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The Cardiomyopathy-Associated K15N Mutation in Tropomyosin Alters Actin Filament Pointed End Dynamics

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Abstract

Correct assembly of thin filaments composed of actin and actin-binding proteins is of crucial importance for properly functioning muscle cells. Tropomyosin (Tpm) mediates the binding of tropomodulin (Tmod) and leiomodin (Lmod) at the slow-growing, or pointed, ends of the thin filaments. Together these proteins regulate thin filament lengths and actin dynamics in cardiac muscle. The K15N mutation in the *TPM1* gene is associated with familial dilated cardiomyopathy (DCM) but the effect of this mutation on Tpm's function is unknown. In this study, we introduced the K15N mutation in striated muscle α -Tpm (Tpm1.1) and investigated its interaction with actin, Tmod and Lmod. The mutation caused a ~3-fold decrease in the affinity of Tpm1.1 for actin. The binding of Lmod and Tmod to Tpm1.1-covered actin filaments also decreased in the presence of the K15N mutation. Furthermore, the K15N mutation in Tpm1.1 disrupted the inhibition of actin polymerization and affected the competition between Tmod1 and Lmod2 for binding at the pointed ends. Our data demonstrate that the K15N mutation alters pointed end dynamics by affecting molecular interactions between Tpm1.1, Lmod2 and Tmod1.

Keywords

actin; cardiomyopathy; tropomyosin; leiomodin; tropomodulin; circular dichroism (CD)

Introduction

Sarcomeres are the smallest contractile units of muscle cells and they are composed of thick and thin filaments. Thin filaments are formed by polymerization of monomeric actin (G-actin) into filamentous actin (F-actin). Various actin-binding proteins dynamically regulate actin polymerization and depolymerization at the fast-growing (barbed) and slow-growing

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(pointed) ends to modulate the length of thin filaments. Correct thin filament lengths are vital for properly functioning muscle cells (for reviews, see [1, 2]). Mutations in actin-binding proteins that are responsible for thin filament lengths can result in detrimental functional effects and cause myopathies [3-5]. Understanding the effect of mutations on the structural and functional properties of actin-binding proteins can shed light on the causes of myopathy development.

The length of thin filaments in sarcomeres is regulated by interactions between tropomyosin (Tpm) and tropomodulin (Tmod) or leiomodulin (Lmod) at the filament pointed ends. Tmod maintains correct thin filament lengths by capping the pointed ends in a Tpm-dependent manner to inhibit polymerization or depolymerization [6]. Lmod, a member of the Tmod protein family, also binds at the pointed end but it acts as a “leaky cap” and allows elongation of thin filaments [7, 8].

The K15N mutation in the *TPM1* gene is associated with familial dilated-cardiomyopathy [3, 5]. It was previously shown that the K15N mutation in striated muscle α -Tpm (Tpm1.1) decreased its affinity for the Tpm-binding site of Lmod2 and the first Tpm-binding site of Tmod1 [9]. Tpm1.1 is a coiled-coil dimeric protein that binds along thin filaments in cardiac muscle cells (for review see [10]). Uncovering the molecular mechanisms of how the K15N mutation effects the interactions between Tpm1.1 and Tmod or Lmod is important for understanding the basis for DCM development.

In muscle cells, several isoforms of Tmod and Lmod, specifically Tmod1, Tmod4, Lmod2 and Lmod3, regulate the thin filament lengths [8, 11-19]. Tmod1, Lmod2 and Lmod3 are found in both skeletal and cardiac muscle cells, whereas Tmod4 is explicitly expressed in skeletal muscle cells. Tmod1 and Lmod2 are major cardiac isoforms essential for normal cardiac function; the knockout of either one of these proteins in mice resulted in morphological and functional abnormalities in the heart followed by embryonic lethality [12, 15, 19, 20].

The domain structures of Tmod and Lmod are remarkably similar (Figure 1). Tmod has two Tpm-binding sites and two actin-binding sites [21-24]. Lmod shares a similar domain arrangement, but it has only one Tpm-binding site [7, 25]. Additionally, Lmod has a ~150 amino acid C-terminal extension that contains a proline-rich region and a third actin-binding site, which includes a Wiskott-Aldrich syndrome protein (WASP)-homology 2 (WH2) domain [17, 26, 27].

In this study, we utilized circular dichroism spectroscopy, pyrene-actin polymerization and actin co-sedimentation assays to test the binding of Tpm1.1, either WT or containing the K15N mutation, to F-actin, Tmod1 and Lmod2. The presence of the K15N mutation in Tpm1.1 decreased its affinity for all of its binding partners tested here. The mutation decreased the abilities of Tmod1 and Lmod2 to bind F-actin and to inhibit polymerization at the pointed end in the presence of Tpm1.1. In addition, the K15N mutation affected the competition between Tmod1 and Lmod2 for binding at the pointed ends. We propose that the K15N mutation in Tpm impairs the regulation of actin polymerization at the pointed

ends by Tmod1 and Lmod2 in the heart, potentially providing the mechanism at the molecular level to explain the development of DCM in patients with this mutation.

