0.5 to 1.0 μM. In other experiments with different tropomodulin and actin preparations (data not shown), saturating concentrations of tropomodulin increased the critical concentration about twofold, to values between 1.0 and 1.4 μM for a range of control critical concentrations from 0.5 to 0.7 μM. Saturation of tropomodulin's effect on the critical concentration at the pointed end was achieved at tropomodulin concentrations between 4 and 10 μM (data not shown).

There is no internal inconsistency between the tropomodulin-induced increase in the critical concentration for gelsolin-capped actin and the absence of any tropomodulin effect without gelsolin. Because the on-rate constant for actin binding at the barbed filament end is 10 times greater than the pointed end on-rate constant, the critical concentration of actin when both ends are uncapped is close to the value for the barbed filament end alone (Walsh et al., 1984*b*). The predominant influence of free barbed ends on the critical concentration is emphasized by our previous observation that the critical concentration increases relatively little, even when 50% of the barbed ends are capped (Northrop et al., 1987; Young et al., 1990). Indeed, the critical concentration approaches the value for the pointed end only when the extent of barbed end capping exceeds 90%.

In summary, tropomodulin only binds to actin that is incorporated into the pointed end of the filament. In the absence of tropomyosin, tropomodulin partially inhibits elongation and depolymerization, and it increases the concentration of free actin monomer at steady state with the pointed filament end.

The Effect of Tropomodulin on Polymerization in the Presence of Tropomyosin

Elongation in the Presence of Tropomyosin and Tropomodulin. One might expect tropomodulin to bind more tightly to the pointed end if the actin filaments are saturated with tropomyosin since tropomyosin offers a second binding site for tropomodulin with a similar affinity (K_d = 0.2 μM; Fowler, 1987; Babcock and Fowler, 1994) as the binding site on the terminal actin molecule for tropomodulin (K_d for inhibition of elongation = 0.1–0.4 μM, 0.1 μM in Fig. 1 *B*). Thus, the tropomodulin concentration necessary for half maximal inhibition of the elongation of tropomyosin-actin

