Published in final edited form as:

J Mol Biol. 2007 September 21; 372(3): 608–618.

Molecular basis of tropomyosin binding to tropomodulin, an actin capping protein

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Summary

The tropomodulins are a family of proteins that cap the pointed, slow-growing end of actin filaments and require tropomyosin for optimal function. Earlier studies identified two regions in Tmod1 that bind the N terminus of tropomyosin, though the ability of different isoforms to bind the two sites is controversial. We used model peptides to determine the affinity and define the specificity of the highly-conserved N termini of three short, non-muscle tropomyosins (α , γ , δ -TM) for the two Tmod1 binding sites using circular dichroism spectroscopy, native gel electrophoresis, and chemical crosslinking. All tropomyosin peptides have high affinity to the second Tmod1 binding site (within residues 109–144; α -TM, 2.5 nM; γ -TM, δ -TM, 40–90 nM), but differ >100- fold for the first site (residues 1–38; α -TM, 90 nM; undetectable at 10 μ M, γ -TM, δ -TM). Residue 14 (R in α ; Q in γ , δ), and to a lesser extent, residue 4 (S in α ; T in γ , δ) are primarily responsible for the differences. The functional consequence of the sequence differences is reflected in the more effective inhibition of actin filament elongation by full-length α -TMs than γ -TM in the presence of Tmod1. The binding sites of the two Tmod1 peptides on a model tropomyosin peptide differ, as defined by comparing $^{15}\text{N}/^1\text{H}$ HSQC spectra of a ^{15}N -labeled model tropomyosin peptide in the absence and presence of Tmod1 peptide. The NMR and circular dichroism studies show that there is an increase in α -helix upon Tmod1-tropomyosin complex formation, indicating that intrinsically disordered regions of the two proteins become ordered when they bind. A proposed model for the binding of Tmod to actin and tropomyosin at the pointed end of the filament shows how the tropomodulin-tropomyosin accentuates the asymmetry of the pointed end and suggests how subtle differences among tropomyosin isoforms may modulate actin filament dynamics.

Keywords

tropomyosin; tropomodulin; actin filament; circular dichroism; nuclear magnetic resonance

Introduction

The reversible polymerization of actin is essential for many cellular functions including determination of shape, directional movement, cytokinesis and intracellular transport¹. Proteins that physically stabilize or regulate the exchange of subunits at the filament ends allow formation of stable cellular structures. Members of two protein families, tropomyosin and

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tropomodulin, form a complex that binds at the pointed end of sarcomeric actin filaments in striated muscle and filaments in the erythrocyte membrane cytoskeleton². The complex forms a tight cap *in vitro*, but *in vivo* actin subunits at the pointed end are exchangeable³.

Both tropomyosin and tropomodulin are multigene families, and expression is cell, tissue, and developmentally regulated. More than 40 tropomyosin isoforms are the products of four genes in vertebrates⁴. Their coiled-coil structure and the ability to bind to the sides of the actin filament are held in common, but alternatively-expressed N-terminal and C-terminal, as well as internal exons, contribute to isoform-specific functions and cellular localizations. For example, long tropomyosins expressed in muscle as well as non-muscle cells have a highly-conserved N terminus. In short, non-sarcomeric tropomyosins expressed primarily in non-muscle cells the sequence encoded by the first two exons of the long isoforms is replaced by a single exon. All four genes encode short isoforms, some of which have specific cellular localizations⁴.

The tropomodulin gene family also has four members, whose products Tmod1-Tmod4, have specific expression patterns, and isoform-specific affinities for tropomyosin. Of these, Tmod1, found in muscle cells and erythrocytes, is the best studied. It has different binding specificities for tropomyosin than Tmod4, found in skeletal muscle⁵. Tmod1 is essential for cardiac myofibril assembly, and a null mutant is lethal in mouse^{6;7}. Tmod3, ubiquitous tropomodulin, unlike other tropomodulins binds monomeric actin⁸.

Tmod is an elongated molecule; the functional domains and binding specificity have been most extensively analyzed in Tmod1. The C-terminal half contains alternating α -helices and β -strands that form a LRR (leucine-rich repeat)⁹. The N-terminal half is mostly disordered^{10;11}, but has three of four known binding sites: two binding sites for the N terminus of tropomyosin (res. 1–38 and 109–144 in Tmod1) and a tropomyosin-dependent capping site^{12;13;14}.

Our goal is to understand the structural basis for capping the tropomyosin-actin filament by Tmod. Using model peptides for α -tropomyosins, we previously reported that Tmod1 cooperatively binds two tropomyosin molecules¹⁴. We proposed a working model for the pointed end of the actin filament in which one Tmod1 molecule uses the two TM-binding sites to bind to the N-termini of the tropomyosins on each side of the actin filament at the pointed end. The model is consistent with our tropomyosin binding and actin polymerization studies with Tmod1 together with both long and short α -tropomyosins. However, previous studies with a non-muscle tropomyosin encoded by the γ -TM gene, TM5NM1, indicated that this isoform binds only to the second binding site on Tmod1^{15;16}. Since the N-terminal sequences of the short tropomyosins are highly conserved, a comparative analysis of the tropomyosin-Tmod1 affinities and effect on polymerization offers the opportunity to understand the structural basis for tropomodulin binding and isoform specificity. Our results show that the highly-conserved N-termini of short tropomyosins have high affinity for the second TM-binding site in Tmod1, but the isoforms differ more than 100-fold for the first site, and that the TM structures in the two complexes are different.