Materials and Methods

Protein sequences

Sequences of Lmod2 (NP_001186644.2), Lmod3 (NP_001291347.1), Tmod1 (NP_990358.1), Tmod4 (NP_057921.1) and tropomyosin α -1 chain isoform Tpm 1.1st (NP_001288265.1) were downloaded from UniProt, Ensembl or NCBI.

Plasmid construction

The construct for α TM1a₁₋₂₁Zip was prepared as described previously [9]. The α TM1a₁₋₂₁Zip peptide contained the 21 N-terminal residues of the striated muscle α -Tpm (Tpm1.1) and 18 residues of the leucine zipper domain of the yeast transcription factor GCN4 [28] to stabilize a coiled-coil structure in solution [29]. To mimic the N-terminal acetylation of Tpm, a Gly residue was added to the N-terminus of α TM1a₁₋₂₁Zip peptides.

The construct for the α TM1a₁₋₂₁Zip[K15N] peptide was prepared by site-directed mutagenesis [9]. The construct for rat striated muscle α -Tpm (Tpm1.1) was a generous gift from Dr. Sarah Hitchcock-DeGregori (Rutgers University, Piscataway, NJ). Tpm1.1 had an Ala-Ser extension on its N-terminus to mimic acetylation of Tpm. Recombinant Tpm1.1 with Ala-Ser extension binds actin well in the absence of troponin [30]. The K15N mutation was introduced into full-length Tpm1.1 with PCR using the oligonucleotides and the protocol described previously [9].

Protein expression and purification

The fragments that correspond to the first or second Tpm-binding sites (s1 or s2) of Tmod1 (Tmod1s2, residues 109-144), Tmod4 (Tmod4s1, residues 2-39; Tmod4s2, residues 111-148) and Lmod3 (Lmod3s1, residues 6-44) were synthesized and purified at Tufts University Core Facility (Boston, MA). Full-length Tmod1 was expressed in *Escherichia coli* BL21 (DE3) cells (Invitrogen) and purified according to [31]. Lmod2₁₋₅₁₄ was a generous gift from Dr. Carol Gregorio (University of Arizona, Tucson, AZ).

G-actin was purified from actin acetone powder [32] as in [33]. Pyrene-iodoacetamide labeled G-actin (P-actin) was prepared as in [34], labeling ratios were calculated according to [35] and the labeled protein was stored in liquid nitrogen. P-actin was defrosted at 37°C and aggregates were removed by centrifugation at 100,000 RPM (Beckman-Coulter TLA-120.1 Rotor), 4°C, 1 hour before experiments.

WT Tpm1.1 and Tpm1.1[K15N] were expressed in *E. coli* BL21 (DE3) and purified as described previously [36] with some modifications according to [37].

Recombinant human gelsolin was a generous gift from Dr. John H. Hartwig (Brigham and Women's Hospital & Hematology Division, Boston, MA).

The concentrations of all peptides and proteins were determined either spectroscopically by measuring their difference spectra as in [38] and/or by BCA Protein Assay Kit (Pierce) according to manufacturer's instructions.

Actin binding assay

The affinity of Tpm1.1 for actin filaments was measured according to a previously published protocol [39] with modifications in buffer composition and F-actin preparation. 5 μ M G-actin in G-buffer (2 mM Tris-HCl pH 8.0, 0.2 mM CaCl₂, 0.01% NaN₃, 0.2 mM ATP and 0.5 mM DTT) was aliquoted in centrifuge tubes. 20 $\times$ polymerization buffer was added to the final concentration of 1 $\times$ (25 mM Imidazole pH 7.0, 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA) and actin was polymerized for 1 hour at room temperature. Under these conditions the steady state has to be reached within 20 minutes [40]. 0-4 μ M WT Tpm1.1 or Tpm1.1[K15N] was added to F-actin and incubated for 20 minutes at room temperature. F-actin was sedimented by centrifugation at 65,000 RPM (Beckman Coulter TLA-100 Rotor), 30 minutes at room temperature. The pellets were washed with F-Buffer (25 mM Imidazole pH 7.0, 100 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 2 mM Tris-HCl, 0.2 mM CaCl₂, 0.01% NaN₃, 0.2 mM ATP and 0.5 mM DTT), and then solubilized in 8 M Urea at room temperature for 30 minutes. The pellet and supernatant samples were mixed with 2 $\times$ Laemmli Sample Buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.004% bromophenol blue and 10% 2-mercaptoethanol), heated at 100°C for 2 minutes and loaded on 10% acrylamide SDS gels. Additionally, gels with known concentrations of Tpm1.1 were run in parallel to generate standard curves and to estimate the free Tpm1.1 concentration in the supernatants. Gels were stained with Coomassie Brilliant Blue R-250. The images were captured with Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA). Quantitative analysis of the background-corrected band densities of the pellet, supernatant and standard Tpm1.1 samples was done with ImageJ Software (NIH).