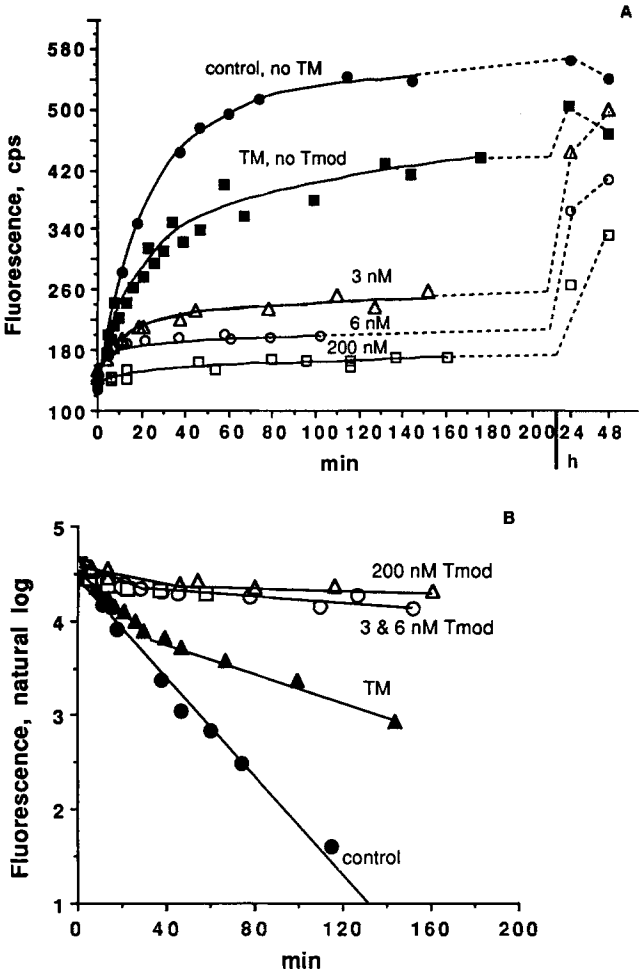

Figure 5. Effect of tropomodulin on elongation at the pointed end of tropomyosin-actin filaments. (*A*) Time course of F-actin formation. The elongation assay was carried out as described for Fig. 1, except that the assay medium contained tropomyosin (0.2 μM in excess over that bound to actin) and tropomodulin (numbers on the curves represent nanomoles of tropomodulin) and the nuclei for elongation consisted of tropomodulin-containing tropomyosin-actin filaments (75 actin molecules per filament) that had been copolymerized overnight. The concentration of nuclei in the assay (i.e., pointed filament ends) was 2.7 nM and the final actin concentration in the elongation assay was 1.4 μM G-actin (10% pyrenyl-actin). 3 and 6 nM total tropomodulin were introduced into the assay along with the actin nuclei for elongation, while 200 nM tropomodulin was added at the start of the elongation assay. The controls did not contain any tropomyosin. (*B*) Time course of G-actin disappearance replotted from *A*. The ordinate values [ln (*final fluorescence − fluorescence at time t*)/(*final fluorescence*] are proportional to polymerizable G-actin (*total G-actin − critical concentration*) at different time points as a fraction of the initial polymerizable actin at time 0.

filaments was very low: 3 nM total tropomodulin reduced the rate to <10% of the control (Fig. 5 *A*). In this experiment, the concentration of ends was 2.7 nM; therefore, most of the tropomodulin must have been bound to the pointed ends, and the free concentration of tropomodulin must have been even lower than 3 nM.

At these low concentrations, tropomodulin does not weaken the association of tropomyosin with actin as previously reported (Fowler, 1990). This effect requires considerably higher concentrations of free tropomodulin than used in these experiments ($K_i \sim 0.5$ μM), and it is presumably caused by tropomodulin binding to the end of tropomyosin molecules and weakening their head-to-tail overlap.

Surprisingly, the presence of tropomyosin alters the time course of elongation at the pointed end (Fig. 5 *A*). This is quite noticeable when the filaments contain tropomyosin alone and is accentuated further by the addition of tropomodulin. The time course of elongation at the pointed ends of filaments made from pure actin can be predicted from the expression

$$rate = k_+ (uncapped\ pointed\ ends)\ [(G\text{-}actin) - critical\ concentration],$$

where k_+ (*uncapped pointed ends*) is constant and [(*G-actin* − *critical concentration*], i.e., polymerizable actin, decreases exponentially with time. Thus, in the absence of tropomyosin, a plot of ln [(*G-actin*) − *critical concentration*] vs time is a straight line (Fig. 5 *B*). (Only when the critical concentration is being approached the rate deviates as if a change in rate constant had occurred [Weber et al., 1987*b*].) By contrast, in the presence of tropomyosin, the slope decreases with time, and if tropomodulin is also present, the slope eventually becomes quite flat. The data suggest a change in the behavior of the pointed filament ends during the assay, presumably a decrease in k_+ during the elongation measurements. This is quite unexpected, considering that the filaments had been preincubated in the same medium with tropomyosin and tropomodulin for 20 h before the elongation assay. A decrease in the concentration of filament ends by annealing is unlikely in view of the capping of the barbed filament ends by gelsolin.