Results

Characterization of γ TM binding to Tmod1

Tmod1 cooperatively binds two molecules of a short α -tropomyosin N-terminal model peptide that contains the Tmod1 binding site (α TM1bZip). Both tropomyosin binding sites are required for the most effective capping by Tmod1 with a short α -TM (α TM5a) that expresses exon α TM1b, or a long α -TM that expresses the alternative first exon, α TM1a (α stTM)¹⁴. Others have reported that tropomyosin expressing exon γ TM1b (γ TM5NM1) binds with high affinity

only to the second site on Tmod1^{15; 16; 17}. Isoform-specific binding of tropomyosin to Tmod1 would have consequences for capping the pointed end of filaments and regulation of actin dynamics in cells.

The conservation of the N-terminal sequence encoded by exon 1b among the three vertebrate tropomyosin genes, which encode short tropomyosins (Table 1), allows analysis of the sequence requirements for binding to the two sites on Tmod1. We used synthetic model peptides in which the first 19 residues are exon 1b-encoded tropomyosin sequence, followed by 18 residues of the GCN4 leucine zipper to stabilize coiled coil formation, a model we used in our previous studies and whose structure we determined¹⁸. The binding of the model peptides to Tmod1 and its fragments was analyzed using non-denaturing polyacrylamide gel electrophoresis, chemical crosslinking, and circular dichroism spectroscopy (CD).

To learn if there are one or two binding sites for the short γ -TM, γ TM5NM1, on the tropomodulin molecule, we studied complex formation between full-length Tmod1 and the TM peptide, γ TM1bZip, using non-denaturing gel-electrophoresis and CD. Figure 1 illustrates the gel-electrophoresis results. Tmod1 forms a complex with γ TM1bZip as well as with the α -TM peptide, α TM1bZip, as a control. However, the complexes have different mobilities. The mobility of the Tmod1- γ TM1bZip complex is intermediate between Tmod1 and the Tmod1 - α TM1bZip complex. The intermediate mobility was observed for a complex between one Tmod1 molecule and one molecule of α TM1bZip¹⁴. Therefore, in these conditions Tmod1 forms a stable complex with only one molar equivalent of the γ TM1bZip peptide.

To characterize the affinity of γ TM1bZip for the two tropomyosin binding sites on Tmod1, we synthesized peptides that correspond to the two sites: Tmod1₁₋₃₈ and Tmod1₁₀₉₋₁₄₄^{11; 14}. We measured the interaction between γ TM1bZip and the two Tmod peptides using CD (Figure 2). At peptide concentrations of 10 μ M, there was an increase in helicity and T_m in the γ TM1bZip/ Tmod1₁₀₉₋₁₄₄ mixture (Figure 2A, Table 2), but no change in the γ TM1bZip/ Tmod1₁₋₃₈ mixture (Table 2), indicating γ TM1bZip binds the second Tmod1 site (with lower affinity than α TM1bZip), but not the first one. However, at 30 μ M peptide there were modest increases in helicity and T_m (Figure 2B), showing that γ TM1bZip does bind the first Tmod1 site, but that the binding constant is 100-fold weaker than α TM1bZip (~2.3 μ M versus 0.0025 μ M at 10 μ M, Table 2). As calculation of dissociation constants requires the assumption that all peptides in the mixture are tightly bound, the true K_d may be even weaker. Complex formation in mixtures of Tmod1₁₋₃₈ and α TM1bZip or γ TM1bZip was also assayed by native gel-electrophoresis and binding only to α TM1bZip was detected (Figure 5, lanes 1-3), supporting our CD data, and the mutagenesis studies from other laboratories^{15; 16}.

Tmod1/ γ TM1bZip interaction was also assayed using covalent cross-linking, since an unstable complex may not have been detected using non-denaturing gel electrophoresis. Mixtures of N-terminal Tmod fragments that contain the first tropomyosin binding site, Tmod1₁₋₃₈ or Tmod1₁₋₉₂, with γ TM1bZip were cross-linked with glutaraldehyde (0.4 nmol each peptide). α TM1bZip was used as a positive control. An additional band appeared when Tmod fragments were mixed with TM1b peptides and treated with glutaraldehyde (Fig. 3A). There was a small amount of crosslinked γ TM1bZip-Tmod1₁₋₃₈ compared to α TM1bZip-Tmod1₁₋₃₈ (Figure 3A, lanes 4 and 6), but the complex with Tmod1₁₋₉₂ yielded more crosslinked product (Figure 3A, lane 9). The positions of the crosslinked bands differed from the peptide dimer which migrated as a faint band above the uncrosslinked peptide (Figure 3B, lane 4). As a negative control for non-specific cross-linking, we used a C-terminal fragment of TM that does not have a Tmod binding site, α TM9d₂₅₂₋₂₈₄. No additional band appeared in the mixture of Tmod1₁₋₉₂ with α TM9d₂₅₂₋₂₈₄ (Figure 3B, lane 3). We conclude that γ TM1bZip can bind to the first binding site in Tmod1, but its affinity is low. If true, Tmod1₁₋₉₂, the N-terminal fragment, which contains the first TM-binding site as well as the TM-dependent actin-capping site, should be

able to inhibit actin polymerization in the presence of a full-length γ 1b tropomyosin, γ TM5NM1.