The apparent binding constant (K_{app}) between actin and Tpm1.1 were determined by fitting the data to the Hill equation as in [36] using SigmaPlot 12.0:

$$\Phi = \frac{n \cdot [Tpm1.1]^{\alpha H} \cdot K_{app}^{\alpha H}}{1 + ([Tpm1.1]^{\alpha H} \cdot K_{app}^{\alpha H})},$$

where Φ is the Tpm/actin band density ratio in the pellet (fraction maximal binding), n is the maximal Tpm1.1 bound, $[Tpm1.1]$ is the free Tpm1.1 concentration in the supernatant and αH is the Hill coefficient of binding. Φ was normalized by dividing data by the maximal band density ratio of Tpm/actin at saturation.

The affinity of Tpm1.1 for an isolated binding site (K_0) was determined by fitting the data to the McGhee & von Hippel equation [41] using Prism 7:

$$\frac{v}{L} = K_0 \cdot (1 - n \cdot v) \cdot \left(\frac{(2 \cdot \omega + 1) \cdot (1 - n \cdot v) + v - R}{2 \cdot (\omega - 1) \cdot (1 - n \cdot v)} \right)^6 \cdot \left(\frac{1 - (n+1) \cdot v + R}{2 \cdot (1 - n \cdot v)} \right)^2,$$

where $R = \sqrt{(1 - (n+1) \cdot \nu)^2 + 4 \cdot \omega \cdot \nu \cdot (1 - n \cdot \nu)}$, L is the free Tpm1.1 concentration in the supernatant, ν is the number of moles of Tpm bound per mol of actin, n is the stoichiometry of Tpm binding to actin ($n=7$ for muscle Tpm), ω is the increase in the association constant for the binding of Tpm1.1 with a contiguous binding site (cooperativity).

Circular dichroism (CD) measurements

The binding of 40 μ M Tmod1s2, Tmod4s1, Tmod4s2 and Lmod3s1 to equimolar concentrations of α TM1a₁₋₂₁Zip peptides, either WT or containing the K15N mutation, was assayed in 10 mM sodium phosphate pH 7.0, 100 mM NaCl in 1 mm cuvettes using an Aviv model 400 spectropolarimeter (Lakewood, NJ) as in [9]. The change in helical content of individual proteins and their complexes as a function of temperature was monitored at 222 nm.

Unfolding curves of complexes were fitted to the experimental data as in [42] to extract the thermodynamic parameters of melting. The dissociation constants (K_d) of the Tmod/Tpm or Lmod/Tpm complexes were calculated from the equation:

$$K_d = \exp\left(\frac{-\Delta\Delta G}{R \cdot T}\right),$$

where G is the difference between free energy of the folding of the complex and the folding of the α TM1a₁₋₂₁Zip alone, R is the universal gas constant, and T is the absolute temperature (K).

Pointed end binding assay

The binding of Tmod1 or Tmod4 to F-actin in the presence of Tpm1.1 was assayed as in [37], except the protein concentrations were 5 μ M for G-actin, 50 nM for gelsolin, 1.25 μ M for WT Tpm1.1, 3.50 μ M for Tpm1.1[K15N] and 200, 300, 400 or 500 nM for Tmod1 or Tmod4.

For experiments with Lmod2₁₋₅₁₄, 5 μ M G-actin in G-buffer was added to centrifuge tubes. Saturating concentrations of Tpm1.1 (1.25 μ M for WT, 3.50 μ M for K15N) were added to G-actin and incubated at room temperature for 10 minutes. Then, 100 nM Lmod2₁₋₅₁₄ was added and incubated at room temperature for 10 minutes. G-actin was polymerized for 20 minutes at room temperature and sedimented as described above.

For competition experiments, after incubating the seeds with 50 nM Tmod1, different concentrations of Lmod2₁₋₅₁₄ (50, 100, 200 nM) were added and incubated for 30 minutes at room temperature. For control (0 nM Lmod2₁₋₅₁₄), G-buffer was added to the seeds instead of Lmod2₁₋₅₁₄. F-actin was sedimented and the pellet samples were prepared for SDS-PAGE as described above. Tpm1.1, Tmods and Lmod2₁₋₅₁₄ were centrifuged at 65,000 RPM (Beckman-Coulter TLA-100 Rotor), room temperature, 30 minutes prior to all co-

sedimentation experiments to eliminate nonspecific sedimentation. The amounts of pelleted Tpm1.1, Tmod or Lmod2₁₋₅₁₄ in the absence of actin were negligibly small.

Western blot

The pellet samples of co-sedimentation experiments were run on SDS-PAGE, transferred to membranes for immunoblotting as described previously [37]. 1×50,000 dilution of the primary polyclonal rabbit antibodies against Tmod1 (custom made by Thermo Fisher Scientific, Waltham, MA), Tmod4 (custom made by Pacific Immunology, Ramona CA) or 1×100 dilution of primary polyclonal rabbit antibodies against Lmod2 (S-12, Santa Cruz Biotechnology).

Densitometric analysis of Western Blot membranes and Coomassie Blue-stained gels was done using ImageJ Software (NIH). For internal normalization, the background-corrected densities of Tmod or Lmod bands on the membranes were divided by that of the control (100%) to calculate the relative amount of Tmod or Lmod that co-sediments with actin filaments.

