Structural Requirements of Tropomodulin for Tropomyosin Binding and Actin Filament Capping†

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ABSTRACT: Regulation of actin filament dynamics underlies many cellular functions. Tropomodulin together with tropomyosin can cap the pointed, slowly polymerizing, filament end, inhibiting addition or loss of actin monomers. Tropomodulin has an unstructured N-terminal region that binds tropomyosin and a folded C-terminal domain with six leucine-rich repeats. Of tropomodulin 1's 359 amino acids, an N-terminal fragment ($Tmod1_{1-92}$) suffices for in vitro function, even though the C-terminal domain can weakly cap filaments independent of tropomyosin. Except for one short α-helix with coiled coil propensity (residues 24−35), the $Tmod1_{1-92}$ solution structure shows that the fragment is disordered and highly flexible. On the basis of the solution structure and predicted secondary structure, we have introduced a series of mutations to determine the structural requirements for tropomyosin binding (using native gels and CD) and filament capping (by measuring actin polymerization using pyrene fluorescence). $Tmod1_{1-92}$ fragments with mutations of an interface hydrophobic residue, L27G and L27E, designed to destroy the α-helix or coiled coil propensity, lost binding ability to tropomyosin but retained partial capping function in the presence of tropomyosin. Replacement of a flexible region with α-helical residues (residues 59−61 mutated to Ala) had no effect on tropomyosin binding but inhibited the capping function. A mutation in a region predicted to be an amphipathic helix (residues 65−75), L71D, destroyed the capping function. The results suggest that molecular flexibility and binding to actin via an amphipathic helix are both required for tropomyosin-dependent capping of the pointed end of the actin filament.

The actin cytoskeleton is central to many biological functions including muscle contraction, determination of cellular architecture, cell migration, and organelle transport (*1*, *2*). Actin filaments are dynamic and are the downstream targets of extracellular and intracellular signals that influence the state of assembly. To carry out these functions, actin filaments bind to other proteins that determine the rates of assembly and disassembly, mechanical stability, and assembly into higher order structures.

Actin filaments are polar; the fast growing (barbed or plus) and slow growing (pointed or minus) ends differ in structure and dynamic properties (*3*). The rapid polymerization of actin filaments can lead to protrusion of lamellipodia or filopodia during processes such as cell crawling and chemotaxis (*2*). Filaments in other regions of the cell, as in stress fibers, the terminal web of epithelial cells, and the sarcomeres of striated muscle, are more stable. Proteins that cap the barbed or pointed end can stabilize actin filaments, as can proteins that bind along the length, such as tropomyosin (TM)[1] (*4*−*6*). At present, several capping proteins are known for the barbed end, including gelsolin and capZ, but tropomodulin is the only known capping protein for the pointed end (*7*, *8*).

Originally discovered as a TM-binding protein in erythrocytes (*9*), tropomodulin is a family of widely expressed proteins, encoded by four genes, that exhibit isoform-specific affinities for different TM isoforms (*10*−*13*). Tropomodulin is localized at the pointed ends of actin filaments in the sarcomeres of striated muscle, in erythrocytes, and in cultured cells (*14*, *15*). Its presence is required for regulation of thin filament length in striated muscle (*16*). Although it can effectively cap the pointed end in vitro (with TM), in living cardiac myocytes both actin and tropomodulin may exchange with free molecules at the pointed end of sarcomeric thin filaments (*17*).

The most extensive analysis has been of the erythrocyte form (Tmod1, E-Tmod), the form expressed in the membrane cytoskeleton of erythrocytes and the sarcomeres of striated muscle. Tropomodulin 1 is an elongated molecule consisting of two domains (*18*). The C-terminal half of the tropomodulin molecule consists of one compact, cooperatively melting domain. The structure of the C-terminal domain, determined using X-ray crystallography, contains a leucine-rich repeat motif and is a right-handed super helix composed of alternate α-helices and *β*-strands (*19*). The C-terminal domain binds actin; at high concentrations (280 nM) it can cap the pointed end of filaments in the absence of TM, and weakly nucleates polymerization (*20*).

