Regulation of the actin cytoskeleton by filament capping proteins is critical to myriad dynamic cellular functions. The ability of these proteins to bind both filaments as well as monomers is often central to their cellular functions. The ubiquitous pointed end capping protein Tmod3 (tropomodulin 3) acts as a negative regulator of cell migration, yet mechanisms behind its cellular functions are not understood. Analysis of Tmod3 effects on kinetics of actin polymerization and steady state monomer levels revealed that Tmod3, unlike previously characterized tropomodulins, sequesters actin monomers with an affinity similar to its affinity for capping pointed ends. Furthermore, Tmod3 is found bound to actin in high speed supernatant cytosolic extracts, suggesting that Tmod3 can bind to monomers in the context of other cytosolic monomer binding proteins. The Tmod3-actin complex can be efficiently cross-linked with N-hydroxysulfosuccinimide in a 1:1 complex. Subsequent tryptic digestion and liquid chromatography/tandem mass spectrometry revealed two binding interfaces on actin, one distinct from other actin monomer binding proteins, and two potential binding sites in Tmod3, which are independent of the previously characterized leucine-rich repeat structure involved in pointed end capping. These data suggest that the Tmod3 isoform may regulate actin dynamics differently in cells than the previously described tropomodulin isoforms.

Cells regulate a number of important processes in space and time via the actin cytoskeleton. The actin cytoskeleton and its dynamic reorganization are in turn regulated by a variety of actin-binding proteins, each of which participates in one or more of the aspects of the polymerization and depolymerization of filaments (1). One of the least understood components in this system is regulation of the monomer pool (2). In part this is because of the complication that although many actin-binding proteins perform functions specific to filaments (e.g. capping and severing), they interact with actin in both the monomer and polymer states. For example, gelsolin, a multifunctional actin- regulatory protein, severs filaments and caps their barbed ends but also binds to actin monomers (3). For some classes of actin regulatory proteins such as the coifin/ADF family, the ability to bind to both monomer and polymer is critical to their known cellular functions (4, 5). In other cases, actin filament-binding proteins bind to monomers but with uncertain functional or biological consequence, as with capping protein/CapZ (6). In each of these cases, the actin-binding protein in question forms an interface with the actin monomer that dictates how the complex interacts with actin and with other actin regulators and therefore its effects on actin polymerization dynamics (7).

Further complicating the understanding of these multifunctional roles are the divergent functions of actin-binding proteins among members of a given family. For example, formins have evolved very different activities with respect to actin dynamics, despite great homology between the various isoforms (8). Even among actin monomer-binding protein families, much diversity of action exists, as illustrated by the WH2 domain containing proteins, which can either promote or inhibit actin assembly (9).

Tropomodulins (Tmods) are a unique family of ~40-kDa proteins that cap filament pointed ends in many types of actin networks and structures and are found in nearly all tissues of vertebrates, as well as in flies and worms (10). Tmods also bind tropomyosin and cap pointed ends of tropomyosin-coated filaments with over a 1000-fold greater affinity than pointed ends of tropomyosin-free actin filaments (11, 12). The biochemical properties of Tmods have been best studied for the Tmod1 isoform, which caps actin filament pointed ends but does not bind actin monomers (11). Tmods contain two major structural domains with unique functions in regulating actin polymerization. First, the C-terminal half of Tmods consists of a series of leucine-rich repeats (LRR) that form a compact, globular domain that caps pointed ends of actin filaments, independent of tropomyosin (13–15). This structure has been defined by x-ray crystallography and is conserved from Caenorhabditis elegans through vertebrates (16, 17). Second, the N-terminal portion of Tmods is flexible and extended in solution (14, 18, 19), and contains the tropomyosin binding activity as well as the ability to cap tropomyosin-actin filament pointed ends with high affinity (18, 20). Recently, a structure determination of Tmod1 residues 1–90 by two-dimensional NMR spectroscopy

---

*This work was supported by National Institutes of Health Grants EY014972 (to R. S. F.), 5K25AR048918 (to M. R. B., and E. G. Y.), GM34225/HL083464 (to V. M. F.), and HL38794 (to D. W. S.). 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 Cell Biology, The Scripps Research Institute, CB163, La Jolla, CA 92037. Tel: 858-784-9839; Fax: 858-784-8753; E-mail: bfischer@scripps.edu.

§ Received for publication, July 3, 2006, and in revised form, September 11, 2006. Published, JBC Papers in Press, October 1, 2006, DOI 10.1074/jbc.M606315200
Tmod3 Binds Actin Monomers

has revealed the presence of a short α-helix that is important for tropomyosin binding and another short region with α-helical propensity that is involved in high affinity capping of tropomyosin-actin filaments (21, 22). Despite the fact that this N-terminal region is well conserved across isoforms (greater than 75% homology), Tmod isoforms demonstrate significant differences in their binding affinities for different tropomyosin isoforms (18).

In vertebrates there are four Tmod isoforms (Tmods1–4), each of which are expressed in a tissue-specific and developmentally regulated fashion. The cell types and actin networks these isoforms are found in can be characterized as either relatively dynamic (Tmod2 and Tmod3) or stable (Tmod1 and Tmod4). The cellular function of Tmod depends on the actin network in which it is found. For example, in stable actin structures like the cardiac muscle sarcomere, Tmod1 regulation of actin exchange at pointed ends controls thin filament lengths and stability (23), such that the lack of Tmod1 in mice is lethal because of defective sarcomere assembly and aborted cardiac development (24, 25). Conversely, in dynamic actin networks such as the lamellipodia of motile endothelial cells, Tmod3 is present in concentrations similar to other actin regulators, such as ADF/cofilin, and acts as a negative regulator of cell migration (26). As yet, Tmod3 is the only protein known to perform this negative regulatory function in dynamic actin networks like those found in lamellipodia (10).

One potential mechanism of negative regulation of cell migration by Tmod3 is reduction in pointed end disassembly (10). This hypothesis is attractive when considered with the presumption that disassembly of the pointed ends is likely to be the rate-limiting step in network turnover (1). However, a puzzling observation has been that Tmod3 levels in the endothelial cells were inversely correlated with levels of F-actin and free barbed ends in lamellae. Based on the ability of Tmod3 to cap pointed ends and slow down pointed end disassembly in vitro, it might have been expected that Tmod3 levels would be directly correlated with F-actin levels, i.e. more Tmod3 would lead to more F-actin and less Tmod3 to less F-actin. Moreover, despite the presence of a large soluble pool of Tmod3 at endogenous levels, these motile cells contained an abundance of free pointed ends (26). These observations suggested the existence of novel actin regulatory mechanisms for Tmod3, and/or other binding partners available to Tmod3 that prevented it from capping a subset of the actin pointed ends in cells.