Inhibition of actin filament elongation in the presence of γ TM5NM1 and Tmod1 fragments

Actin polymerization on gelsolin-capped seeds was assayed using pyrene-actin fluorescence in the presence of γ TM5NM1 and Tmod1_{1–344} or Tmod1_{1–92}. We used Tmod1_{1–344} instead of full-length Tmod1 because removal of the C-terminal 15 residues destroys tropomyosin-independent capping without influencing the capping ability in the presence of tropomyosins¹⁹.

Gelsolin-capped actin filaments were combined with saturating concentrations of γ TM5NM1 (2.5 μ M) and elongation was measured as a function of Tmod1 concentration (see Materials and Methods). The concentration of γ TM5NM1 required for saturation is 5-fold higher than for α TM5a and 3-fold higher than for α stTM^{13; 19}. Both Tmod1_{1–344} and Tmod1_{1–92} inhibited elongation of actin filaments in the presence of γ TM5NM1, although Tmod1_{1–92} was less effective, reaching 50% inhibition of maximal Tmod1_{1–344} inhibition at ~65 nM compared to ~15 nM with Tmod1_{1–344}. The poor inhibition by γ TM5NM1 even at the highest Tmod1_{1–92} concentrations compared to α stTM or α TM5a presumably reflects its lower affinity for the first binding site.

Identification of residues responsible for isoform specific binding of tropomyosin to Tmod1

The differences in affinity of the TM1bZip peptides for the first Tmod1 binding site offer the opportunity to learn how the sequence in this conserved region of tropomyosin encodes information for binding Tmod1. The contrasts between α TM1bZip and γ TM1bZip binding to the first Tmod1 site and the ability to block elongation with Tmod1_{1–92} are striking considering 12 of 19 residues are identical, and most of the changes are conservative (Table 1). These sequences are encoded by exon 1b of the α - and γ -TM genes, respectively, and are more similar to each other than either one is to the N-terminus of stTM, encoded by exon 1a of the α -TM gene. In an effort to learn which residues in short tropomyosins define the affinity for Tmod1 we designed several TM1bZip peptides and analyzed their binding to Tmod1. To help solve the puzzle, we also made δ -TM1bZip peptide that has the sequence of another widely-expressed short non-muscle tropomyosin (δ TM4) and shares some of the differences of γ TM1bZip from α TM1bZip (Table 1).

The interaction of δ TM1bZip to Tmod1_{1–38} and Tmod1_{109–144} was assayed by non-denaturing gel electrophoresis and CD. The binding characteristics of δ TM1bZip were similar to those of γ TM1bZip. There was no band corresponding to a complex with Tmod1_{1–38} in native gels (Figure 5, lane 4), and there were small increases in helicity and T_m only at 30 μ M peptide (Figure 2C, Table 2). Like γ TM1bZip, it bound to Tmod1_{109–144} but with lower affinity than α TM1b (Table 2). The differences are striking considering there are five sequence differences between the δ TM1bZip and γ TM1bZip and six with α TM1bZip (Table 1).

Based on the sequences of α TM1bZip and γ TM1bZip, the differences in Tmod1 binding must be caused by changes in one or more of the following residues: 4, 10, 14, 15, 18. The importance of these residues was tested using an iterative loss-of-function approach by introducing γ TM1bZip residues into the α TM1bZip sequence in synthetic peptides. Two peptides were synthesized with changes in the α TM1b sequence: α TM1bZip(S15V/E18Q) and α TM1bZip(S4T/R14Q). The peptide with the S15V/E18Q mutation bound Tmod1_{1–38}, but the peptide with the S4T/R14Q mutation lost its binding ability (Figure 5, lanes 7–8). To determine the residue responsible for the loss of binding, two other peptides were synthesized: α TM1bZip(S4T) and α TM1bZip(R14Q). The peptide with the S4T mutation bound to Tmod1_{1–38}, but the peptide with the R14Q mutation had greatly reduced binding (Figure 5, lanes 5–6). The

changes in affinity of all mutants for both TM-binding sites were assayed using CD. The changes in T_m values and dissociation constants, calculated from the CD curves, are given in Table 2. According to these data, the change of S4 to T decreased the affinity of the α TM1b peptide for Tmod1_{1–38} fourfold, whereas the change of R14 to Q resulted in almost complete loss of binding in these conditions.

NMR studies of the α TM1bZip/Tmod1_{1–38} and α TM1bZip/Tmod1_{109–144} complexes

In order to define the binding sites of the Tmod1 peptides on the N terminus of tropomyosin, we compared ^{15}N - ^1H HSQC spectra of ^{15}N - α TM1bZip in the presence and absence of unlabeled Tmod1 peptides (Figure 6). We previously determined the structure of ^{15}N - α TM1bZip¹⁸. The first five residues are disordered, and starting with L6 the structure is canonical coiled coil until the C-terminal three residues. For the NMR study, the N-acetyl group in the synthetic peptide was substituted with an initial Gly to allow isotopic labeling; its affinity for Tmod1_{1–38} was within experimental error of the N-acetylated peptide (0.13 vs 0.09 μM). The complexes were stoichiometric with 1 Tmod1 peptide (one chain):1 α TM1bZip (two chains), and therefore are, by definition, asymmetric. Both complexes showed more crosspeaks than the unbound peptide, indicating complex formation. Comparison of the ^{15}N - ^1H HSQC spectrum of the unbound peptide (in which the crosspeaks have been assigned) with those in the complexes gave information about the extent of the binding site of the Tmod1 peptide on tropomyosin.