Fluorescence measurements

Actin polymerization was measured by the change in P-actin fluorescence using a PTI fluorometer (Lawrenceville, NJ) (excitation, 366 nm, and emission, 387 nm, with 2 nm slit width) as in [33]. F-actin seeds were prepared by polymerization of 3 μM G-actin in the presence of 30 nM gelsolin to measure inhibition rates of actin polymerization at the pointed end by Tmod1 or Lmod2₁₋₅₁₄. Polymerization was monitored by the increase in fluorescence when the filaments were diluted 5-fold with 1 μM G-actin (10% pyrenyl-actin) in F-buffer in the presence of saturating concentrations of Tpm1.1 (1 μM for WT, 3 μM for K15N) and/or 5 nM Tmod1 or Lmod2₁₋₅₁₄. Initial rates (R) were calculated as the first derivatives at time zero using *Exponential Growth to Maximum* or *Exponential Growth* fit settings in SigmaPlot 12.0. The inhibition of actin polymerization by Tmod1 or Lmod2₁₋₅₁₄ was calculated as $R_{\text{exp}}/R_{\text{control}}$. R_{control} is the rate of actin polymerization (control), and R_{exp} is the rate of actin polymerization in the presence of Tpm1.1 and Tmod1 or Lmod2₁₋₅₁₄.

All graphs were created and all statistical analyses (one-way ANOVA) were performed with SigmaPlot 12.0 or Prism 7.0b.

Results

The K15N mutation in Tpm1.1 decreases its affinity for F-actin

Lys-15 in Tpm1.1 is located next to Glu-16, which was shown to have close contact with Arg-147 of actin [43]. Since the K15N mutation disrupts the coiled-coil structure of Tpm1.1 [9], we hypothesized that this mutation may affect the interaction between Tpm1.1 and F-actin. To test this hypothesis, we compared the affinities of WT Tpm1.1 and Tpm1.1[K15N] for F-actin using a co-sedimentation assay (Figure 2). Data were fit to the Hill and the McGhee & von Hippel equations to calculate the binding constants (K_{app} and K_0 , respectively) for the interaction of F-actin and Tpm1.1 (Figure 2B,C and Table 1). Using

both approaches we demonstrated that the mutation caused an approximately 3-fold decrease in Tpm1.1's affinity for F-actin.

The K15N mutation in Tpm1.1 decreases its affinity for the second Tpm-binding site of Tmod1

We previously showed that the K15N mutation in Tpm1.1 weakened its interaction with the only Tpm-binding site of Lmod2 (Lmod2s1, residues 2-41) and the first Tpm-binding site of Tmod1 (Tmod1s1, residues 1-38) [9]. To test the effect of the K15N mutation on Tpm1.1's binding to the second Tpm-binding site of Tmod1 (Tmod1s2, residues 109-144) [24], we performed temperature-dependent unfolding experiments using circular dichroism (CD) with α TM1a₁₋₂₁Zip, a model peptide that contains the 21 N-terminal residues of Tpm1.1 [9]. The N-terminus of α TM1a₁₋₂₁Zip contained a Gly residue to mimic the N-terminal acetylation of Tpm. α TM1aZip with the N-terminal Gly and the acetylated Tpm peptide bind Tmod similarly [44].

Temperature-dependent unfolding of α TM1a₁₋₂₁Zip alone and in a complex with Tmod1s2 was monitored by CD at 222 nm. The thermodynamic parameters of unfolding of the Tpm/Tmod complex were determined from the melting curves and then used to calculate the dissociation constants (K_d) as in [45].

The melting temperatures (T_m) and the calculated K_d values for the complexes are presented in Table 2. The unfolding curve of the Tpm/Tmod peptide complex was different than the sum of the unfolding curve of the individual peptides (Figure 3). Increased helicity and higher melting temperature of the peptide mixture compared with their sums indicated complex formation. The K15N mutation reduced the T_m of the α TM1a₁₋₂₁Zip/Tmod1s2 complex. The decrease in T_m suggests formation of a weaker and less stable complex between Tmod1s2 and α TM1a₁₋₂₁Zip[K15N] compared with the WT Tpm peptide. We calculated K_d s from the melting curves and found that the affinity of Tmod1s2 for α TM1aZip decreased ~2.5-fold when the K15N mutation was present.

Additionally, we studied the effects of the K15N mutation on α TM1a₁₋₂₁Zip's binding to other Tmod and Lmod muscle isoforms, specifically Tmod4 and Lmod3 (Figure S1 of Supplementary Material). The peptides for the first and second Tpm-binding sites of Tmod4 (Tmod4s1, residues 2-39; Tmod4s2, residues 111-148) [46] and the Tpm-binding site of Lmod3 (Lmod3s1, residues 6-44) [25] were used for the measurements. The K_d values of complexes between α TM1a₁₋₂₁Zip and Tmod4s1, Tmod4s2 or Lmod3s1 are reported in Table S1 (Supplementary Material). We found that similarly to Tmod1 and Lmod2, the K15N mutation also decreased the binding of Tpm1.1 to the Tpm-binding sites of Tmod4 and Lmod3.