Here we show that Tmod3, unlike the Tmod isoforms found in stable actin structures, binds to actin monomers both in vivo and in vitro. Tmod3 competitively inhibits thymosin β4 (tβ4) binding to actin monomers, with an affinity for the monomer similar to its affinity for filament pointed ends. Kinetic modeling further shows that the Tmod3-actin monomer complex is surprisingly stable in contrast to the association of Tmod3 with actin filament pointed ends. Using zero length cross-linkers, we find that Tmod3 forms a 1:1 complex with actin monomer. Tryptic digestion and LC-MS/MS of this cross-linked complex identified two putative Tmod3-binding peptides, both contained in the N-terminal domain of Tmod3, which was alone sufficient to sequester monomers. These data indicate that Tmod3, unlike other Tmods, binds efficiently to actin monomers as well as filament pointed ends.

EXPERIMENTAL PROCEDURES

Proteins—Rabbit skeletal muscle actin was prepared from acetone powder as described previously (27). Purified non-muscle actin was purchased from Cytoskeleton, Inc. (Denver, CO). Pyrene-labeled actin was prepared and stored as described (12). Prior to use in assays, actin was dialyzed several times against freshly prepared buffer A (2 mM Tris, pH 8.0, 0.2 mM CaCl₂, 0.02% NaN₃, 2 mM dithiothreitol (DTT), 0.5 mM ATP). Purified recombinant chicken Tmod1 and -4, rat Tmod2, and human Tmod1 and Tmod3 were expressed as glutathione S-transferase (GST) fusion proteins in BL21 Escherichia coli and purified as described previously (28). Tmod3 (1–92) and Tmod3 (1–189) were made by mutating codons 93 or 190 and 191 into stop codons, within the context of the glutathione S-transferase fusion expression plasmid, and purified as above. Protein concentrations were determined spectrophotometrically by absorption as described (15).

Actin Polymerization Assays—Pyrenyl-actin was converted to Mg²⁺-actin as described previously (29), and polymerization reactions were performed in a polymerization buffer (10 mM imidazole, pH 7.0, 0.1 M KCl, 2 mM MgCl₂, 1 mM NaN₃, 1 mM DTT, 0.5 mM ATP, and 0.1 mM CaCl₂). Polymerization was initiated by the addition of 10X polymerization buffer to achieve the final buffer concentration. Fluorescence measurements (λₑₓ = 366.5 nm, λₑₘ = 407 nm) were made using a Fluoromax 3 fluorimeter (Jobin Yvon, Edison, NJ). Nucleation reactions were performed by addition of various amounts of Tmod3 to 2 μM G-actin (8% pyrenyl), prior to the addition of salts (15). For pointed end elongation measurements, gelsolin-capped actin filaments (1:10 gelsolin:actin) (11) were added to 2 μM G-actin (8% pyrenyl) in the presence or absence of Tmod3 followed by addition of polymerization salts as described (11). For barbed end elongation experiments, spectrin-actin seeds (30) or F-actin seeds (31) were prepared and added to a total concentration of 1.5 μM G-actin (8% pyrenyl) in the presence or absence of Tmod3 followed by addition of polymerization salts. In the case of steady state reactions, the final concentration of actin was as stated in the figure legends. For steady state measurements, filament seeds (~9 μM barbed ends, as determined by kinetic modeling; see below) or spectrin-actin seeds (200 nM) were added to 3 μM actin (10% pyrenyl), with or without Tmods, and allowed to polymerize to steady state for 30–36 h at room temperature prior to measurement of total fluorescence. At times longer than 60 h, a slight decrease in F-actin as reported by pyrene fluorescence was observed, probably due to accumulation of ADP-actin monomers. Tmods were included in the polymerization mixture either at the beginning or after 12 h. We observed no significant difference between these two regimes. We also observed no measurable difference in G-actin fluorescence in the presence of Tmod3 (data not shown). Nucleation, pointed end, and barbed end elongation experiments were performed similarly, except that the fluorescence was monitored immediately upon addition of polymerization salts. Representative experiments from each series are shown for clarity. Averages from different days are
not reported, because of variation in pyrenyl-actin from day to day (32).

**Cross-linking of Actin and Tmod3**—Purified proteins were dialyzed three times against 1 liter of Buffer A and then against cross-linking buffer (2 mM KHPO₄, pH 8.0, 1 mM CaCl₂, 1 mM DTT, 0.5 mM ATP) at 4 °C. To test if divergent cation differences were important for complex formation, some samples were briefly dialyzed against cross-linking buffer with 1 mM MgCl₂ at pH 7.5 (because the optimum pH for the EDC cross-linking reaction is 7.5 or lower). In our experience, this did not dramatically affect the cross-linking reaction. Immediately before the reactions, the proteins were dialyzed against the appropriate cross-linking buffer without ATP and DTT for 2 h at 4 °C (33).

Each protein sample was centrifuged at 300,000 × g at 4 °C for 20 min to remove aggregates, and concentrations were determined by spectrophotometry. The proteins were combined at a 1:1 molar ratio at concentrations between 10 and 20 μM. L-Ethyl-3-(3-dimethylaminopropyl)-carboadiimide (EDC; Pierce) and N-hydroxysulfosuccinimide (NHS; Pierce) stock solutions were prepared immediately before addition to reactions and were added to a final concentration of 1 mM. The reaction was carried out at room temperature for 20 min. The reactions were stopped by addition of either 200 mM (final concentration) glycine or 10 mM hydroxyxylamine. Samples were boiled in Laemmli sample buffer, separated by SDS-PAGE on either 10 or 7.5–15% gradient gels. Gels were either stained with Coomassie Brilliant Blue R-250 or transferred to nitrocellulose for immunoblotting with antibodies to Tmod3 or actin, as described (26).

**In-gel Trypsin Digestion, HPLC Peptide Mapping, and LC-MS/MS**—Coomassie Blue-stained gel bands were excised and digested with modified trypsin essentially as described previously (34) with minor modifications. Briefly, gel bands were: destained using 200 μl of 200 mM ammonium bicarbonate, 50% acetonitrile for 30 min at 37 °C; dried under vacuum; reduced using 100 μl of 20 mM tris(2-carboxyethyl)phosphine in 25 mM ammonium bicarbonate, pH 8.0, for 15 min at 37 °C; alkylated using 100 μl of 40 mM iodoacetamide in 25 mM ammonium bicarbonate, pH 8.0, for 30 min at 37 °C; washed twice with 200 μl of 25 mM ammonium bicarbonate for 15 min each and washed once with 200 μl of 50% acetonitrile, 25 mM ammonium bicarbonate; dried under vacuum, and digested with 20 μl of 0.02 μg/μl modified trypsin (Promega) in 40 mM ammonium bicarbonate overnight with shaking at 37 °C. The supernatant was removed to a clean tube, and 20 μl of 40 mM ammonium bicarbonate was added for 30 min with shaking at 37 °C. The supernatants were combined, and 4 μl of neat acetic acid was added for LC-MS/MS samples or 4 μl of 5% trifluoroacetic acid for subsequent microbore HPLC analysis. All samples were stored at −20 °C until analyzed.