In the presence of tropomyosin, the action of tropomodulin on elongation (and depolymerization) was very sensitive to the manner of assembling the three proteins. Inhibition of elongation and depolymerization was maximal when tropomyosin and tropomodulin were copolymerized with actin, usually during a 15–20-h period. Incubation of preassembled gelsolin-capped actin filaments with tropomyosin and tropomodulin during the same length of time produced a weaker and more variable inhibition (data not shown). This is presumably because during copolymerization rapid binding of two tropomyosin molecules to each newly assembled stretch of 14 actin monomers of an elongating actin filament insures the formation of an uninterrupted tropomyosin polymer on each actin strand. By contrast, the addition of tropomyosin to preformed actin filaments can result in the simultaneous formation of head-to-tail oligomers at many different sites all along the actin filament. In this case, it is likely that many or most oligomers cannot fuse into one polymer because the vacant spaces between them are too small to accommodate a connecting tropomyosin molecule. The oligomers cannot easily rearrange themselves along the length of the filament, a situation that has been referred to as "the parking problem" (Wegner, 1979). Thus, the requirement for copolymerization of actin with tropomyosin suggests that blocking of elongation by the combined action of tropomodulin and tropomyosin might require the presence of an uninterrupted tropomyosin polymer on the actin filament. This interpretation is supported by our observations many years ago (Bremel et al., 1972) that the reconstitution of fully calcium regulated actin filaments, which depends on the presence of an uninterrupted tropomyosin polymer on the actin filament, required the copolymerization of actin with tropomyosin and troponin and could not be accomplished by the addition of these regulatory proteins to polymerized actin.

Depolymerization in the Presence of Tropomyosin and Tropomodulin. It has been shown previously that in high concentrations tropomyosin alone or combined with troponin strongly inhibits depolymerization from the pointed filament end (Broschat et al., 1989, 1990; Weigt et al., 1990). At a tropomyosin concentration too low to block depolymerization, the further addition of tropomodulin completely arrests depolymerization after the initial relatively rapid depolymerization of a small amount of actin (Fig. 6). In one experiment (data not shown), we followed the fluorescence for >3 d and found that there was no further decrease between 24 and 96 h. Centrifugation confirmed that the remaining fluorescence reflected polymerized actin and was not caused by denaturation of pyrenyl-actin (which can raise pyrenyl fluorescence about threefold): the fluorescent material could be spun down by a 2-h centrifugation at 120,000 *g*.

We have also observed cessation of depolymerization at 10 times lower concentrations of tropomodulin than shown here

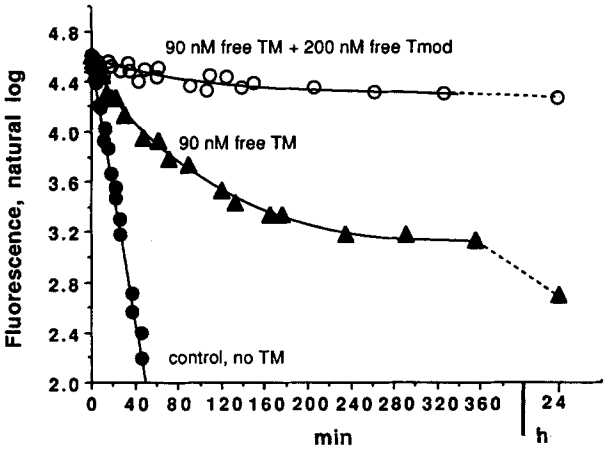

Figure 6. Time course of depolymerization at the pointed end of tropomyosin − actin filaments with and without tropomodulin. The ordinate (fluorescence) gives the natural logarithm of the fraction of the remaining F-actin. The assay was carried out as described for Fig. 2, except that the actin filaments (average length of 100 molecules per filament) had been copolymerized with tropomyosin and, when present, tropomodulin (2.4 μM during copolymerization). Final concentrations in the assays were: actin 0.5 μM, tropomyosin and tropomodulin, 90 nM and 200 nM, respectively, in excess over the bound proteins. The 24-h endpoints correspond to 15% remaining F-actin in the presence of tropomyosin and to 70% remaining F-actin in the presence of tropomyosin and tropomodulin.

(15 and 25 nM, data not shown). At these lower tropomodulin concentrations, depolymerization started out relatively fast, although slower than the control rate, and more actin had been depolymerized before depolymerization was arrested.