The K15N mutation affects Tmod1's and Lmod2's binding to F-actin in the presence of Tpm1.1

The CD experiments demonstrated that the interactions between the peptides that correspond to the individual binding sites of Tmod or Lmod isoforms and Tpm1.1 were weakened when the K15N mutation was present in Tpm1.1. The only Tpm-binding site of Lmod2 bound to the Tpm1.1 peptide with the K15N mutation ~6 times weaker than to the WT peptide [9],

while Tmod1s1 and Tmod1s2 showed ~4- [9] and ~2.5-fold decreases in binding affinity, respectively. One could expect that the cumulative decrease in binding of the full-length Tmod1 will be even higher than the decrease for either of the Tpm-binding sites. However, it is difficult to derive the quantitative effect of this mutation on the interaction between full-length proteins from the data on fragments. To find out how binding of full-length Tmod to F-actin would be affected by the presence of the K15N mutation in Tpm1.1, co-sedimentation experiments were performed. Actin filaments capped with gelsolin at their barbed ends (seeds) were prepared and WT Tpm1.1 or Tpm1.1[K15N] was added to the seeds at saturating concentrations determined from the F-actin binding experiments (Figure 2). Tmod1 was added to the seeds at different concentrations and sedimented. The amount of Tmod1 in the pellets increased in a concentration-dependent fashion in the presence of WT or mutated Tpm1.1 (Figure 4). The amount of Tmod1 was ~2-fold less in the pellet samples of F-actin with Tpm1.1[K15N] compared to the amount of Tmod1 in the samples with WT Tpm1.1. Surprisingly, when the same experiment was performed using Tmod4, the differences between the amounts of Tmod4 that were co-sedimented in the presence of WT or mutated Tpm1.1 were not statistically significant (Figure S2 of Supplementary Material), although both Tmod4s1 and Tmod4s2 demonstrated decreased binding to α TM1a₁₋₂₁Zip[K15N] in CD experiments. Therefore, our experiments show that the K15N mutation in Tpm1.1 disrupts Tmod1's binding at the pointed end.

We also studied the binding of Lmod2 to F-actin in the presence or absence of Tpm1.1. Unlike Tmod1, Lmod2 is a strong nucleator of actin polymerization [17]. Since after polymerizing gelsolin-capped seeds, a fraction of G-actin (equal to the critical concentration of actin for the pointed end) remains in solution, using Lmod2 under the same assay conditions as with Tmod1 may result in nucleation of remaining G-actin and creation of new uncapped barbed ends. Therefore, we changed the co-sedimentation assay conditions for experiments with Lmod2. Instead of nucleating actin polymerization using gelsolin, we nucleated actin polymerization by adding Lmod2 to G-actin. This resulted in formation of seeds with Lmod2 already bound at the pointed end. In addition, we used Lmod2₁₋₅₁₄, which lacks the C-terminal WH2 domain (Figure 1). This truncated Lmod2 was previously shown to nucleate G-actin, although weaker than full-length Lmod2 and it inhibits actin polymerization from the pointed ends in a Tpm-dependent fashion [8].

To observe how Lmod2's binding at the pointed ends is affected in the presence of WT or mutated Tpm1.1, the seeds were prepared by polymerizing G-actin in the presence of Tpm1.1 and Lmod2₁₋₅₁₄. We analyzed the pellets of sedimented F-actin samples to see if the amount of Lmod2₁₋₅₁₄ co-sedimented with F-actin would change in the presence of the K15N mutation in Tpm1.1. (Figure 5A). Our results showed that the amount of Lmod2₁₋₅₁₄ co-sedimented with F-actin decreased by ~40% in the presence of Tpm1.1[K15N]. Interestingly, there was no statistically significant difference between this amount and the amount of Lmod2₁₋₅₁₄ co-sedimented with F-actin in the absence of Tpm1.1 (Figure 5B).

Tmod1 and Lmod2 compete for binding F-actin

Tmod1 and Lmod2 share similar binding domains (Figure 1) and one can expect them to compete with each other for interacting with the same binding partners. Therefore, in order

to understand if Tmod1 and Lmod2 compete for binding F-actin, we performed co-sedimentation experiments. Tmod1 was allowed to bind actin seeds in the presence of Tpm1.1. Then, increasing concentrations of Lmod2₁₋₅₁₄ were added to see if there would be a change in the amount of co-sedimented Tmod1 with F-actin in the presence of Lmod2₁₋₅₁₄.

The amount of Tmod1 co-sedimented with F-actin decreased when the Lmod2₁₋₅₁₄ concentration was increased (Figure 6). When 200 nM Lmod2₁₋₅₁₄ was added, ~50% of Tmod1 remained in F-actin pellets in the presence of WT Tpm1.1. On the other hand, only ~30% of Tmod1 was detected in F-actin pellets in the presence of Tpm1.1[K15N]. Together these findings suggest that Tmod1 and Lmod2₁₋₅₁₄ compete for binding to F-actin in the presence of Tpm1.1 and the K15N mutation in Tpm1.1 favors Lmod2₁₋₅₁₄'s ability to remove bound Tmod from F-actin.

The K15N mutation in Tpm1.1 decreases the abilities of Tmod1 and Lmod2₁₋₅₁₄ to inhibit pointed end polymerization

To find out if the K15N mutation in Tpm1.1 affects the inhibitory abilities of Tmod1 or Lmod2₁₋₅₁₄, a pyrene-actin pointed end polymerization assay was performed. Gelsolin-capped F-actin seeds were prepared and G-actin polymerization at the pointed end was measured as the increase in fluorescence in the presence of Tpm1.1 and Tmod1 or Lmod2₁₋₅₁₄. Exponential growth curves of actin polymerization were fit to the data and initial rates were calculated as derivatives at time 0.