In contrast, the N-terminal half has no cooperatively melting structure; it is flexible and disordered and becomes more structured upon binding TM (*13*, *21*, *22*). The N-

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[1] Abbreviations: TM, tropomyosin.

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terminal domain is required to maintain stable thin filaments in cardiac myocytes (*17*). Fragments as short as the first 92 residues can cap the pointed end of actin filaments in the presence of TM, whereas a fragment of the first 48 residues can bind TM, but does not cap actin filaments (*23*).

The structure of the first 92 residues ($Tmod1_{1-92}$), determined using NMR and CD (*24*), is mainly disordered with one helical region, residues 24−35, within a sequence with coiled coil propensity (residues 23−38). Residues 55−62, a region with prolines that exhibit cis/trans isomerization, and the C-terminal 17 residues are flexible. In addition, residues 65−75 have the sequence of an amphipathic helix, but they are not helical in the solution structure. NMR resonances from residues 1−38 become greatly displaced and/or broadened upon addition of a TM peptide that binds to $Tmod1_{1-92}$, showing that this is the TM-binding site. Destruction of the coiled coil propensity by mutation of an interface Leu to Glu or Gly results in the loss of TM binding. On the basis of our data, we suggested that residues 48−92 may be important for capping.

In the present paper we have used the insights gained from the NMR structure, and the predicted motifs, to address the requirements of tropomodulin for binding to TM and for TM-dependent capping of the actin filament. Using site-directed mutagenesis, we have defined the requirement for an α-helical coiled coil in the TM-binding domain and the importance of flexibility and the amphipathic helix within the predicted actin-capping region. The results suggest that mutations critical for TM/tropomodulin binding in the absence of actin have only small effects on capping activity, and that molecular flexibility and binding to actin via an amphipathic helix are both required for TM-dependent capping of the pointed end of the actin filament.

MATERIALS AND METHODS

Constructions of Expression Vectors of Mutated Tropomodulin N-Terminal Fragments. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The plasmids were amplified by PCR using *PfuTurbo* DNA polymerase and two complementary sets of oligonucleotides. The plasmids for the N-terminal fragment of chicken E-tropomodulin (Tmod1), named *pET(His)*$Tmod1_{1-92}$ (*23*) and *pET(His)*$Tmod1_{1-92}$ *(L27G)* (*24*) were used as the templates.

To change Leu71 to aspartate, the oligonucleotides were 5′-GAA GAA CTC ATG GCC CAC *GAT* GAG CAG CAG GCG AAA GAC-3′ and 5′-GTC TTT CGC CTG CTG CTC *ATC* GTG GGC CAT GAG TTC TTC-3′. To change Thr59, Gly60, and Pro61 to alanines, the oligonucleotides were 5′-CG CAA AAG CCG CCA *G*CC G*C*C *G*Cg TTC AAA AGG G-3′ and 5′-C CCT TTT GAA CG*C* G*G*C GG*C* TGG CGG CTT TTG CG-3′. Mutated nucleotides are italic.

The original plasmid was digested using *Dpn*I, and the mixture was used to transform *Escherichia coli* (DH5α). After plasmid purification, the presence of the mutations was confirmed by sequencing of the full tropomodulin fragment. Synthesis of all oligonucleotides and sequence determination were done at the UMDNJ DNA Synthesis and Sequencing Facility (Robert Wood Johnson Medical School (RWJMS), Piscataway, NJ).