For microbore HPLC with UV detection, typically 80% of the extracted tryptic peptide solution was injected onto a Beckman Coulter Gold System 126 with a 5-μm, 2.1 × 150-mm Zorbax 300 SB-C18 column. Peptides were eluted using a trifluoroacetic acid:acetonitrile gradient at 200 μl/min using detection at 214 nm. Peaks were collected into precleaned microcentrifuge tubes, and selected peaks of interest were analyzed by MALDI-MS using conventional methods.

LC-MS/MS peptide mapping and identification was performed using either an LTQ ion trap mass spectrometer or LTQ FT-ICR mass spectrometer (Thermo Electron Corp., San Jose, CA). The tryptic peptides were separated by reverse phase-HPLC on a nanocapillary column, 75 μm inner diameter × 15 cm PicoFrit (New Objective, Woburn, AM), packed with MAGIC C18 resin, 5-μm particle size (Michrom BioResources, Auburn, CA). The mass spectrometers were set to repetitively scan m/z from 375 to 2000 m/z followed by data-dependent MS/MS scans on the six most abundant ions with dynamic exclusion enabled. The resulting masses and MS/MS spectra were searched using SEQUEST Browser (Thermo Electron Corp.) with a custom unindexed data base consisting of the exact sequences of the two proteins. Separate searches using trypsin specificity with one allowed missed cleavage and no enzyme were performed. In general, searches were performed using a static modification of Cys as the carboxamidomethyl derivative and dynamic modifications as follows: Met to methionine sulfoxide (+16 Da), methylation of His (+14 Da) for the methylated actin His, and pyroglutamic acid (−17 Da) for the partial N-terminal cyclization of tryptic peptides with N-terminal glutamines. Resulting data were filtered to eliminate all matches where S was <0.20, 0.50, and 0.70 for +1, +2, and +3 charge stages, respectively, and p > 25 for all charge states. All assigned sequences that affected potential interpretations were manually inspected to confirm the assigned peptide sequence.

**tβ4 Fluorescence Anisotropy Competition Assays**—Measurement of Tmod3 competition for actin monomer binding with tβ4 was measured by fluorescence of tetramethylrhodamine-5-maleimide-labeled tβ4, as described previously (2). In addition to full-length tβ4, a synthetic, truncated ε-amino rhodamine-labeled peptide (1–25 rhod-tβ4 peptide) was also used in some assays. Standard curves of the fluorescence anisotropy of rhodamine-labeled tβ4 peptide as a function of Mg²⁺·actin concentration in F buffer (5.0 mM Tris-HCl, 40 mM KCl, 2.0 mM MgCl₂, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.1 mM CaCl₂, 0.125 mM EGTA, and 0.01% sodium azide, pH 7.8) were generated with vertically excited polarized light at 546 nm in an L or T format steady state fluorimeter. The horizontal (Iₕ) and vertical (Iₐ) components of the emitted light were detected at 568 nm for 0.3-ml samples in glass cuvettes. The fluorescence anisotropy, r, was calculated as described previously (2). Because the bound actin does not change the total fluorescence intensity of the rhodamine-labeled tβ4, the observed anisotropy is a linear function of the fraction of tβ4 that is bound to actin.

**Modeling of Barbed End and Pointed End Elongation Rates**—We used BerkeleyMadonna version 8.01 to generate ordinary differential equation models of the barbed end elongation rates. In the case of barbed end elongation experiments, we determined the number of free barbed ends added to the reaction by using the published on and off rates for actin monomers (35) onto the barbed ends, and we allowed the amounts of free barbed ends to vary to fit the experimental data of polymerization reactions in the presence of no Tmod3. We then used this amount of free barbed ends for all subsequent barbed end elongation models of data from experiments performed the same day. Similarly, for pointed end elongation reaction models, even though we used a known amount of seeds for the reaction, we
Tmod3 Binds Actin Monomers

FIGURE 1. Tmod3 sequesters actin monomers in pyrenyl-actin polymerization assays. A, nucleation of free barbed ends by Tmod3. Tmod3 was added to 2 µM total actin (8% pyrenyl), and polymerization salts were added to initiate the reaction. Polymerization following spontaneous nucleation of filaments was monitored by increased pyrenyl-actin fluorescence, as described under "Experimental Procedures." B, Tmod3 decreases elongation from free barbed ends. Filaments with free barbed ends (~9 nM) were allowed to elongate in the presence of 1.5 µM G-actin (8% pyrenyl), with varying amounts of Tmod3. C, Tmod3 increases the concentration of unpolymerized actin in a concentration-dependent manner at steady state. Actin was polymerized to steady state, diluted to the appropriate concentration, and incubated in the presence of various concentrations of Tmod3. Interpolation of data points by linear regression is shown to indicate the apparent critical concentration at the intercept with the G-actin curve. D, polymerized actin at steady state as a function of Tmod3 concentration. 2 nM unlabeled filaments with free ends were incubated in the presence of 1 µM total actin (8% pyrene-labeled) to steady state in the presence of Tmod3. At low (<50 nM) levels of Tmod3, increasing Tmod leads to increased actin polymerization, as pointed ends are capped, reducing the critical concentration to that of the barbed ends. At higher levels of Tmod3, monomers are sequestered, decreasing the polymerized actin.
Tmod3 Binds Actin Monomers

![Graph showing fluorescence anisotropy of actin monomers with and without Tmod3](image)

**Figure 2.** Comparison of monomer sequestering ability of vertebrate Tmod isoforms. Actin (8% pyrenyl) at the indicated concentrations was incubated to steady state in the absence or presence of 3 μM Tmods, as shown in Fig. 1C. Linear interpolation shows the intercept with G-actin fluorescence curve to approximate the critical concentration. For this data set, no linear interpolation can be plotted for Tmod3. No difference between control and Tmod1 or Tmod4 plots is observed, indicating a lack of monomer binding, whereas a small but reproducible effect on steady state F-actin is observed with Tmod2. Note scale difference for actin range from Fig. 1C.