Figure 6A illustrates the ^{15}N - ^1H HSQC spectra of the ^{15}N - α TM1bZip, free (red) and in complex with Tmod1_{1–38} (cyan). In the unbound TM peptide there are 43 crosspeaks, 35 from backbone amides (two overlap) and 8 from sidechain amides. In the complex, there were 63 crosspeaks, and the major shifts were in peaks from the tropomyosin region of the peptide (versus the GCN4 region). Of these, it was possible to assign the additional crosspeaks for sidechain amides (Q17 is shifted) and the backbone amide crosspeaks for residues 1–5. All were shifted (greatest in S3, S4, S5) and res. 1, 3–5 became doublets. This means that the first five TM residues that are disordered in the free peptide have a regular structure in the complex. The doublets show that the environments of the chains are different (expected in an asymmetric structure) and that the chains are in slow exchange, evidence for formation of a tight complex. While the binding engaged all 19 residues in the TM region, the GCN4 domain was not involved in a major way.

The first 92 residues of Tmod1 are naturally unfolded, the only ordered structure being the α -helix formed by res. 24–35. Binding of α TM1bZip to ^{15}N -Tmod1_{1–92} shifts residues 1–38, showing they all change upon binding to tropomyosin¹¹. A Tmod1 peptide containing only the helical region (res. 23–38) was less helical and did not bind α TM1bZip, as assayed by native gel electrophoresis and circular dichroism, giving further support for the designation of res. 1–38 as a tropomyosin binding site (data not shown).

The binding of Tmod1_{109–144} to α TM1bZip caused more changes in the ^{15}N - ^1H HSQC spectrum, indicating a more extensive interface than with Tmod1_{1–38} (Figure 6B). There were 42 additional crosspeaks arising from changes in the tropomyosin, 16 from side chain amides (Q17, Q19, N20, N25) and 26 from backbone amides (19 from tropomyosin, 7 from GCN4). The changes were greatest in the tropomyosin region, but the interface must extend into the GCN4 region. As with Tmod1_{1–38}, the crosspeaks assigned to S3, S4, and S5 were doublets, evidence for an ordered, stable structure.

The ^{15}N - ^1H HSQC spectra give no specific structural information, but they show that the binding sites on tropomyosin for the two Tmod1 peptides are different. Since a single tropomodulin binds two molecules of tropomyosin at the pointed end of an actin filament, the interactions add to the intrinsic asymmetry of the filament end.

Discussion

In this work we show that all tropomyosins bind to two sites on Tmod1, and that isoform-specific differences in affinity for the two sites contribute to the efficiency in capping the pointed end of the actin filament. Using NMR we defined the regions of tropomyosin involved in both tropomodulin binding sites and show that the tropomyosin structures are different in the two complexes. Together the results illustrate how the tropomyosin-tropomodulin cap enhances the asymmetry of the pointed end. We also show that subtle sequence differences among tropomyosin isoforms can have major effects on the affinity for the first Tmod1 binding site and thereby modulate the dynamics of the actin filament's pointed end.

The analysis resolves a long-standing debate in the literature concerning the location of tropomyosin binding sites on tropomodulin. Babcock et al.¹⁷ showed that the binding site for γ -TM5NM1 on Tmod1 is within residues 90–184, recently localized to the region including residue 135^{15; 16}. The suggestion that it is a solitary binding site contradicts our model that one tropomodulin molecule binds two tropomyosin molecules at the pointed end, shown to be true for both short and long α -tropomyosins (α -TM5a and α -stTM)¹⁴. We have now resolved the question by showing that γ -tropomyosin and δ -tropomyosin can bind to both Tmod1 sites, but with much lower affinity than α -tropomyosin to the first site. Further evidence for γ TM5NM1 binding the first site is that it inhibits elongation with Tmod1_{1–92}, even though the second TM-binding site is absent in this Tmod1 fragment. However, the inhibition is incomplete compared to Tmod1_{1–344}, showing that the lower affinity for the first site makes γ -tropomyosin a less effective capper of the pointed end.

The NMR studies indicate the extent of the two Tmod1 binding sites on α TM1bZip and show that the tropomyosin structures are different in the two complexes. While the structures of Tmod1-TM complexes remains to be solved, we have put forth hypotheses about the secondary structure and orientations of the chains in the complexes. The models shown in Figure 7, which we emphasize are cartoons, are based on circular dichroism spectra of the complexes, the effects of complex formation on the ¹H-¹⁵N HSQC spectra of α TM1bZip, and mutagenesis studies^{11; 14; 15; 16}. The circular dichroism spectra of the complexes of both Tmod peptides with α TM1bZip are >80 % helical, suggesting that upon binding there is formation of a large amphipathic helical or coiled-coil interface.