Protein Expression and Purification. Wild-type $Tmod1_{1-92}$ and mutant tropomodulin fragments $Tmod1_{1-92}$ (L27G) and $Tmod1_{1-92}$ (L27E) (*24*), $Tmod1_{1-92}$ (T59A/G60A/P61A), $Tmod1_{1-92}$ (L71D), and $Tmod1_{1-92}$ (L27G/L71D) were overexpressed in *E. coli* BL21(DE3)pLysE and purified on Ni−NTA agarose according to the method previously described (*23*). The presence of mutated amino acids in the expressed peptides used for our experiments was confirmed by electrospray mass spectroscopy (Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT). The molecular masses of mutant tropomodulin fragments were found to be 11820 Da for $Tmod1_{1-92}$ (L27E), 11748 Da for $Tmod1_{1-92}$ (L27G), 11763 Da for $Tmod1_{1-92}$ (T59A/G60A/P61A), 11807 Da for $Tmod1_{1-92}$ (L71D), and 11751 Da for $Tmod1_{1-92}$ (L27G/L71D), which was in good agreement with the corresponding calculated masses 11820.1, 11748.1, 11762.1, 11806.1, and 11750 Da.

Chicken pectoral skeletal muscle actin was purified from acetone powder as described (*25*). G-actin was purified on a Sephacryl S-100 column (*26*) and was stored in liquid nitrogen. Actin was labeled with pyrenyliodoacetamide, and the labeling ratios were calculated according to refs *27* and *28*. The degree of the labeling was 80−99%. Before experiments, G-actin (labeled or unlabeled) was defrosted in a 37 °C water bath and then centrifuged at 100000 rpm (TLA-100, Beckman) for 30 min at 4 °C.

Rat recombinant α-tropomyosin TM5a was expressed in *E. coli* and purified as previously described (*29*). $AcTM1b_{1-19}$-Zip is a designed chimeric protein that contains 19 residues of short rat α-tropomyosin encoded by exon 1b and the 18 C-terminal residues of the GCN4 leucine zipper domain (*30*). It was synthesized by SynPep (Dublin, CA).

Protein purity was evaluated using SDS−PAGE (*31*). Native gel electrophoresis was done in 9% polyacrylamide gels that were polymerized in the presence of 10% glycerol without SDS (*24*).

Concentrations of proteins were determined by measuring their difference spectra in 6 M guanidine−HCl between pH 12.5 and pH 6.0 (*32*) using the extinction coefficients of 2357 M^{-1} cm^{-1} per tyrosine and 830 M^{-1} cm^{-1} per tryptophan (*33*).

Recombinant human gelsolin was a generous gift from Dr. Philip G. Allen (Confocal and Multiphoton Imaging Facility, Brigham and Women's Hospital & Hematology Division, LMRC, Boston, MA).

Fluorescence Measurements. The rates of actin polymerization were measured using the change in pyrene actin fluorescence (*27*) using a PTI fluorimeter (Lawrenceville, NJ) (excitation 366 nm and emission 387 nm with a 1 nm slit). To measure polymerization of actin at the pointed end, short filaments capped at the barbed ends with gelsolin were prepared by polymerization of 3 μM G-actin in the presence of 28 nM gelsolin. The G-actin cation used for polymerization was changed from Ca^{2+} to Mg^{2+} by incubation with 50 μM $MgCl_2$ and 0.2 mM EGTA for 10 min before the experiment. Polymerization was monitored by the increase in fluorescence when the filaments were diluted 5-fold with G-actin (10% pyrenylactin) in F-buffer (100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM NaN_3, 10 mM imidazole, pH 7.0) containing TM and/or tropomodulin fragments. The final concentrations of F- and G-actin were 0.6 and 1.5 μM, correspondingly.

Gelsolin-capped filaments were prepared in sets of four, and fluorescence measurements were carried out in parallel

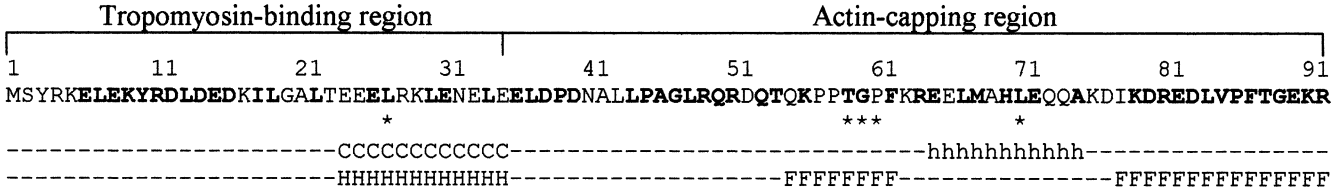

FIGURE 1: Amino acid sequence of chicken Tmod1$_{1-92}$ aligned to the secondary structure predicted (second row) and obtained from NMR data (third row): C, predicted coiled coil; H, observed helix; h, predicted helix; F, observed flexible fragment; bold, amino acids conserved among tropomodulin isoforms; asterisks, mutated amino acids.