---

Comparison of Tmod isoforms reveals that the ability to sequester actin monomers may be unique to the Tmod3 isoform. As observed previously (11, 12), Tmod1 does not alter the actin critical concentration in the presence of free barbed ends (Fig. 2), nor does it affect elongation rates from free barbed ends (data not shown). Tmod4, which in mammals is expressed only in skeletal muscle (38), is similar to Tmod1 in that it does not bind detectably to monomers (Fig. 2). Tmod2 is predominantly expressed in neurons (39). Relative to Tmod3, Tmod2 causes a much smaller but reproducible decrease on the total F-actin (Fig. 2). These data suggest that Tmod3 has evolved a monomer binding function specific to its role in dynamic actin systems.

**Tmod3 Competes with tβ4 for Actin Monomer Binding**—To obtain a measurement of Tmod3 affinity for actin monomers, we used a tβ4 fluorescence anisotropy assay (2). In this assay, the anisotropy of a fluorescently tagged tβ4 molecule is used to report the free actin concentration in a sample, because the anisotropy of the tβ4-actin complex is greater than free tβ4. Thus, the total anisotropy is a direct function of the proportion of bound and free tβ4 in the assay (2). We observed that increasing concentrations of Tmod3 resulted in a dramatic decrease in the fluorescent anisotropy of mixtures of actin with fluorescent tβ4, indicative of the dissociation of the tβ4-actin complex (Fig. 3A). At saturating Tmod3 concentrations, the anisotropy was equivalent to free tβ4 (not shown), indicating that Tmod3 was able to competitively inhibit tβ4 binding to actin. To rule out the possibility that Tmod3 could have formed a ternary complex with actin and tβ4 that has the same anisotropy as free tβ4, we placed the dye at the carboxyl end of a truncated form of tβ4 (40), in the cleft between subdomains 1 and 3. Notably, Tmod3 was also able to compete with the truncated tβ4 for binding to actin monomers (Fig. 3A, inset), indicating that Tmod3 directly inhibits tβ4 binding to actin monomers, most likely by forming a Tmod3-actin monomer complex.

Comparison of the effect of Tmod3 on fluorescent anisotropy of tβ4 with ATP-Mg2+-actin or ADP-Mg2+-actin revealed that Tmod3 was somewhat more effective at competing for tβ4 binding to ATP-Mg2+-actin (Fig. 3A) than to ADP-Mg2+-actin.
Transfected
Antibody
Latrunculin A

Actin
Flag

Mg²⁺-actin (Fig. 3B). From these data, we calculated the $K_d$ value of Tmod3 for binding to ATP-Mg²⁺-actin to be 0.13 μM, and the $K_d$ value of Tmod3 for ADP-Mg²⁺-actin to be 0.6 μM. This 4-fold decrease in affinity of Tmod3 for ADP-Mg²⁺-actin monomers (as compared with ATP-Mg²⁺-actin monomers) is considerably smaller than other monomer binding proteins. For instance, t²4 binds to ATP-Mg²⁺-actin with an affinity at least 50-fold higher than for ADP-Mg²⁺-actin (36), whereas proteins such as ADF or Srv2/CAP bind preferentially to ADP-Mg²⁺-actin with an affinity 100-fold higher than for ATP-Mg²⁺-actin (37, 41). However, the $K_v$ value of Tmod3 for binding to either ATP- or ADP-Mg²⁺-actin is within an order of magnitude of these other monomer binding proteins for their preferred actin-nucleotide binding partner. Moreover, the $K_d$ values of Tmod3 for binding to ATP- or ADP-Mg²⁺-actin monomers are near the endogenous Tmod3 concentration in cells (0.5 μM; see Ref. 26), suggesting that a significant portion of the Tmod3 could be associated with actin monomers in cells.

Tmod3 Binds Actin Monomers in the Cytosol—Cells contain a constellation of cytosolic actin monomer binding proteins, including profilin, t²4, and cofillin, that are present in micro-molar concentrations and function to regulate actin monomer-polymer dynamics and levels (7). To investigate whether Tmod3 can bind actin monomers in the context of these other actin monomer binding proteins, HEK 293 cells were transfected with FLAG-tagged Tmod3, lysed with detergent, and all filamentous actin removed by ultracentrifugation (see “Experimental Procedures”). When the FLAG-tagged Tmod3 in the supernatant was immunoprecipitated, Western blotting revealed that actin was present in the pellet containing the antibody-coated beads (Fig. 4). Preincubation of the cleared extracts with 10 μM LatA to ensure depolymerization of all the actin had no effect on the ability of FLAG-tagged Tmod3 to co-precipitate actin, indicating that FLAG-Tmod3 was interacting with monomeric actin in the cytosol. In reactions with untransfected lysates or without primary antibody, little to no actin was detected in association with the beads indicating that nonspecific binding of actin was not a factor in this assay. It should be noted that the FLAG-Tmod3 is expressed from a cytomegalovirus promoter and is therefore likely expressed at higher concentrations than the endogenous Tmod3. However, in similar experiments using GFP-Tmod3 overexpressing constructs in endothelial cells, we have found that the exogenous Tmod3 is overexpressed ~3–5-fold over the endogenous Tmod3 (26). In any case, these data indicate that Tmod3 can bind to actin monomers in the cellular context of other actin monomer binding proteins found in the cytosol, many of which exist at much higher concentrations than the endogenous or overexpressed Tmod3 (2). Furthermore, this experiment demonstrates that Tmod3 can bind to either β- or γ-actin monomers, which are the isoforms found in non-muscle cells.

Kinetic Modeling of the Tmod3-Actin Interactions—To determine the on and off rates for the Tmod3-actin complex, we performed kinetic modeling of the actin polymerization reactions in filament elongation assays. This approach is based on the fact that polymerization rates from either the barbed or pointed ends are linearly dependent on actin monomer concentration (35). First, we obtained kinetic constants for Tmod3 binding to actin monomers by modeling the barbed end elongation rates to eliminate the contribution of Tmod3 binding to filament pointed ends. Using ordinary differential equations to model the barbed end capping reactions in the presence of Tmod3, the best fit curves to our experimental data predict that the Tmod3 has slow on and off rates for the actin monomer (~0.003/μM/s and 0.001/sec, respectively), resulting in a $K_d$ of ~0.5 μM (Fig. 5), which is reasonably close to the measured $K_d$ of 0.13 μM for ATP-Mg²⁺-actin determined from the fluorescent-t²4 anisotropy assays (Fig. 3A).