We first compared the effect of the K15N mutation on the pointed end polymerization in the presence of Tpm1.1 only (Figure 7A, D). There was no statistically significant difference between the rates of actin polymerization in the presence of WT Tpm1.1 and Tpm1.1[K15N]. Next, we compared the ability of Tmod1 or Lmod2₁₋₅₁₄ to inhibit actin polymerization at the pointed end in the presence of WT or mutated Tpm1.1. Actin polymerization was monitored in the presence of saturating concentrations of WT Tpm1.1 or Tpm1.1[K15N], and 5 nM Tmod1 or Lmod2₁₋₅₁₄ (Figure 7B, C). 5 nM was chosen, since Tmod1 and Lmod2₁₋₅₁₄ exhibited ~50% inhibition of polymerization in the presence of Tpm1.1 at this concentration [8, 33]. We expected that a possible difference in their capping abilities due to the K15N mutation in Tpm1.1 should be more evident at this concentration.

Previously Tmod1 was shown to be more effective in capping than Lmod2₁₋₅₁₄ [8]. As expected, at a concentration of 5 nM, Tmod1 capped the pointed ends of F-actin more effectively than Lmod2₁₋₅₁₄ in the presence of WT Tpm1.1 (Figure 7D and Table S2 of Supplementary Material). The K15N mutation in Tpm1.1 decreased the capping ability of both proteins at comparable levels (~1.5 fold) and Tmod1 was still more effective in inhibiting actin polymerization from the pointed ends than Lmod2₁₋₅₁₄ in the presence of Tpm1.1[K15N]. In conclusion, weakening of the molecular interactions between Tpm1.1 and Tmod1 or Lmod2 by the K15N mutation in Tpm1.1 ultimately disrupts the regulation of actin polymerization at the pointed end.

Discussion

The K15N mutation in the *TPM1* gene is associated with DCM [3, 5] but the molecular mechanism for how this mutation leads to cardiomyopathy development is unknown. We previously showed that this mutation disrupted the coiled-coil structure of α TM1a₁₋₂₁Zip, a peptide containing the N-terminal region of Tpm1.1 [9]. Here, we introduced the K15N mutation in full-length Tpm1.1 to study the effects of this mutation on the assembly of the pointed end, which is crucial to maintain correct lengths of thin filaments [2]. First, we demonstrated that the mutation affected Tpm1.1's binding to F-actin. We used two different approaches to calculate Tpm1.1's affinity for actin using the Hill and the McGhee & von Hippel models [41, 47, 48]. By both methods, we showed that the mutation caused a ~3-fold decrease in the Tpm1.1's affinity for F-actin. The K15N mutation is located in close proximity to Glu-16, which has a close contact with Arg-147 of actin [43]. The decrease in actin-binding affinity of Tpm1.1 may be explained by disruption of the coiled-coil structure of Tpm1.1 caused by the K15N mutation. The mutation may induce a conformational change on the neighboring Glu-16, which in turn may affect the molecular interactions with actin. Another explanation is that the mutation may alter the head-to-tail interactions between Tpm1.1 molecules. Tpm1.1 binds F-actin cooperatively in a head-to-tail fashion and the 11 N-terminal residues of Tpm1.1 are involved in the formation of the overlap junction with the C-terminus of the adjacent Tpm1.1 molecule [49]. A change in the structure of Tpm1.1 caused by the K15N mutation may affect the conformation of the N-terminus of Tpm1.1 and therefore cooperativity of Tpm1.1 binding to actin filaments.

A decrease in Tpm1.1's affinity to actin alone can affect the stability of F-actin in cardiac muscle cells and lead to DCM development, as previously shown for Tpm1.1's with different mutations, such as M8R [4]. Similar to Met-8, Lys-15 is in the position *a* of the heptad repeat (Figure 1B). However, the decrease in Tpm1.1's affinity for actin caused by the K15N mutation is significantly smaller compared with that of the M8R mutation (3-fold and 100-fold, respectively) [50].

In our previous study, we demonstrated decreased binding between the Tpm-binding site of Lmod2 (Lmod2s1) or the first Tpm-binding site of Tmod1 (Tmod1s1) and α TM1a₁₋₂₁Zip[K15N] compared with the WT peptide [9]. Our CD results in this study showed that binding of the second Tpm-binding site of Tmod1 (Tmod1s2) to Tpm1.1 also decreased in the presence of the K15N mutation. Therefore, this mutation affects the binding of both Tpm-binding sites of Tmod1 to Tpm1.1.

The K15N mutation also weakened the interactions between α TM1a₁₋₂₁Zip and Lmod3s1, Tmod4s1 and Tmod4s2. However, Tmod4 is not expressed in cardiac muscle [26] and the hearts of the Lmod3-knockout mice maintain normal function [18]. Therefore, the decreases in the affinities between Lmod3 or Tmod4 and the mutated Tpm1.1 are unlikely to be associated with DCM development.