in a four-cuvette holder with actin as a control in each set as described (*23*). Exponential curves were fit to the polymerization data using SigmaPlot, and initial rates (*R*) were calculated as the first derivatives at time zero.

Circular Dichroism Measurements. CD measurements were made using an Aviv model 215 spectropolarimeter (Lakewood, NJ) as previously described (*34*, *35*). The protein concentrations were 10 μM in 100 mM NaCl, 10 mM sodium phosphate, pH 6.5. The conformation of the Tmod peptides did not change as a function of temperature, and the folding and unfolding of the AcTM1b$_{1-19}$Zip peptide and the Tmod/TM peptide complexes were fully reversible. Therefore, it was possible to determine the binding constants of the Tmod/AcTM1b$_{1-19}$Zip complexes, *K*, using the relationship $K = \exp(-\Delta\Delta G/RT)$, where $\Delta\Delta G$ is the difference in free energy of folding of the complex minus that of the AcTM1b$_{1-19}$Zip peptide alone, *R* is the gas constant, and *T* is the temperature (*36*). The equations used for determining the binding constants and the thermodynamics of folding of the two-chain TMZip peptide and the three-chain TMZip/Tmod peptide complex from the changes in their circular dichroism as a function of temperature have been described in detail previously (*13*, *35*).

RESULTS

Mutant Design. The focus of our investigation is Tmod1$_{1-92}$, the shortest known fragment that retains the major function of tropomodulin, which is the inhibition of actin filament elongation at the pointed end in the presence of TM (*23*). We designed a series of mutations based on the amino acid sequence and solution structure of Tmod1$_{1-92}$ to investigate the structural parameters important for TM binding and actin filament pointed-end capping. Figure 1 shows the TM-binding and actin-capping regions of Tmod1$_{1-92}$ with known and predicted structural information. The only defined structure in solution is the helical region, residues 24−35, which is within a region with a coiled coil pattern of hydrophobic residues. We mutated the conserved Leu27, at an "a" interface position, to Gly (L27G) to interrupt the α-helix, or Glu (L27E) to destroy the hydrophobic interface while maintaining the α-helical conformation. We previously reported that these two mutations result in loss of binding to a TM model peptide (*24*).

The Tmod1$_{1-92}$ sequence contains a predicted amphipathic helix, a motif often involved in protein−protein interactions, in the C-terminal part of the peptide required for capping. One face of the helix contains side chains that are hydrophobic (L67, A68, L71, A75), while the other face contains side chains that are hydrophilic except for A69. To test the importance of this predicted helix for Tmod1 function, a conserved Leu in the predicted hydrophobic surface was mutated to Asp (L71D). This type of mutation destabilizes amphipathic helices and interferes with hydrophobic interactions (*37*). The L71D mutation was also introduced into one of the "coiled coil" mutants (L27G/L71D).

A third mutation was designed to learn the importance of the flexibility observed in residues 55−62 for pointed-end capping. Two of the three prolines in this region, Pro58 and Pro61, exhibit cis/trans isomerization (*24*), and the intervening Thr59 and Gly60 are conserved in all tropomodulins; Pro61 is conserved in most. Alanines are the best residues to promote helix formation and stabilization, while glycines and prolines are the worst (*38*). Recently, Lin et al. (*39*) showed that even very short alanine sequences have unique helix-stabilizing effects. Therefore, to make this region less flexible, three residues, T59, G60, and P61, were mutated to Ala.