Tropomodulin Has No Effect on Actin Association with the Barbed End of the Actin Filament in the Absence or Presence of Tropomyosin

The interaction of tropomodulin with the pointed end is specific since tropomodulin does not cap the barbed ends of actin filaments. For these experiments, we used spectrin-actin complexes that have free barbed ends that can serve as nuclei for elongation (Fig. 7 *A*). To evaluate the effect of tropomodulin on tropomyosin-containing actin filaments, we preelongated spectrin-actin complexes to a filament length that accommodated, on the average, about three tropomyosin molecules per filament. These experiments showed that tropomodulin did not inhibit the rate of elongation at the barbed filament end (Fig. 7 *A*), even when combined with tropomyosin (Fig. 7 *B*), which greatly lowers the concentration of tropomodulin necessary for the inhibition of elongation at the pointed filament end (see above). Furthermore, tropomodulin did not increase the critical concentration, i.e., decrease the final extent of polymerization (Fig. 7), as would have been expected if it had capped the barbed filament ends (see also Fig. 3).

These data also serve as a control for the validity of the fluorescence measurements: tropomodulin did not affect the fluorescence of pyrenyl-actin in either the monomeric or polymeric state.

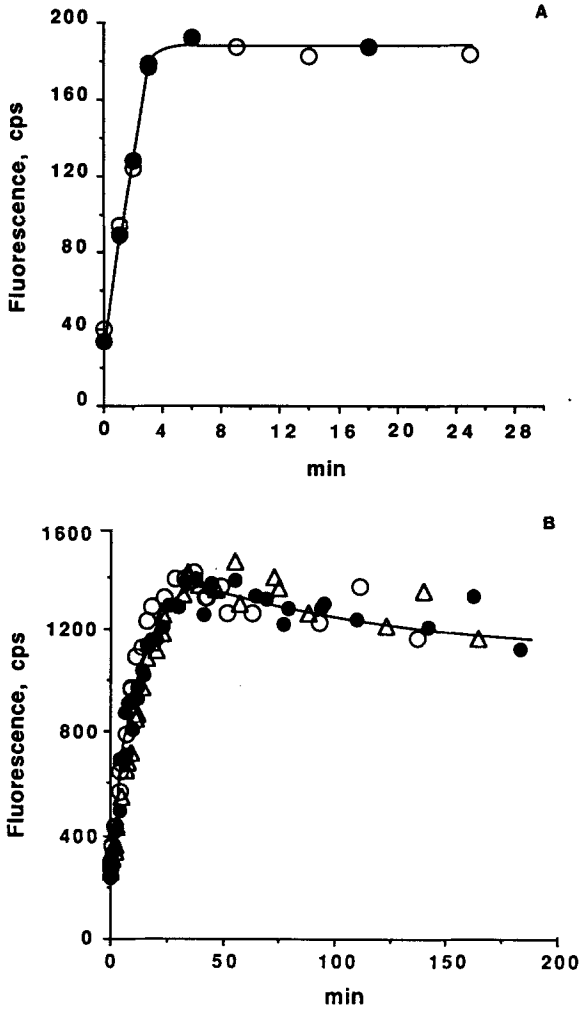

Figure 7. Effect of tropomodulin on the time course of elongation of actin filaments at the barbed end. (*A*) In the absence of tropomyosin. Elongation was started by the simultaneous addition of spectrin-actin complexes and polymerizing salts to a solution containing 0.5 μM G-actin (10% pyrenyl-actin) in the absence (*closed circles*) or presence of 10 μM tropomodulin (*open circles*). (*B*) In the presence of tropomyosin. Elongation was started by the simultaneous addition of polymerizing salts and of preelongated spectrin-actin complexes (1.6 nM final concentration) (see Materials and Methods) to a solution containing 1.6 μM G-actin (10% pyrenyl-actin), 200 nM tropomyosin, and 500 nM tropomodulin when present, both in excess over that introduced with the preelongated spectrin-actin complexes. (*Closed circles*) controls; (*open triangles*) tropomyosin only; (*open circles*) tropomyosin and tropomodulin.