Our affinity measurements showed significant decreases in Tmod and Lmod's binding to Tpm1.1. We tested the binding of full-length Tmod1 and Lmod2₁₋₅₁₄ to actin filaments in the presence of WT or mutated Tpm1.1. The K15N mutation in Tpm1.1 decreased the

pointed end-binding and –capping abilities of Tmod1 and Lmod2₁₋₅₁₄. By weakening the molecular interactions between Tpm1.1 and Tmod1 or Lmod2, the K15N mutation impairs the regulation of actin polymerization at the pointed end.

We also demonstrated that Lmod2 is able to displace Tmod1 bound to F-actin in the presence of Tpm1.1 *in vitro*. This observation suggests that a competition mechanism for binding F-actin exists between these proteins. This finding is consistent with the proposed models for the antagonistic function of Tmod1 and Lmod2 in modulating thin filament lengths in cardiomyocytes [7, 8]. Tmod1 has two Tpm-binding sites and the K15N mutation in Tpm1.1 affects the binding of both of these sites. Therefore, the K15N mutation in Tpm1.1 can be expected to have a greater effect in the Tmod1's binding at the pointed end compared with Lmod2, which has only one Tpm-binding site. Furthermore, Lmod2 contains an extended first actin-binding site compared with Tmod1 [7] and an additional third actin-binding site [17, 27]. Indeed, the K15N mutation in Tpm1.1 seemed to enhance Lmod2's ability to replace Tmod1 bound at the pointed end. Therefore, relatively strong actin-binding by Lmod2 may preserve Lmod2's ability to compete with Tmod1 for binding at the pointed ends, while Tmod1's binding at the pointed end is weakened by the K15N mutation in Tpm1.1. To summarize, the differences between the binding sites of Tmod1 and Lmod2 can explain the unequal effects of the K15N mutation on the competition between Tmod1 and Lmod2.

Our data demonstrate that the K15N mutation in Tpm1.1 alters the balance between Tmod1 and Lmod2 for binding to the pointed ends of thin filaments. Enhanced displacement of Tmod1 by Lmod2 from the pointed end may result in longer thin filaments in the hearts of DCM patients with this K15N mutation. We predict that this change may be an important factor leading to K15N-associated DCM development in the heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The K15N mutation in Tpm1.1 weakens its binding to F-actin, Tmod1 and Lmod2.
- Tmod1 and Lmod2's binding to F-actin is decreased when Tpm1.1 is mutated.
- The K15N mutation impairs inhibition of actin polymerization at the pointed end.
- Tmod1 and Lmod2 compete for binding F-actin and Tpm modulates their competition.

Figure 1. The schematic view of the binding sites of Tmod, Lmod and Tpm1.1

A: Domain structure of Tmod and Lmod. Tpm1, Tpm2: tropomyosin-binding sites; A1, A2, A3: actin-binding sites, LRR: leucine-rich repeats. The dashed line shows where Lmod2 was truncated in this study. B: Amino acid sequence of the N-terminus of Tpm1.1. The location of the DCM-associated K15N mutation is shown in bold. The binding site for Tmod and Lmod [9] is shown in the box. The letters below the sequence demonstrate the positions of the coiled-coil heptad repeat.

Figure 2. Effect of the K15N mutation on Tpm1.1's binding to actin

Binding of Tpm1.1 (WT or K15N) was measured by co-sedimentation with F-actin. A: 0-4 μM of Tpm1.1 was added to 5 μM F-actin and sedimented at 25 $^{\circ}C$ in 25 mM Imidazole pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.01% NaN_3 , 0.2 mM ATP and 0.5 mM DTT. B: Stoichiometric binding of one Tpm1.1 per seven actins is represented by fraction maximal binding of 1. The data (obtained in triplicates) were fit to the Hill equation to estimate the apparent binding constant (K_{app}). C: Evaluation of the binding between Tpm1.1 and actin using the McGhee & von Hippel approach. The lines represent the best fits for estimating the association constant (K_D) from nonlinear regression analysis. The binding constants are presented in Table 1.

Figure 3. Temperature dependent unfolding of the α TM1a₁₋₂₁Zip/Tmod1s2 complex
 Binding of Tmod1s2 to α TM1a₁₋₂₁Zip (WT) or α TM1a₁₋₂₁Zip[K15N] (K15N) was assessed with CD spectroscopy. The graphs demonstrate the unfolding curves of the individual peptides (Tmod1s2, WT, K15N), their mixtures (Tmod1s2/WT, Tmod1s2/K15N) and the sums of the individual unfolding curves (Tmod1s2+WT, Tmod1s2+K15N). The lines represent fitted curves to the melting data.