Folding and TM-Binding Ability. Binding of tropomodulin fragments to a TM model peptide was directly and qualitatively analyzed using native polyacrylamide gel electrophoresis (Figure 2A, Table 1). In this assay, tropomodulin fragments are electrophoresed on nondenaturing gels alone and in combination with AcTM1b$_{1-19}$Zip, a peptide that contains the first 19 amino acids of a short TM followed by 18 C-terminal residues of a GCN4 leucine zipper sequence to stabilize the coiled coil conformation. This peptide binds tightly to wild-type Tmod1$_{1-92}$, resulting in a slower migrating band. The TM peptide alone is positively charged and does not enter the gel.

Tmod1$_{1-92}$ with mutations in the amphipathic helix (L71D) or the flexible region (T59A/G60A/P61A) formed a complex with AcTM1b$_{1-19}$Zip (Figure 2A, lanes 2 and 6), showing that these regions are not involved in TM binding, which is consistent with the NMR study. The double mutant L27G/L71D, like the L27E and L27G mutants (*24*), lost TM binding though the smear in the mixture of L27G/L71D and AcTM1b$_{1-19}$Zip (lane 4) indicates there may be residual weak binding similar to that seen with the L27G mutation alone.

The conformation and TM binding were quantitatively analyzed using CD. The helical contents calculated from the CD spectra of the Tmod1$_{1-92}$ mutants were close to that of the wild type except for L27G, which had a helical content 17 ± 1% slightly lower than that of the wild type (22%), indicating the mutation destabilized the α-helical region, as designed. In contrast, the replacement of residues 59−61 with alanine increased the helical content to 26%.

Analysis of the binding using circular dichroism spectroscopy showed that the fragments with the L71D and T59A/G60A/P61A mutations bind to the TM model peptide (Figure 2B, C). The dissociation constants estimated from the changes in ellipticity, 0.35 ± 0.08 μM for L71D and 0.36 ± 0.22 μM for T59A/G60A/P61A, were within experimental error of the wild type (0.22 ± 0.1 μM). On the basis of these