Discussion

Tropomodulin is a capping protein for the pointed filament ends of tropomyosin-containing actin filaments. That is, tropomodulin in concentrations stoichiometric to the concentration of filament ends blocks elongation and depolymerization in the presence of tropomyosin. At much higher concentrations, tropomodulin alone partially inhibits elongation and depolymerization of pure actin filaments, indicating that it also has a binding site for actin. K_d values derived from the inhibition of the elongation rates of pure actin filaments range between 0.1 and 0.4 μM. Since the K_d of tropomodulin binding to tropomyosin is also 0.2 μM (Babcock and Fowler, 1994), the tight capping of the tropomyosin-containing actin filaments (K_d < 1 nM) suggests that tropomodulin binds to both the terminal tropomyosin and the terminal actin molecule.

Tropomodulin binds to actin molecules only when they are incorporated into the pointed filament ends: tropomodulin does not sequester actin monomers as shown here, and it does not bind alongside actin filaments as shown previously by sedimentation experiments with spontaneously polymerized actin (Fowler, 1990). Since actin forms very long filaments in the absence of a nucleating protein, the amount of tropomodulin bound to the pointed filament ends would have been undetectable in these experiments.

One way to explain partial inhibition of elongation and depolymerization of pure actin filaments at saturating concentrations of tropomodulin is that tropomodulin does not cap both actin strands at the pointed filament end. For instance, if tropomodulin binds loosely to only one of the two actin molecules at the pointed filament end and dissociates faster than the rate of actin monomer addition or loss, elongation or depolymerization will continue at a reduced rate. Indeed, one tropomodulin per filament end was found in a preliminary binding experiment using gelsolin-capped actin filaments (Fowler, V. M., unpublished observations). This may be because there is not enough room at the filament end for two tropomodulin molecules, as seems to be the case for the other pointed-end–capping protein, DNase I (Weber et al., 1994). Alternatively, tropomodulin may prefer one of the

two actin molecules at the pointed filament end over the other. For example, tropomodulin may prefer the monomer added last to the pointed end because it might contain a different nucleotide (ATP or ADP.Pi) than the penultimate monomer. Partial capping has been observed previously only for insertin, a barbed end-capping protein whose mechanism of action is not fully understood (Ruhnau et al., 1989; Gaertner and Wegner, 1991).

An unusual effect of tropomodulin, which has not been observed previously for any other capping protein, is the doubling of the critical concentration at the pointed filament end. (Effects of barbed-end capping proteins on critical concentration are caused by the shift of the critical concentration from that of the barbed filament end to that of the pointed filament end when the barbed ends are completely capped.) The extent of the tropomodulin-induced increase in critical concentration is consistent with the different extents of inhibition of actin monomer binding and release at the filament end: tropomodulin inhibits the rate of elongation about twice as much as the rate of depolymerization. It is intriguing to speculate that these effects could result from the sensitivity of tropomodulin-actin binding to the nucleotide content of actin, as proposed above, since the rate constants for monomer binding and release and the critical concentrations are different for ADP-actin than for ATP-actin (Carlier, 1991; Pollard, 1990). In this regard, a number of other actin-binding proteins have been shown to be sensitive to the nucleotide content of actin. Gelsolin, for example, binds more strongly to ADP- than to ATP-actin (Laham et al., 1993), and thymosin-β_4 prefers ATP-actin monomers 50-fold over ADP-actin monomers (Carlier, 1991).