Next, using the experimentally measured $K_v$ value of 0.13 μM for Tmod3-actin monomer (Fig. 3), we modeled the pointed end elongation reactions, fitting the modeled curves to our experimental data as before (Fig. 6). This analysis generated a $K_d$ value for capping pointed ends of ~0.17 μM, close to the $K_d$ of ~0.11 μM determined experimentally for Tmod3 (26) and for other Tmod3s (11, 12, 38). Most strikingly, the on and off rates for Tmod3 at pointed ends are both predicted to be at least 3 orders of magnitude faster than the on and off rates for Tmod3 from actin monomers. Thus, once formed, the Tmod3-actin monomer complex may be fairly stable as compared with the labile association of Tmod3 with pointed ends. Such slow on and off rates are similar to those for gelsolin binding to actin monomers (42). Given the slow on rate for monomers relative to pointed ends, low concentrations of Tmod3 are still able to cap pointed ends in elongation assays, as these assays only measure initial rates. However, the slowly formed Tmod3-actin monomer complex may not participate directly in capping pointed ends, because the model predicts a very low affinity for the binding of this complex to pointed ends ($K_d$ ~125 μM).

Despite kinetic rate differences, the similar affinities of Tmod3 for actin monomers and pointed ends suggest that at steady state, relatively stable Tmod3-actin monomer complexes may coexist with transiently capped Tmod3 actin pointed ends. However, the relative amounts of Tmod3 bound to pointed
**Tmod3 Binds Actin Monomers**

**FIGURE 5. Modeling of Tmod3-actin binding kinetics.** Using BerkeleyMadonna, forward and reverse rate constants for capping and monomer binding were modeled to fit experimental data over various Tmod3 concentrations (indicate in μM on graphs). Either barbed end (A) or pointed end (B) elongation reactions were modeled individually. Smooth black lines represent modeled reactions, which are superimposed onto the experimental data. The reactions modeled are shown with Tmod concentrations listed adjacent to each curve. C, kinetic constants derived from each reaction set modeled. The barbed end reactions were modeled to derive monomer interaction kinetics (see "Experimental Procedures"). These values for Tmod3:monomer binding were then used to model pointed end capping. The values for Tmod3:actin P-end Cap are those derived for the Tmod3-actin monomer complex (TA) to cap pointed ends. For pointed end elongation models, average maximum relative error = 1.8 x 10^-2, average root mean square (relative) = 3.13 x 10^-5. For barbed end reactions average maximum relative error = 8 x 10^-5, root mean square (relative) = 2.52 x 10^-2. Values represent averages with ranges derived from fits of multiple experimental curves. D, kinetic equations used for the modeling process. T is Tmod3 concentration at time t; A is free monomer concentration at time t; TA is Tmod3-actin complex, and NA and Nb represent free pointed end and barbed end concentrations, respectively.

ends versus actin monomer would depend greatly on the concentrations of free monomer and free tropomyosin-actin pointed ends, as compared with filament ends without tropomyosin. Our model also predicts a slightly slower on and off rate for Tmod3 at the filament pointed end than was previously calculated for Tmod1 (12).

**Tmod3 Is Efficiently Cross-linked to Actin Monomers with EDC/NHS**—The stability of the Tmod3-actin monomer complex suggested that we could use chemical cross-linking to prepare covalent Tmod3-actin complexes for structural interaction analyses. Therefore, we performed cross-linking experiments using EDC and NHS to form zero-length covalent cross-links. When combined in equimolar concentrations, Tmod3 and actin can be covalently cross-linked within 20 min at room temperature to a complex with an apparent molecular mass of ~80 kDa, consistent with a 1:1 stoichiometry of the Tmod3-actin complex (Fig. 6A). The broad mobility of the cross-linked complexes is most likely because of variable reaction with the EDC/NHS leading to formation of multiply cross-linked species. Furthermore, both Tmod3 and actin are found in this complex, as demonstrated by immunoblot (Fig. 6A). Compared with other actin monomer binding proteins such as profilin (33), the cross-linking of actin and Tmod3 occurred with remarkable efficiency, often approaching 100%. This efficiency also suggests that a 1:1 complex is the predominant species being cross-linked, rather than small oligomers of actin. This is further supported by the fact that Tmod3 was efficiently cross-linked to either Ca^2+^-actin or Mg^2+^-actin (Fig. 6A, lane set 1 versus 2), in low salt conditions unfavorable for filament formation. By contrast, Tmod1 is not cross-linked to actin under these conditions (Fig. 6B).

To further preclude the possibility of filament nucleation occurring during the cross-linking reaction, reactions were also performed in the presence of high concentrations of LatA. LatA prevents actin polymerization via monomer sequestration, through binding to the cleft between subdomains 2 and 4 resulting in a "closed" monomer conformation (43, 44). Preincubation with 10 μM LatA had no effect on formation of the Tmod3-actin cross-linked complex (data not shown), suggesting that Tmod3 does not require nucleation of small filaments to be able to cross-link to actin.

For most work using purified actin, skeletal muscle α-actin is used, because of the ease of isolation of large quantities of α-actin from muscle (27). However, because non-muscle cells contain a mixture of β- and γ-actin, we confirmed that Tmod3 could be efficiently cross-linked to purified non-muscle actin under the same conditions as above. As shown in Fig. 6B, Tmod3 but not Tmod1 is efficiently cross-linked to non-muscle (β,γ)-actin as well as α-actin. The data further support the fact that Tmod3 is able to bind to non-muscle cytoplasmic actin monomers, consistent with our observations in cell lysates (Fig. 4).

**The Pointed End Capping LRR Domain Is Not Required for Monomer Binding**—All Tmod isoforms found to date contain a conserved LRR domain, which is structurally conserved from *C. elegans* to humans (15–17). This domain enables Tmod1 to cap actin filament pointed ends in the absence of tropomyosin (15). To determine whether this domain was required for actin monomer binding by Tmod3, we made two truncation mutants of Tmod3 that lacked this domain. We based our mutagenesis
Tmod3 Binds Actin Monomers

strategy on the known structural features of Tmod1 (22). In the N-terminal portion of Tmod1, there are two α-helices that may be important for tropomyosin binding, and sequence analysis suggested that these helices are likely to occur in Tmod3 as well. Therefore, one of the truncation mutants is terminated shortly after the second helix (Tmod3-(1–92)). The other truncation mutant codes for the entire N-terminal portion of the Tmod3 protein but lacks the C-terminal LRR domain. In cross-linking assays, both of these truncated forms of Tmod3 were cross-linked efficiently to actin, each with an apparent stoichiometry of 1:1 (Fig. 6C), as indicated by the expected molecular weights (~62 kDa for Tmod3-(1–189) and ~50 kDa for Tmod3-(1–92)). The cross-linked species observed with the Tmod3-(1–92) fragment produced a more focused SDS-polyacrylamide gel band, suggestive of less molecular heterogeneity, as compared with complexes formed with either the full-length Tmod3 or the 1–189 fragment.