When Tmod1_{1–38} binds ¹⁵N-labeled TM1bZip there are changes in the crosspeaks of the 19 tropomyosin residues in the ¹H-¹⁵N HSQC spectrum, with minor perturbations of the GCN4 residues. The only ordered structure in Tmod1_{1–92} is an α -helix in residues 24–35, yet in the HSQC spectrum of ¹⁵N-¹H-Tmod1_{1–92} almost all the residues between 1 and 38 are broadened and shifted upon complex formation with α TM1bZip. This observation, together with the large increase in helicity upon complex formation, suggests that the binding site is more extensive than the residue 24–35 helix and that the intrinsically disordered region of Tmod1 becomes ordered upon binding. Mutagenesis of L27 to E greatly reduces the affinity of Tmod1_{1–38} for α TM1bZip suggesting a requirement for an amphipathic helix that may form a coiled coil interface with the tropomyosin coiled coil starting at L6¹¹. Figure 7a,c illustrates a possible model in which residues 24–38 of Tmod1 are oriented parallel to residues 2–16 of α TM1bZip helix, since in an antiparallel arrangement of the coiled coil interface would extend into the GCN4 portion of the molecule. Since the first 22 residues of Tmod1 are also involved in binding α TM1bZip and must contribute to the increase in helix upon complex formation, they might interact in an antiparallel sense with residues 2–17 on the opposite side of the tropomyosin coiled coil. Possible hydrophobic interactions are consistent with such a model (Figure 7).

The model gives insight into a possible explanation for the importance of R14 in α TM1bZip for binding the first Tmod1 binding site. When the chains are aligned as in Figure 7a, R14 of

tropomyosin could form an intermolecular salt bridge with E33 of Tmod1. Mutation of R14 to Q as in γ TM1bZip or δ TM1bZip would weaken this interaction. Replacing S4 with a bulkier threonine also may explain the observed 4-fold loss in affinity.

The model shown in Figure 7c illustrates only one possible structure. In an alternative model, residues 1–38 could form an antiparallel two helix bundle, with a turn between residues 18 and 21 that could lie on one face of the tropomyosin molecule forming a structure similar to a four helix bundle. Such an interface would have fewer hydrophobic interactions between tropomodulin and tropomyosin, but would allow strong salt bridges between residues R10 and R14 of tropomyosin with E33 and E35 of tropomodulin.

The Tmod1_{109–144}- α TM1bZip complex is also highly helical suggesting formation of an extensive coiled coil interface upon complex formation. Mutagenesis of γ TM5NM1 shows that the *a* and *d* interface residues L6, V9 and I13 are critical for binding the second Tmod site²⁰, consistent with a coiled coil interface. Mutagenesis studies have shown that the Tmod1 residues I131¹⁴ and L135¹⁶ are critical for binding. The Tmod1 sequence has a coiled coil periodicity between residues 121 and 135 suggesting it could form a helix that would be long enough to extend into the GCN4 portion of the α TM1bZip molecule. Together with the NMR study showing that the binding site extends into the GCN4 region of α TM1bZip, we suggest that Tmod1_{109–144} and α TM1bZip form an antiparallel three-helix coiled coil that is interrupted at P114, near the C-terminal end of the GCN4 region. Residues 30 to 36 of the α TM1bZip are unperturbed when Tmod1_{109–144} binds, suggesting that the residues 109–114 do not bind the peptide. A possible binding scheme for binding α TM1bZip, as well as native α TM1b, and a hypothetical structure for the Tmod1_{109–144}/ α TM1bZip complex are shown in Figure 7b,d. The sequence shows some possible interactions; in the three helix bundle the R14 residue is exposed. We stress that Figure 7 illustrates only hypothetical models; we will learn the arrangement of the helices and understand the significance of specific residues only by solving the structures of the complexes.

The position of a single Tmod molecule at the pointed end of the actin filament that forms fundamentally different complexes with the tropomyosin molecules on the two sides of the filament helix augments the asymmetry of the end. Differences in tropomyosin affinity for the two binding sites in Tmod may regulate its correct positioning at the pointed end. Since small sequence variations in the N terminus of tropomyosin can have major effects on Tmod1 binding and the ability to cap the pointed end, the end becomes a significant regulatory site. Tropomyosins are recognized to be a major regulator of the actin filaments in cells, having the ability to protect filaments against severing and branching^{21; 22; 23}, to recruit specific myosins²⁴, alter cell shape, and now to regulate the pointed end. Regulation of the cooperativity as well as the effectiveness of capping by specific tropomyosins may have significant consequences for local filament dynamics in cells.

Materials and methods

Protein Expression and Purification

Full-length Tmod1 and Tmod1_{1–344} were overexpressed in *E. coli* BL21(DE3)pLysE using the method described in²⁵ and purified according to²⁶. Tmod1_{1–38} was synthesized by the Keck Facility at Yale University (New Haven, CT). Other Tmod fragments were synthesized by the Tufts University Core Facility (Boston, MA). Chicken pectoral skeletal muscle actin was purified from acetone powder as described²⁷. G-actin was purified on a Sephacryl S-300 column²⁸ and was stored in liquid nitrogen. Actin was labeled with pyrenyl-iodoacetamide and the labeling ratios were calculated according to^{29; 30}. 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 100,000 rpm (TLA-100, Beckman) for 30 min at 4°C. γ TM5NM1, a

short rat γ -tropomyosin, was purified according to ³¹. TM peptides, α TM1bZip (previously named AcTM1bZip ¹⁸), γ TM1bZip and δ TM1bZip are designed chimeric proteins that contains 19 residues of short α , γ or δ -tropomyosin correspondingly, encoded by exon 1b and the 18 C-terminal residues of the GCN4 leucine zipper domain. The peptides are N-acetylated. These peptides as well as mutated α TM1bZip peptides were synthesized by the Tufts University Core Facility (Boston, MA). ¹⁵N-Gly α TM1bZip, used in the NMR studies was expressed and purified as described previously ¹⁸. Recombinant human gelsolin was a generous gift from Dr. John H. Hartwig (Brigham and Women's Hospital & Hematology Division, Boston, MA).