In the presence of tropomyosin, tropomodulin completely caps the pointed filament ends and does not increase the critical concentration at the pointed filament end. As discussed above, it is likely that tropomyosin provides a second binding site for tropomodulin at the pointed end, thus increasing the affinity of tropomodulin for the pointed end ($K_d \leq 1$ nM). In this case, a very slow rate of tropomodulin dissociation with respect to the rate of actin monomer addition would be expected, thus blocking elongation completely. Although we find that tropomyosin alone partially reduces elongation at the pointed filament end[2] (for contrary results see Broschat, 1990; Hitchcock-DeGregori et al., 1988), the extent of inhibition is not as large as in the presence of tropomodulin. Thus, it seems likely that tropomodulin participates directly in the blocking of elongation at the pointed ends of tropomyosin containing actin filaments.

On the other hand, complete arrest of depolymerization from the pointed filament end can be caused by high concentrations of tropomyosin considerably in excess of the amount necessary to saturate the actin filaments (Broschat, 1990).[2] The presence of tropomodulin lowers the concentration of tropomyosin needed to arrest depolymerization. This might be explained by tropomodulin strengthening the binding of the terminal tropomyosin molecule to the pointed end of the actin filament. With only one instead of two head-to-tail overlaps, the terminal tropomyosin molecule is bound considerably more weakly to the actin filament than are the tropomyosin molecules in the interior of the tropomyosin polymer.

How can one extrapolate from these properties of tropomodulin to its role in the cell? The data presented in this report together with the results from previous immunolocalization experiments show that tropomodulin is specifically associated with the pointed ends of the tropomyosin-containing actin filaments in skeletal muscle (Fowler et al., 1993), cardiac muscle (Gregorio, C. C., and V. M. Fowler, unpublished observations), and in human red blood cells (Ursitti and Fowler, 1994). In cardiac muscle cells, the length of the tropomyosin-actin filaments varies over about a twofold range (Robinson and Winegrad, 1979; Kruger et al., 1991). Tropomodulin might play a role in maintaining this length distribution, which is relatively narrow compared to the exponential length distribution of pure actin filaments in vitro. In skeletal muscle, the virtually identical length of the actin filaments has been proposed to be caused by the presence of nebulin (Labeit et al., 1991; Kruger et al., 1991). However, tropomodulin could protect against filament elongation under conditions, should they occur, where the monomer concentration exceeds the critical concentration for the pointed end.

In the red blood cell, the actin filaments are thought to contain only ~15–20 actin molecules each, 8–10 per filament strand (Shen et al., 1986). Such short filaments can accommodate only two tropomyosin molecules, one for each strand (Fowler and Bennett, 1984). Thus in this case, tropomodulin could increase the strength of tropomyosin binding to these filaments. Without head-to-tail overlap, direct tropomyosin binding to the minifilament is expected to be very weak. Although the barbed ends of these tropomyosin-containing actin filaments are not reported to be capped (Pinder et al., 1986), capping of their pointed ends by tropomodulin could prevent filament annealing and length redistribution that might otherwise occur.

In motile cells such as neutrophils, which polymerize a large amount of actin within 15 s after exposure to formyl-peptides (Wallace et al., 1984), one can envision a function for the tropomodulin-induced increase in the critical concentration at the pointed filament end of pure actin filaments. When the barbed filament ends are capped, e.g., in resting neutrophils, the tropomodulin-induced increase in the concentration of free actin monomer would lead to an increase in the size of the pool of bound actin monomer. Since the actin–thymosin-β_4 complex is in equilibrium with the concentration of free monomeric actin, the amount of actin monomer bound by a given total concentration of thymosin-β_4 would increase with increasing free monomeric actin (Weber et al., 1992). A larger pool of thymosin-β_4–bound actin monomer would provide more actin for rapid polymerization after stimulation of the neutrophils.

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2. The inhibitory effects of tropomyosin on elongation and depolymerization at the pointed filament end are not caused by trace contamination by tropomodulin since preincubation of tropomyosin at 85°C (which abolishes tropomodulin's activity) has no effect on the activity of the tropomyosin (Weber, A., and V. M. Fowler, unpublished observations).

Muscle Institute) to A. Weber. V. M. Fowler is an Established Investigator of the American Heart Association.

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