Figure 4. Co-sedimentation of Tmod1 with F-actin in the presence of Tpm1.1

A: 200-500 nM Tmod1 was added to gelsolin-capped 5 μ M F-actin in the presence of saturating concentrations of WT (1.25 μ M) or mutant Tpm1.1 (3.5 μ M) and sedimented. Pellets were loaded on 10% acrylamide SDS-gels and transferred to PVDF membranes for Tmod1 detection using antibodies. Parallel Coomassie-stained gel shows co-sedimented F-actin and Tpm1.1. B: Background-corrected densities of Tmod1 bands were divided by that of control (500 nM Tmod1 co-sedimented with F-actin and WT Tpm1.1) to calculate relative Tmod1 band densities. Columns show averages and errors bars show the standard deviations (n=4). Asterisks demonstrate statistically significant differences between groups that were determined by One-Way ANOVA post hoc Holm-Sidak test (** p <0.01, *** p <0.001).

Figure 5. Co-sedimentation of Lmod2₁₋₅₁₄ with F-actin in the presence of Tpm1.1

A: 5 μ M G-actin was polymerized in the presence of saturating concentrations of WT (1.25 μ M) or mutant Tpm1.1 (3.5 μ M) and 100 nM Lmod2₁₋₅₁₄. After sedimenting F-actin, the pellets were loaded on 10% acrylamide SDS-gels and transferred to PVDF membranes for Lmod2 detection using antibodies. Parallel Coomassie-stained gel shows co-sedimented F-actin and Tpm1.1. B: Background-corrected densities of Lmod2₁₋₅₁₄ bands were divided by that of control (100 nM Lmod2₁₋₅₁₄ co-sedimented with F-actin only) to calculate relative Lmod2₁₋₅₁₄ band densities. Columns show averages and errors bars show the standard deviations (n=3). Asterisk demonstrates statistically significant difference between the groups that was determined by One-Way ANOVA post hoc Holm-Sidak test (* p <0.05).

Figure 6. The effect of the K15N mutation in Tpm1.1 on the competition between Tmod1 and Lmod2₁₋₅₁₄ for binding F-actin

A: 50 nM Tmod1 was added to gelsolin-capped 5 μ M F-actin in the presence of saturating concentrations of WT (1.25 μ M) or mutant Tpm1.1 (3.5 μ M). 0-200 nM Lmod2₁₋₅₁₄ was then added and F-actin was sedimented. Pellets were loaded on 10% acrylamide SDS-gels and transferred to PVDF membranes for Tmod1 and Lmod2 detection using antibodies. Parallel Coomassie-stained gel shows co-sedimented F-actin and Tpm1.1. B: Background-corrected densities of Tmod1 bands were divided by of control (50 nM Tmod1 co-sedimented with F-actin in the presence of WT Tpm1.1 or Tpm1.1[K15N]) to calculate relative Tmod1 band densities. Columns show averages and errors bars show the standard deviations (n=4). Asterisks demonstrate statistically significant differences between groups that were determined by One-Way ANOVA post hoc Holm-Sidak test (* p <0.05, ** p <0.01, *** p <0.001).

Figure 7. The effect of the K15N mutation on Tpm1.1-dependent inhibition of actin polymerization by Tmod1 and Lmod2₁₋₅₁₄

The pointed-end capping ability of Tmod1 and Lmod2 was assayed with gelsolin-capped actin filaments (0.6 μ M) in the presence of saturating concentrations of Tpm1.1 (1 μ M for WT, 3 μ M for K15N). Representative pointed end polymerization curves of actin-filaments A: in the absence and B, C: in the presence of 5 nM Tmod1 or Lmod2₁₋₅₁₄ were measured as an increase in fluorescence. Lines represent fitted curves to polymerization data. D: Initial rates were calculated as the first derivatives at time zero after fitting. The inhibition of actin polymerization by Tmod1 or Lmod2₁₋₅₁₄ was calculated as $R_{exp}/R_{control}$. Control is actin polymerization in the absence of Tpm1.1, Tmod1 and Lmod2₁₋₅₁₄. Columns are averages and error bars are standard deviations (n=3-8). Letters above columns (a, b, c, d) represent the groups that show statistically significant differences determined by One-Way ANOVA post hoc Holm-Sidak test (see Table S2 of Supplementary Material for the p values).

Table 1

Parameters of Tpm1.1 binding to F-actin.

	Hill model	McGhee & von Hippel model
	K_{app} (μM^{-1})	K_{θ} (μM^{-1})
WT	2.69 ± 0.13	0.060 ± 0.014
K15N	0.98 ± 0.05	0.020 ± 0.002

Table 2

Melting temperatures (T_m) and dissociation constants (K_d) of the Lmod2/Tpm1.1 or Tmod1/Tpm1.1 peptide complexes.

Peptide	α TM1a ₁₋₂₁ Zip		α TM1a ₁₋₂₁ Zip[K15N]	
	T_m (°C)	K_d (30°C, μ M)	T_m (°C)	K_d (30°C, μ M)
Lmod2s1 ^a	36.4 ± 0.1	0.37 ± 0.05	28.0 ± 0.2	2.31 ± 0.07
Tmod1s1 ^a	32.5 ± 0.7	1.00 ± 0.13	26.1 ± 0.2	3.77 ± 0.01
Tmod1s2	31.3 ± 0.5	1.50 ± 0.14	25.2 ± 0.8	3.96 ± 0.52

The thermodynamics of unfolding of the complexes between Tpm1.1 and Tmod1 or Lmod2 peptides and the Tpm1.1 peptide alone were used to estimate the K_d values.

^aReported earlier in [9].