Protein purity was evaluated using SDS-PAGE ³². Concentrations of proteins were determined by using BCA protein assay kit (Pierce) or by measuring their difference spectra in 6 M guanidine-HCl between pH 12.5 and 7.0 ³³ using the extinction coefficients of 2357 per tyrosine and 830 per tryptophan ³⁴.

Binding Experiments

Binding was visualized using native polyacrylamide gel electrophoresis in 9 % polyacrylamide gels that were polymerized in the presence of 10% glycerol without SDS were used ¹¹. To prepare complexes for loading onto gels, Tmod fragments were mixed with TM peptides in a 1:2 molar ratio.

Fluorescence measurements

The rates of actin polymerization were measured using the change in pyrene actin fluorescence ²⁹ 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 polymerizing 3 μ M G-actin in the presence of 15 nM gelsolin according to ¹⁹. 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 MgCl₂, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM CaCl₂, 1 mM NaN₃, 10 mM imidazole, pH 7.0) containing TM and Tmod1. The final concentrations of F- and G-actin after dilution were 0.6 μ M and 1.1 μ M respectively.

Gelsolin-capped filaments were prepared in sets of four and fluorescence measurements were carried out in parallel in a four cuvette holder with actin as a control in each set as previously described ¹⁹. Exponential curves were fit to the polymerization data using SigmaPlot, and initial rates (R) were calculated as the first derivatives at time zero. The inhibition of polymerization was calculated as $R_{\text{exp}}/R_{\text{control}}$, where $R_{\text{control}}=1$ (in the absence of tropomyosin and tropomodulin). To determine at what concentration Tmod1₁₋₃₄₄ reaches 50% inhibition, the curves from the graph were normalized (inhibition without Tmod1₁₋₃₄₄ was 0 and maximal Tmod1₁₋₃₄₄ inhibition was 100 %).

Circular dichroism measurements

CD measurements were made using an Aviv model 215 spectropolarimeter (Lakewood, NJ) as previously described ^{35; 36}. The dissociation constants of the Tmod1/TM peptide complexes, K_d , were determined using the relationship: $K_d = \exp(-\Delta\Delta G/RT)$, where $\Delta\Delta G$ is the difference in free energy of folding of the complex minus that of the TM fragment alone, R is the gas constant and T is the temperature ³⁷. The equations used for determining the dissociation constants and the thermodynamics of folding of the two-chain TM peptide and the three-chain TM/Tmod1 peptide complex from the changes in their circular dichroism as a function of temperature are described in detail in ^{5; 36}.

Crosslinking

fragments of Tmod1 and TM were mixed in 10 mM Na-phosphate buffer, pH 7.0, containing 100 mM NaCl. Glutaraldehyde was added to a final concentration of 0.01 %. After incubation for 30 min at room temperature, 100 mM Tris-HCl, pH 8.0, was added to terminate the reaction. Samples were analyzed by SDS-PAGE and quantified using a Molecular Dynamics model 300A computing densitometer (Sunnyvale, CA).

NMR studies

^{15}N - ^1H -heteronuclear single quantum coherence (HSQC) NMR spectra of ^{15}N -labeled Gly $\alpha\text{TM1bZip}$ in the absence and presence of unlabeled Tmod1_{1–38} and Tmod1_{109–144} were collected at 10°C at 800 MHz on a Bruker BioSpin NMR spectrometer.

Molecular modeling

Cartoons of the of complexes of Tmod1_{1–38} and Tmod1_{109–144} with $\alpha\text{TM1bZip}$ were constructed using the programs MolMol³⁸ and Sybyl version 17.2, Tripos Inc. Saint Louis MO. To construct the cartoon of one possible complex between $\alpha\text{TM1bZip}$ and Tmod1_{1–38}, a four-helix coiled coil, with one chain running antiparallel and three chains running parallel were constructed by taking chains A and C from the X-ray structure of a mutant of GCN4 that forms an antiparallel three-chained coiled coil³⁹ (PDB 1RB6) and chains A and C from a structure of a mutant of GCN4, which forms a parallel three coiled coil⁴⁰ (PDB 1IJ3). The strands were aligned so that the four strands were equidistant from each other and formed a four-helix coiled coil using the program MolMol. Residues 1–16 of the GCN4 sequence from chain A of each mutant were replaced with residues 5–20 from $\alpha\text{TM1bZip}$. The N-terminus was extended with three helical serines and three non-helical residues, Gly-Ala-Gly, at the extreme N-terminus. Residues 1–15 were removed from chain C of 1RB6 and residues 16–32 were replaced with residues 3–19 from Tmod1. M1 and S2 were added at the N-terminus with a random conformation. Residues 1–13 of chain C from 1IJ3 were replaced with residues 26 to 38 of Tmod1 and LTEE were added at the N-terminus.

To construct the cartoon of one possible structure of the Tmod1_{109–144}/ $\alpha\text{TM1bZip}$ complex, the antiparallel three-stranded coiled coil from the 1RB6 file was used as a template. The parallel strands, A and B were mutated to the sequence of $\alpha\text{TM1bZip}$ as describe above. Residues 1–3 and 33 of the chain C were deleted and residues 4–32 were replaced with residues 116 to 144 of Tmod1 and residues 109 to 115 were added at the N-terminus without constraining the conformation. The energies of the hypothetical structures were minimized using Sybyl.