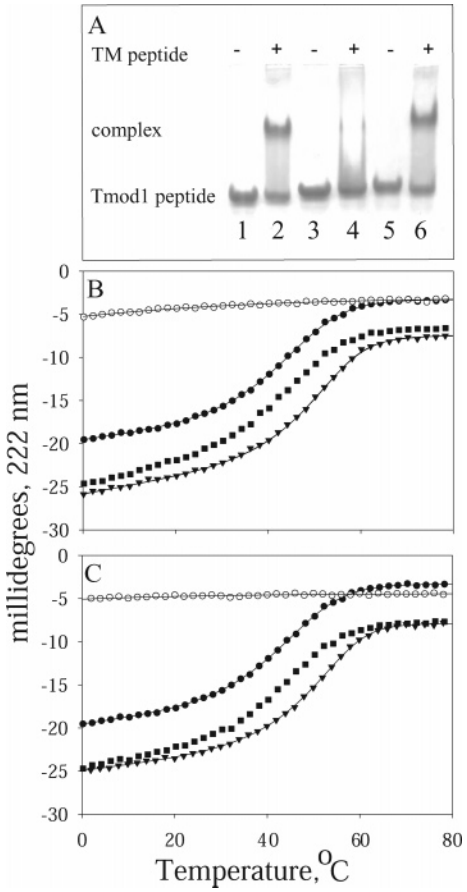

FIGURE 2: (A) Complex formation between AcTM1b$_{1-19}$Zip and Tmod1$_{1-92}$ mutants monitored by nondenaturing polyacrylamide gel electrophoresis: lane 1, L71D; lane 2, L71D and AcTM1b$_{1-19}$Zip; lane 3, L27G/L71D; lane 4, L27G/L71D and AcTM1b$_{1-19}$Zip; lane 5, T59A/G60A/P61A; lane 6, T59A/G60A/P61A and AcTM1b$_{1-19}$Zip. AcTM1b$_{1-19}$Zip is positively charged and does not enter the gel. (B, C) Binding of Tmod1$_{1-92}$ mutants to a TM model peptide measured using circular dichroism spectroscopy. The temperature dependence of the ellipticity at 222 nm was measured for unmixed and mixed AcTM1b$_{1-19}$Zip. Key: (B) T59A/G60A/P61A; (C) L71D. (○) Tmod1$_{1-92}$ mutant alone; (●) AcTM1b$_{1-19}$Zip; (■) sum of the folding curves of the AcTM1b$_{1-19}$Zip and Tmod1$_{1-92}$ mutants alone; (▼) folding of the mixture of the AcTM1b$_{1-19}$Zip and Tmod1$_{1-92}$ mutants.

results, and the direct binding assay (Figure 2), we conclude that mutations in the C-terminal part of the Tmod1 fragment do not affect TM binding. As with the L27G and L27E mutants (*24*), the L27G/L71D mutant had no detectable effect on the unfolding of AcTM1bZip (data not shown), indicating a lack of interaction.

Capping Activity of Tropomodulin Mutant Fragments. In the absence of TM Tmod1$_{1-92}$ has no effect on actin polymerization. We assayed the capping activity of Tmod1$_{1-92}$ (wild-type and mutated fragments) and Tmod1$_{38-92}$ (lacking the TM-binding site) by measuring inhibition of pointed-end elongation in the presence of a short nonmuscle TM, TM5a, that effectively blocks actin polymerization with Tmod1$_{1-92}$ (*23*). Pointed-end polymerization was nucleated by short actin filaments with gelsolin blocking the barbed ends. The actin seeds contained unlabeled actin, and the polymerization rate was measured using the initial rate of increase in fluorescence during polymerization of pyrenylactin (Figure 3).

Table 1: Tropomyosin-Binding and Capping Abilities of Tropomodulin Fragments[a]

fragment	TM binding	concn of fragment at 50% inhibition (nM)	inhibition effect at 100 nM (%)
Tmod1$_{1-92}$	+[b]	5	100
L27G	−[c]	15	93
L27E	−[c]	20	93
T59A/G60A/P61A	+	55	66
L71D	+	not reached	36
L27G/L71D	−	not reached	22
Tmod1$_{38-92}$	−[c]	not reached	none[d]

[a] Concentrations that correspond to 50% inhibition were determined from the curves in Figure 4 at $R_{exp}/R_{control} = [R_{exp}/R_{control}$ (w/o Tmod1$_{1-92}$) $- R_{exp}/R_{control}$ (for 100 nM Tmod1$_{1-92}$)]/2 = (0.89 − 0.04)/2 = 0.425. The inhibition effect (%) was calculated as [1 − [$R_{exp}/R_{control}$ (for 100 nM fragment) − $R_{exp}/R_{control}$ (for 100 nM Tmod1$_{1-92}$)]/[$R_{exp}/R_{control}$ (w/o Tmod1$_{1-92}$) − $R_{exp}/R_{control}$ (for 100 nM Tmod1$_{1-92}$)]] × 100. [b] Reference *23*. [c] Reference *24*. [d] Within experimental error of the control for actin in the presence of TM5a alone.