To test whether either of these truncation mutants was able to bind to and sequester monomers at steady state, actin was polymerized to steady state and incubated in the presence of either full-length or truncated forms of Tmod3 or Tmod1 (Fig. 7, A and B). When excess molar ratios of Tmod3-(1–92) or Tmod3-(1–189) are incubated with a range of actin concentrations, a significant increase in the apparent critical concentration is observed (as determined by the intercept with the G-actin fluorescence), consistent with monomer binding (Fig. 7A), similar to what is observed with the full-length Tmod3 molecule. As anticipated, similar truncation mutants of Tmod1 did not have this effect, consistent with the inability of full-length Tmod1 to bind monomer (Fig. 7A). When the ability of these Tmod3 fragments to sequester monomer is analyzed as a function of concentration (Fig. 7B), the smaller fragment exhibited a decreased ability to sequester monomers, whereas the larger fragment retained essentially full monomer sequestering ability. We therefore conclude that the C-terminal actin capping domain is dispensable for monomer sequestration. Furthermore, the N-terminal region (1–92) in Tmod3 that is homologous to the tropomyosin-actin capping region in Tmod1 is not sufficient to confer full monomer binding activity but requires portions of the central region of Tmod3 between residues 92 and 189.

Identification of Potential Interacting Peptides in the Tmod3-Actin Complex—To identify interaction sites on actin and Tmod3, we took advantage of the high efficiency of the cross-linking reaction to further analyze the complex by in-gel trypsin digestion followed by either microbore HPLC peptide mapping with detection by absorbance at 214 nm and analysis of peaks by MALDI-MS (UV peptide mapping) or by capillary HPLC-tandem mass spectrometry (LC-MS/MS peptide mapping). Data from the cross-linked complex were compared with controls where the individual proteins were reacted with the cross-linker under identical conditions, as well as the individual proteins without any cross-linker. Peptides identified by LC-MS/MS analysis of the cross-linked complex were compared with those identified in the control samples. Most expected tryptic peptides within the size range typically detected by LC-MS were identified by multiple MS/MS spectra in both the complex and the pertinent control samples, with several striking exceptions. Actin tryptic peptides that were not observed in the complex peptide maps were residues 148–177, which forms a β-sheet in subdomain 3, and residues 216–238, which forms an exposed loop in subdomain 4 (Fig. 8, A and B). Although always absent from the peptide maps of the complex, these peptides were consistently observed in the actin with cross-linker alone control, indicating they were modified only in the presence of Tmod3 (i.e. cross-linked to Tmod3). In contrast, all other actin peptides that were observed in the peptide maps of the complex were also observed in the actin only control. Furthermore, UV peptide mapping results were consistent with the LC-MS/MS results. Unfortunately, neither method resulted in direct identification of Tmod3-actin cross-linked peptides. However, this is not surprising based on the sizes of peptides that disappeared after cross-linking, because any cross-linked peptide complexes would be too big to elute efficiently from either the gel slices after digestion or from the reverse phase column. Consistent with this prediction, a series of very low yield, late eluting peaks were consistently observed on UV HPLC peptide maps of all digests of the complex that
were absent from all controls. Repeated attempts to identify these peaks were not successful because of their very low yield.

We also identified two putative cross-link sites within the Tmod3 molecule using the LC-MS/MS peptide mapping approach, the position of which also suggests an extended Tmod3-actin interface. These tryptic peptides were residues 31–51 and residues 149–169 (Fig. 8B). The putative cross-link site was further localized to residues 31–40 because a peptide corresponding to residues 41–51 was detected in both the complex and control when the LC-MS/MS data were analyzed using a no-enzyme specificity control. These shorter peptides presumably formed because of in-source fragmentation at the Asp-Pro bond at this site. The residue 31–51 tryptic peptide was also consistently detected on Tmod3 control peptide maps but not on cross-linked Tmod3-actin complex peptide maps using UV peptide mapping. The cross-linked 31–40 peptide of Tmod3 overlaps with an amphipathic α-helix identified by two-dimensional NMR in the homologous region of Tmod1 (residues 24–35 of Tmod1) that has been shown to be important for interaction with tropomyosin but may be dispensable for tropomyosin-actin capping (21, 22). The 149–169 peptide does not overlap with either the tropomyosin binding domain at the N terminus or the actin pointed end-capping domain at the C terminus of Tmods (Fig. 8B). These data corroborate our mutagenesis experiments and suggest that both regions are involved in actin monomer binding.

DISCUSSION

Our results demonstrate that the broadly expressed pointed end capping protein Tmod3 (10) binds to actin monomers. We have shown that Tmod3 sequesters monomers in both steady state conditions and under conditions of barbed end elongation in pyrenyl-actin assays. In addition, we have demonstrated that Tmod3 binds to cytoplasmic actin monomers in the context of cell lysates. Finally, we demonstrate that Tmod3 can be remarkably efficiently cross-linked to actin monomers. We further show that this monomer binding activity is observed with Tmod3 but not the muscle isoforms Tmod1 or Tmod4. This represents the first significant functional divergence in actin binding observed between Tmod isoforms and therefore suggests that Tmod3 may regulate actin dynamics differently from other Tmods.

Global sequence comparison of Tmod3 and Tmod1 does not reveal obvious large domains or regions of significant divergence to explain their dramatically different monomer binding abilities. Nevertheless, structural data and localized sequence comparison do provide some clues to the isoform differences. For example, there are some potential differences in secondary structure between the two, such as differences in α-helix 1, which is formed by residues 24–35 in Tmod1 (21, 22). By sequence homology this helix is formed by residues 28–39 in Tmod3. When viewed along the helical axis, the Tmod1 helix is amphipathic in organization, whereas the Tmod3 is more disordered in its charge distribution. Similarly, the 149–169-residue peptide from Tmod3 represents a region of diversity between Tmod3 and Tmod1 with 8 nonconservative amino acid substitutions out of 21 total residues (Fig. 8C) (39), and is of previously unknown function. Unfortunately, structural data on this region are currently lacking, and no specific secondary structure has been found. Furthermore, it must be kept in mind that the peptides found only represent those available for cross-linking, and not necessarily the entire binding site interface of the Tmod3-actin complex.

Indeed, our data suggest that Tmod3 interacts with actin over an extended interface, potentially spanning multiple sub-
domains of the actin monomer. On the actin molecule, both the cleft between subdomains 1 and 3 and a portion of subdomain 4 may be bound by Tmod3. The observed potential pairs of cross-linked peptides on actin as well as on Tmod3 each exist at a significant molecular distance from each other. Such an extended interface of interaction between actin and Tmod3 could explain the low on and off rates predicted for complex formation (Fig. 5).