Acknowledgements

We thank Dr. John Hartwig for recombinant human gelsolin and Dr. Peter Gunning for plasmid for γTM5NM1 expression. We thank Brian Rapp for γTM5NM1 purification, Lucy Kotlyanskaya for ^{15}N -Gly $\alpha\text{TM1bZip}$ preparation and Mikhail Gongadze for technical assistance. Supported by the American Heart Association Grant 0535328N and UMDNJ Foundation Grant to Dr. Alla S. Kostyukova and by National Institutes of Health Grants GM63257 to Dr. Sarah E. Hitchcock-DeGregori and GM36326 to Dr. Sarah E. Hitchcock-DeGregori and Dr. Norma Greenfield.

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Figure 1. Complex formation between Tmod1 and TM peptides monitored by non-denaturing polyacrylamide gel-electrophoresis. Lane 1 and 4, Tmod1; lane 2, Tmod1 and α TM1bZip; lane 3, Tmod1 and γ TM1bZip. TM peptides are positively charged and do not enter the gel. Arrowhead indicates Tmod1, arrows indicate complexes.

Figure 2.

Binding of Tmod1 fragments to TM peptides measured using circular dichroism spectroscopy. (A): Tmod1₁₀₉₋₁₄₄ and γTM1bZip, the peptide concentrations were 10 μM in 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. (B): Tmod1₁₋₃₈ and γTM1bZip, and (C): Tmod1₁₋₃₈ and δTM1bZip, the peptide concentrations were 30 μM in 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. (●) Tmod1 peptide; (○) TM peptide; (▽) sum of the folding curves of TM and Tmod1 peptides; (▼) the folding of the mixture of TM and Tmod1 peptides.

Figure 3. The cross-linking of TM model peptides and Tmod1 N-terminal fragments with glutaraldehyde monitored by the SDS-PAGE (15 % polyacrylamide). A: Complex formation: lane 1, Tmod1₁₋₃₈; lane 2, Tmod1₁₋₃₈ treated with glutaraldehyde; lane 3, Tmod1₁₋₃₈/αTM1bZip; lane 4, Tmod1₁₋₃₈/αTM1bZip treated with glutaraldehyde; lane 5, Tmod1₁₋₃₈/γTM1bZip; lane 6, Tmod1₁₋₃₈/γTM1bZip treated with glutaraldehyde; lane 7, Tmod1₁₋₉₂ treated with glutaraldehyde; lane 8, Tmod1₁₋₉₂/γTM1bZip; lane 9, Tmod1₁₋₉₂/γTM1bZip treated with glutaraldehyde; lane 10, Tmod1₁₋₉₂. B: Control for non-specific cross-linking: lane 1, TM9d₂₅₂₋₂₈₄; lane 2, TM9d₂₅₂₋₂₈₄ treated with glutaraldehyde; lane 3, Tmod1₁₋₉₂/TM9d₂₅₂₋₂₈₄ treated with glutaraldehyde; lane 4, γTM1bZip treated with glutaraldehyde. The arrowheads indicate individual proteins, whereas the arrows indicate complexes.

Figure 4.

Dependence of inhibition of pointed end elongation of gelsolin-capped actin filaments on Tmod1₁₋₃₄₄ or Tmod1₁₋₉₂ concentration in the presence of 2.5 μ M γ TM5NM1. Initial rates (R) were calculated as the first derivatives at time zero after fitting exponential growth curves to the data. The inhibition of polymerization was calculated as $R_{\text{exp}} / R_{\text{control}}$, where $R_{\text{control}}=1$ (in the absence of tropomyosin and tropomodulin). (●) with Tmod1₁₋₃₄₄; (○) with Tmod1₁₋₉₂. (■) the value shown for γ TM5NM1 in the absence of tropomodulin is 1.15 ± 0.18 ($n=3$). For comparison initial rates in the presence of 50 nM Tmod1₁₋₉₂ with 0.75 μ M α stTM (◆) or 0.5 μ M α TM5a (◇) are shown.

Figure 5.

Complex formation between Tmod1₁₋₃₈ and TM peptides monitored by non-denaturing polyacrylamide gel-electrophoresis. Lane 1, Tmod1₁₋₃₈; lane 2, Tmod1₁₋₃₈ and α TM1bZip; lane 3, Tmod1₁₋₃₈ and γ TM1bZip; lane 4, Tmod1₁₋₃₈ and δ TM1bZip; lane 5, Tmod1₁₋₃₈ and α TM1bZip(S4T); lane 6, Tmod1₁₋₃₈ and α TM1bZip(R148Q); lane 7, Tmod1₁₋₃₈ and α TM1bZip (S4T/R148Q); lane 8, Tmod1₁₋₃₈ and α TM1bZip(S15V/E18Q). TM peptides are positively charged and do not enter the gel. The difference in mobility of complexes is due to isoelectric points of TM peptides (pI=10.4 for α TM1bZip(S15V/E18Q) and 9.9 for α TM1bZip and α TM1bZip(S4T)). Arrow indicates Tmod1₁₋₃₈, arrowhead indicates complexes.