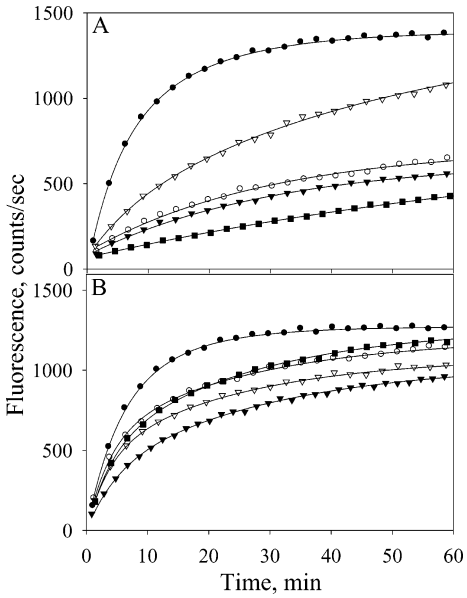

FIGURE 3: Pointed-end elongation of 2.8 nM gelsolin-capped actin filaments in the presence of TM5a and Tmod1$_{1-92}$, wild-type or mutant, in F-buffer (100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM NaN_3, 10 mM imidazole, pH 7.0). G-actin (1.5 μM, 10% pyrene-labeled) was added. Key: (A) (●) control, no TM or tropomodulin; (■) 0.5 μM TM5a and 25 nM wild-type Tmod1$_{1-92}$; (▽) 0.5 μM TM5a and 40 nM T59A/G60A/P61A; (▼) 0.5 μM TM5a and 40 nM L27G; (○) 0.5 μM TM5a and 40 nM L27E; (B) (●) control, no TM or tropomodulin; (■) 0.5 μM TM5a; (▽) 0.5 μM TM5a and 200 nM L27G/L71D; (▼) 0.5 μM TM5a and 200 nM L71D; (○) 0.5 μM TM5a and 200 nM Tmod1$_{38-92}$. Exponential growth curves were fit to the data.

Figure 3 compares pointed-end elongation in the presence of TM5a and single concentrations of wild-type and mutant Tmod1$_{1-92}$. To compare their effectiveness, the dependence of inhibition of the initial rate of polymerization on Tmod1$_{1-92}$ concentration is shown in Figure 4 and summarized in Table 1. The inhibitory activities of the Tmod$_{1-92}$ mutants in decreasing order are WT > L27G ≈ L27E > T59A/G60A/P61A > L71D > L27G/L71D. Tropomyosin alone inhibits the initial rate of polymerization by about 10%, and Tmod1$_{1-92}$ alone has no effect (Figure 4). Mutations in the flexible region (T59A/G60A/P61A) and in the predicted amphipathic helix (L71D) were the most severe, being ~10-

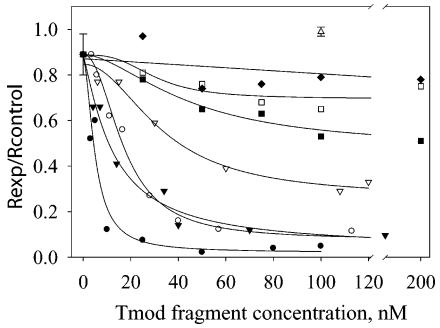

FIGURE 4: Dependence of the inhibition of actin polymerization on $Tmod1_{1-92}$ concentration in the presence of 0.5 μM TM5a. Initial rates (R) were calculated as the first derivatives at time zero after fitting. The inhibition of polymerization was calculated as $R_{exp}/R_{control}$, where $R_{control} = 1$ (in the absence of TM and Tmod1 fragments). Key: (●) $Tmod1_{1-92}$; (▼) L27G; (○) L27E; (▽) T59A/G60A/P61A; (■) L71D; (■) L27G/L71D; (◆) $Tmod1_{38-92}$; (△) the value shown for $Tmod1_{1-92}$ in the absence of TM5a is 0.99 ± 0.02 ($n = 3$); (▲) the value shown for TM5a in the absence of tropomodulin fragments is 0.89 ± 0.12 ($n = 6$).

fold and more than 50-fold, respectively, less effective than the wild type at inhibiting capping activity, even though they retained normal affinity for the TM model peptide. Mutations in the N-terminal helix in the TM-binding domain of $Tmod1_{1-92}$, L27G and L27E, unexpectedly quite effectively inhibited pointed-end polymerization in the presence of TM (~3−4-fold weaker than the wild type), but not in its absence, even though neither bound the TM model peptide (*24*). When the L27G mutation was combined with the mutation in the amphipathic helix, L27G/L71D, inhibitory activity was lost (Figure 3B). A peptide lacking the TM binding site, $Tmod1_{38-92}$, had no capping activity (Figure 3B).