It is interesting to note that although the C-terminal globular domain of Tmod1 is tightly folded into a leucine-rich repeat domain (16), the N-terminal domain, including the regions homologous to those identified in Tmod3 here as likely contact sites with actin, is unstructured and may allow the molecule a 115-Å length in solution (14). This could enable Tmod3 to interact with the monomer in such an extended interface on the actin. However, we cannot exclude the possibility that multiple species of Tmod3-actin complex exist, thus confounding these models.

Although actin monomer binding proteins are numerous (at least 25 in mammals), they can be functionally described in six classes (7). Of these, most use a t/H4/WH2 domain for actin binding, which can be used to either promote assembly or sequester monomers (9). Interestingly, this domain is a structural motif shared by many actin-binding proteins, including perhaps actin itself (45). In addition to the three classes that use t/H4/WH2 motifs, two classes use ADF domains, whereas profilins use a domain unique to profilin (Ref. 7 and references therein). We were able to uncover no linear sequence similarities in any portion of Tmod3 to other monomer binding proteins that would place it in any of these classes. Comparison of the locations of Tmod3 interaction sites on actin with the binding interfaces of other actin monomer binding proteins is more instructive and reveals some commonalities and some differences, suggesting Tmod3 is a novel type of actin monomer binding protein. For example, when the putative Tmod3-binding peptide (residues 148–177) in subdomain 3 of actin is represented on a surface model of either the profilin-actin complex (PDB code 2BTF (46)), the tβ4-actin complex (PDB code 1T44 (47)), or the D-binding protein-actin complex (PDB code 1MA9 (48)), it becomes clear that at least a portion of the site is masked by this

![Diagram of Tmod3 and actin interaction](image)

**FIGURE 8. Putative cross-linked peptides identified from Tmod3-actin complex identified by LC/MS.** Tryptic peptides were eluted from SDS-polyacrylamide gel slices and subjected to LC/MS analyses, leading to the identification of two peptides each from actin and Tmod3 that were likely to be cross-linked. A, the Schutt actin model (PDB code 2BTF (46)), with the candidate peptides highlighted in yellow. Residues 148–177 form a β-sheet surface in subdomain 3, whereas residues 216–238 form an α-helix loop in subdomain 4. Subdomains are noted in roman numerals. B, space-filling model of the actin monomer shown in A, with the same identified residues shown in yellow. C, Tmod3 peptides identified from the same cross-linked complexes, mapped onto the domain structure of Tmod1 based on data from Tmod1. The sequences of the two identified peptides are shown on top, in comparison to the corresponding Tmod1 peptides on bottom. The α-helices listed have been previously identified in structural studies of Tmod1 (16, 21, 22). LRR domain indicates the leucine-rich repeat domain (16). A diagram of the two truncation mutants analyzed in Figs. 6 and 7 is shown.
Tmod3 Binds Actin Monomers

class of proteins. This may partly explain the ability of Tmod3 to compete with tβ4– (1–25), which binds in this pocket between subdomains 2 and 3 (47).

However, unlike these other actin monomer binding proteins, Tmod3 monomer binding affinity was not sensitive to actin nucleotide status (less than 4-fold) or to LatA binding, both of which are known to induce substantial conformational changes in the monomer conformation (45, 49). In addition to the potential shared site with these other actin-binding proteins in subdomain 3, there is at least a second putative Tmod3-binding actin peptide (residues 216–238) in subdomain 4. This region of actin is thought to be involved in subunit-subunit interactions in the filament (5) but has not been shown to be involved in interactions with any known actin monomer binding proteins and is not thought to undergo a dramatic conformational change between nucleotide states (45).

Although Tmod3 binding to these regions is consistent with the ability of Tmod3 to sequester monomers from polymerization, it is somewhat puzzling, because Tmod3 is able to nucleate filaments as well. In a nucleating complex, one would expect that interference between subunits across the filament, as suggested by the binding region on subdomain 4, would be counterproductive. It is likely that the ability of Tmod3 to nucleate actin assembly requires interaction of Tmod3 with sites on actin that were not observed in the cross-linked complex, potentially because of lack of EDC/NHS-reactive residues in close proximity. Interestingly, like Tmod3, Tmod1 was also reported to have a weak actin nucleating activity that was located in the C-terminal LRR domain (15), which our data suggest is dispensable for monomer sequestration.

It is intriguing that Tmod3 binds to monomers, whereas Tmod isoforms 1 and 4 do not. Tmod3 is broadly expressed in cells containing dynamic actin filament systems and lacking actin filament architectures with regulated lengths (10). Because the monomer binding affinity of Tmod3 is very close to that for the pointed ends of pure actin filaments, it is possible that Tmod3 does not significantly bind to actin filament pointed ends in vivo, assuming that the pool of free actin monomers greatly exceeds the number of free pointed ends in the cell. However, when actin filaments are co-polymerized with tropomyosin, the affinity of Tmod3 for actin filament pointed ends is increased more than 1000-fold (11, 12, 38). Therefore, it is possible that Tmod3 capping is targeted specifically to tropomyosin–actin pointed ends and not actin filaments free of tropomyosin. This would tend to stabilize tropomyosin–actin filaments, while allowing actin filaments without tropomyosin to be depolymerized more readily. Given that the free monomer concentration greatly exceeds the concentration of free pointed ends, the predicted slow on and off rates for Tmod3 from the monomer would suggest that a pool of free actin monomers could effectively buffer Tmod3 from capping pointed ends. In fact, in situations where free monomer pools are high, this buffering would be enhanced such that even fewer pointed ends would be capped, creating a negative feedback loop for capping. In situations where free monomer pools are reduced or actin has been stabilized into tropomyosin-coated filaments, Tmod3 capping activity would then be increased. Whether or not this model holds true, it will be interesting to uncover how Tmod3 binding to monomer contributes to the overall monomer turnover and regulation in the context of competing proteins such as profilin, tβ4, and cofillin.

Acknowledgments—We are grateful to J. Moyer and J. Palomique for technical assistance. We thank H. Higgs, J. Cooper, and R. Dominguez for helpful discussions.