Figure 6. ^{15}N -1H HSQC spectra of ^{15}N - $\alpha\text{TM1bZip}$ in the presence and absence of tropomodulin fragments. Panel A, (red) ^{15}N - $\alpha\text{TM1bZip}$, (cyan) ^{15}N - $\alpha\text{TM1bZip}$ + unlabeled Tmod1₁₋₃₈. Panel B, (red) ^{15}N - $\alpha\text{TM1bZip}$, (cyan) ^{15}N - $\alpha\text{TM1bZip}$ + unlabeled Tmod1₁₀₉₋₁₄₄. The peptides were each ~0.5 mM in 100 mM NaCl, 10 mM NaCl, 5% deuterium oxide, pH 6.5. Data were acquired on a Bruker 800 MHz NMR spectrometer. Note in unbound $\alpha\text{TM1bZip}$ Q19 overlaps R11 and V27 overlaps N20.

Figure 7.

Schematic of possible binding modes of Tmod1₁₋₃₈ and Tmod1₁₀₉₋₁₄₄ to α TM1bZip. (a, b) The sequences and possible coiled-coil interactions at *a* and *d* positions of the heptad repeat of the two Tmod binding sites for α TM1bZip. Mutations in Tmod1 that cause loss of binding to TM 11; 13; 14; 15; 16 are outlined in cyan, and mutations in TM5NM1 that cause loss of binding to Tmod1 are outlined in brown²⁰. (c, d) Models of possible structures of the binding interfaces. In the models, the two chains of α TM1bZip are black and the single chains of the two Tmod1 peptides are grey. (a, c) Residues 1–13 of Tmod1₁₋₃₈ bind antiparallel to the α TM1bZip coiled coil on one side of the tropomyosin interface, residues 15 to 26 loop around the N-terminus of TM and residues 24–36 bind parallel to the other side of the tropomyosin coiled coil interface. (b, d) Residues 121–138 of Tmod1₁₀₉₋₁₄₄ bind antiparallel to residues 6 to 23 of α TM1bZip to form a three helix bundle. (e) Cartoon model of the binding of Tmod (terra cotta) to tropomyosin (cyan) at the pointed end of the actin filament (white) to illustrate how one Tmod1 may interact with the N-termini of two TM molecules on opposite sides of the actin helix to cap the pointed end. The TM-dependent capping site includes residue 71 and is shown binding to actin. The TM-independent capping site at the extreme C-terminus of Tmod1 is not shown.

Table 1**Alignment of TMZip sequences**

Sequences of TM chimeric proteins containing the N-termini of short non-muscle TMs: α TM5a, α TM1bZip; γ TM5N M1, γ TM1bZip; and δ TM4, δ TM1bZip, encoded by exon 1b. Residues 20 to 37 of the peptides correspond to the last 18 C-terminal residues (264–281) of the yeast transcription factor GCN4⁴¹. The residues that are coiled coil are labeled with the residue position of the heptad repeats. Bold - residues different between α TM1bZip and two other short TM peptides, underlined – changed residues. α TM1bZip in previous studies was called AcTM1bZip.

TM peptide [source]	Tropomyosin sequence	GCN4 sequence
α TM1bZip [α TM5a]	AGSSSLEAVRRKIRSLOEO	NYHLENEVARLKKLVGER
γ TM1bZip [γ TM5NM1]	AGSTTIEAV KRKIO VLOOO	NYHLENEVARLKKLVGER
δ TM1bZip [δ TM4]	AGLNSLEAV KRKIO ALOQQ	NYHLENEVARLKKLVGER
α TM1bZip (S15V/E18Q)	AGSSSLEAVRRKIRVLOOO	NYHLENEVARLKKLVGER
α TM1bZip p(S4T/R14Q)	AGSTSLEAVRRKIOSLOEO	NYHLENEVARLKKLVGER
α TM1bZip (S4T)	AGSTSLEAVRRKIRSLOEO	NYHLENEVARLKKLVGER
α TM1bZip (R14Q)	AGSSSLEAVRRKIOSLOEO	NYHLENEVARLKKLVGER
heptad repeat	a d a d	a d a d a

Table 2**Binding of tropomodulin peptides to tropomyosin peptides**

The difference in the observed midpoints of the cooperative unfolding transitions (determined from the first derivative of the unfolding curves) of the mixture of the TMZip and Tmod fragments and the additions of the curves for the TMZip and Tmod fragments alone. The K_d values were estimated from the thermodynamics of unfolding of the complexes compared with the Tmod fragments and TMZips alone. All components were 10 μ M in 100 mM NaCl, 10 mM sodium phosphate, pH 7. NC – not calculated.

	Tmod1 ₁₋₃₈		Tmod1 ₁₀₉₋₁₄₄	
	ΔT_m , °C	K_d 30°C, μ M	ΔT_m , °C	K_d 30°C, μ M
α TM1bZip	7.1 $\pm$ 0.5	0.09 $\pm$ 0.02	13.8 $\pm$ 0.6	0.0025 $\pm$ 0.0011 [*]
γ TM1bZip	no change	NC	12.5 $\pm$ 2.2	0.04 $\pm$ 0.03
δ TM1bZip	no change	NC	7.8 $\pm$ 2.1	0.09 $\pm$ 0.02
α TM1bZip (S15V/E18Q)	6.1 $\pm$ 0.4	0.21 $\pm$ 0.01	11.7 $\pm$ 0.2	0.017 $\pm$ 0.003
α TM1bZip (S4T/R14Q)	no change	NC	7.6	0.11
α TM1bZip (S4T)	4.0 $\pm$ 0.3	0.39 $\pm$ 0.01	13.4 $\pm$ 0.1	0.04 $\pm$ 0.02
α TM1bZip (R14Q)	no change	NC	4.9 $\pm$ 0.2	0.26 $\pm$ 0.21

* previously reported ¹⁴