DISCUSSION

The tropomodulin molecule contains two actin-capping regions, one at the N-terminus that is TM-dependent and a second at the extreme C-terminus that is TM-independent (*20*). Functional analysis of the effects of mutations in three regions of the TM-dependent actin-capping domain $Tmod1_{1-92}$ gives new insight into the structural requirements for essential tropomodulin functions.

Capping Activity. Mutations in the region of $Tmod1_{1-92}$ required for capping (residues 39−92) influence capping but not TM binding, consistent with the structural studies showing that the TM-binding domain is within residues 1−38. Although residues 65−75, which have the sequence of an amphipathic helix, do not form a stable structure visualized using NMR, the higher helical content measured using CD suggests there are additional helix-forming regions (*24*). The nearly complete loss of capping with the L71D mutation, one that would destabilize an amphipathic helix, supports the idea that the formation of such a helix upon actin binding is important for capping. Amphipathic helices are often involved in protein−protein interactions. For example, a predicted amphipathic helix was shown to be important for activation of Arp2/3 complex by WASP/Scar proteins (*40*), and an amphipathic helix present in β-thymosin/WH2 domains is important for actin binding (*41*).

The flexibility of $Tmod1_{1-92}$ would be reduced by replacing residues in a flexible region (*24*) by Ala residues with a propensity to promote a stiffer α-helix. Chou/Fasman analysis of the mutated sequence (*42*) suggests that the alanines would initiate a continuous helix from residue 59 through residue 82 that would be propagated to the putative amphipathic helical region. The increase of the helical content in T59A/G60A/P61A confirms this. The T59A/G60A/P61A mutation severely impaired the capping activity, suggesting that turns in the flexible region, which may be allowed by proline isomerization, are necessary for proper tropomodulin positioning at the pointed end. Turns may allow proper orientation of the hydrophobic surface of the amphipathic helix in relation to a binding site on the actin molecule. However important the flexible region and amphipathic helix are for capping, the C-terminal region of $Tmod1_{1-92}$ alone ($Tmod1_{38-92}$) has no effect, showing that binding to TM is required for its function at the pointed end.

Tropomyosin Binding. We previously reported that mutations in the TM-binding domain, which destroy the α-helix or the coiled coil propensity (L27G, L27E), result in a lack of binding to a TM model peptide (*24*). It came as a surprise that these mutants were able to block pointed-end elongation nearly as well as wild-type $Tmod1_{1-92}$, whereas a fragment without the first 37 residues has no capping activity. In our previous studies we showed that although TM unacetylated at the N-terminus binds only weakly to tropomodulin in solution, it enhances the ability of tropomodulin to inhibit actin polymerization at the pointed end (*23*). These results may be explained if we suppose that the binding of tropomodulin and TM to actin is cooperative and binding TM to actin changes the conformation of TM, and/or the conformation of actin, resulting in an additional binding site for tropomodulin. In any case, the TM dependence of capping by $Tmod1_{1-92}$ involves more than just the coiled coil helical region (residues 24−38). Additional residues in $Tmod1_{1-92}$ are conserved in all tropomodulins. Although they are unstructured in solution, they may be important for TM binding, as indicated by the broadening of the entire regions in the NMR with the TM model peptide binding.

The C-terminal domain of tropomodulin (residues 160−344) is well-folded, and the last 15 residues (residues 345−359) are required for low-affinity, TM-independent capping by the C-terminal domain, suggesting direct binding to actin (*20*, *23*). The binding of tropomodulin to the pointed end in the presence of TM is most likely regulated by other proteins. One possibility is that the C-terminus, which contains an LRR motif associated with protein−protein interactions (*43*), binds to proteins that further regulate the actin filament, or the activity of tropomodulin itself. For example, nebulin, the thin filament "ruler" found in skeletal muscle, binds to the C-terminal domain (*19*, *44*). Recently it was reported that thymosin β-10, a G-actin-binding protein, also binds to tropomodulin (*45*). Further investigation into binding partners for tropomodulin may give new insights into tropomodulin function and the mechanisms of regulation of actin dynamics by tropomodulin.

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