REFERENCES

1. Carlier, M. F., Le Clainche, C., Wiesner, S., and Pantaloni, D. (2003) BioEssays 25, 336–345
2. Yarmola, E. G., and Bubb, M. R. (2004) J. Biol. Chem. 279, 33519–33527
3. Babcock, G. G., and Fowler, V. M. (1994) J. Biol. Chem. 269, 364–370
4. Blondin, L., Sapountzi, V., Maciver, S. K., Lagarrigue, E., Benyamin, Y., and Roustan, C. (2002) Eur. J. Biochem. 269, 4194–4201
5. Aguda, A. H., Burtnick, L. D., and Robinson, R. C. (2005) EMBO Rep. 6, 220–226
6. Wear, M. A., and Cooper, J. A. (2004) Trends Biochem. Sci. 29, 418–428
7. Paaviilainen, V. O., Bértling, E., Falck, S., and Lappalainen, P. (2004) Trends Cell Biol. 14, 386–394
8. Harris, E. S., and Higgs, H. N. (2006) Methods Enzymol. 406, 190–214
9. Hertzog, M., van Heijenoort, C., Didry, D., Gaudier, M., Coutant, J., Gigant, B., Dideлот, G., Preat, T., Knossow, M., Guittet, E., and Carlier, M. F. (2004) Cell 117, 611–623
10. Fischer, R. S., and Fowler, V. M. (2003) Trends Cell Biol. 13, 593–601
11. Weber, A., Pennise, C. R., Babcock, G. G., and Fowler, V. M. (1994) J. Cell Biol. 127, 1627–1635
12. Weber, A., Pennise, C. R., and Fowler, V. M. (1999) J. Biol. Chem. 274, 34637–34645
13. Krieger, I., Kostyukova, A. S., and Maeda, Y. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 743–744
14. Fujisawa, T., Kostyukova, A., and Maeda, Y. (2001) FEBS Lett. 498, 67–71
15. Fowler, V. M., Greenfield, N. J., and Moyer, J. (2003) J. Biol. Chem. 278, 40000–40009
16. Krieger, I., Kostyukova, A., Yamashita, A., Nitanai, Y., and Maeda, Y. (2002) Biophys. J. 83, 2716–2725
17. Lu, S., Li, S., Carson, M., Chen, L., Meehan, E., and Luo, M. (2004) Proteins 56, 384–386
18. Greenfield, N. J., and Fowler, V. M. (2002) Biophys. J. 82, 2580–2591
19. Kostyukova, A., Maeda, K., Yamashita, E., Krieger, I., and Maeda, Y. (2000) Eur. J. Biochem. 267, 6470–6475
20. Kostyukova, A. S., and Hitchcock-DeGregori, S. E. (2004) J. Biol. Chem. 279, 5066–5071
21. Greenfield, N. J., Kostyukova, A. S., and Hitchcock-DeGregori, S. E. (2005) Biophys. J. 88, 372–383
22. Kostyukova, A. S., Rapp, B. A., Choy, A., Greenfield, N. J., and Hitchcock-DeGregori, S. E. (2005) Biochemistry 44, 4905–4910
23. Littlefield, R., Almenar-Queralt, A., and Fowler, V. M. (2001) Nat. Cell Biol. 3, 544–551
24. Chu, X., Chen, J., Reedy, M. C., Vera, C., Sung, K. L., and Sung, L. A. (2003) Am. J. Physiol. 284, H1827–H1838
25. Fritz-Six, K. L., Cox, P. R., Fischer, R. S., Xu, B., Gregorio, C. C., Zoghbi, H. Y., and Fowler, V. M. (2003) J. Cell Biol. 163, 1033–1044
26. Fischer, R. S., Fritz-Six, K. L., and Fowler, V. M. (2003) J. Cell Biol. 161, 371–380
27. Pardee, J. D., and Spudich, J. A. (1982) Methods Cell Biol. 24, 271–289
28. Babcock, G. G., and Fowler, V. M. (1994) J. Biol. Chem. 269, 27510–27518
29. Young, C. L., Southwick, F. S., and Weber, A. (1990) Biochemistry 29, 2232–2240
30. Zigmund, S. H., Joyce, M., Yang, C., Brown, K., Huang, M., and Pring, M. (1998) J. Cell Biol. 142, 1001–1012
31. Sampath, P., and Pollard, T. D. (1991) Biochemistry 30, 1973–1980
32. Lal, A. A., Korn, E. D., and Brenner, S. L. (1984) J. Biol. Chem. 259, 8794–8800
33. Vandekerckhove, J. S., Kaiser, D. A., and Pollard, T. D. (1989) J. Cell Biol. 109, 619–626
34. Speicher, M. R., Petersen, S., Uhrig, S., Jentsch, I., Fauth, C., Els, R., and Petersen, I. (2000) Lab. Invest. 80, 1031–1041
35. Kuhn, J. R., and Pollard, T. D. (2005) Biophys. J. 88, 1387–1402
36. Carlier, M. F., Jean, C., Rieger, K. J., Lenfant, M., and Pantaloni, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5034–5038
37. Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H., and Pantaloni, D. (1997) J. Cell Biol. 136, 1307–1322
38. Almenar-Queralt, A., Lee, A., Conley, C. A., Ribas de Pouplana, L., and Fowler, V. M. (1999) J. Biol. Chem. 274, 28466–28475
39. Cox, P. R., and Zoghbi, H. Y. (2000) Genomics 63, 97–107
40. Yarmola, E. G., Parikh, S., and Bubb, M. R. (2001) J. Biol. Chem. 276, 45555–45563
41. Mattila, P. K., Quintero-Monzon, O., Kugler, J., Moseley, J. B., Almo, S. C., Lappalainen, P., and Goode, B. L. (2004) Mol. Biol. Cell 15, 5158–5171
42. Selve, N., and Wegner, A. (1987) Eur. J. Biochem. 168, 111–115
43. Morton, W. M., Ayscough, K. R., and McLaughlin, P. J. (2000) Nat. Cell Biol. 2, 376–378
44. Ayscough, K. R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D. G. (1997) J. Cell Biol. 137, 399–416
45. Dominguez, R. (2004) Trends Biochem. Sci. 29, 572–578
46. Schutt, C. E., Myslik, J. C., Rozyczki, M. D., Goonesekere, N. C., and Lindberg, U. (1993) Nature 365, 810–816
47. Irobi, E., Aguda, A. H., Larsson, M., Guerin, C., Yin, H. L., Burtnick, L. D., Blanchon, L., and Robinson, R. C. (2004) EMBO J. 23, 3599–3608
48. Otterbein, L. R., Cosio, C., Graceffa, P., and Dominguez, R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8003–8008
49. Bubb, M. R., Govindasamy, L., Yarmola, E. G., Vorobiev, S. M., Almo, S. C., Somasundaram, T., Chapman, M. S., Agbandje-McKenna, M., and McKenna, R. (2002) J. Biol. Chem. 277, 20999–21006