Tropomyosin Ends Determine the Stability and Functionality of Overlap and Troponin T Complexes

Thomas Palm, Norma J. Greenfield, and Sarah E. Hitchcock-DeGregori
Department of Neuroscience and Cell Biology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-5635

ABSTRACT Tropomyosin binds end to end along the actin filament. Tropomyosin ends, and the complex they form, are required for actin binding, cooperative regulation of actin filaments by myosin, and binding to the regulatory protein, troponin T. The aim of the work was to understand the isoform and structural specificity of the end-to-end association of tropomyosin. The ability of N-terminal and C-terminal model peptides with sequences of alternate α-tropomyosin isoforms, and a troponin T fragment that binds to the tropomyosin overlap, to form complexes was analyzed using circular dichroism spectroscopy. Analysis of N-terminal extensions (N-acetylation, Gly, AlaSer) showed that to form an overlap complex between the N-terminus and the C-terminus requires that the N-terminus be able to form a coiled coil. Formation of a ternary complex with the troponin T fragment, however, effectively takes place only when the overlap complex sequences are those found in striated muscle tropomyosins. Striated muscle tropomyosins with N-terminal modifications formed ternary complexes with troponin T that varied in affinity in the order: N-acetylated > Gly > AlaSer > unacetylated. The circular dichroism results were corroborated by native gel electrophoresis, and the ability of the troponin T fragment to promote binding of full-length tropomyosins to filamentous actin.

INTRODUCTION

Tropomyosin (TM) is an α-helical protein that forms a two-stranded coiled coil. Tropomyosin molecules overlap at their ends by ~8–11 amino acids (McLachlan and Stewart, 1975) to form long chains along the grooves of the actin filament. This way TM confers structural stability to the actin filament and modulates its function (reviewed in Perry, 2001). In eukaryotic cells TM exists in a large number of isoforms which are expressed by alternative promoters and alternative splicing of multiple genes (Lin et al., 1997).

Two major classes of tropomyosins in higher eukaryotes are the long, ~284-residue tropomyosins expressed in muscle and nonmuscle cells, and the short ~247-residue tropomyosins found in nonmuscle cells, that differ at the N-terminus (Lin et al., 1997). In the short tropomyosin isoforms, which span the length of six actin monomers in the filament, exon 1b (residues 1–44) replaces exon 1a and 2 (residues 1–80) expressed in the long isoforms, which span the length of seven actin monomers. Additional diversity results from alternative splicing of the other coding exons, including exon 9, which encodes the C-terminal 26 amino acids.

Studies of many different tropomyosin isoforms, both wild-type and recombinant forms, as well as nonnatural chimerae, have established that the N- and C-terminal regions of tropomyosin, and modifications to those sequences, alter tropomyosin binding to actin and troponin as well as the cooperative interaction with myosin on the actin filament (Mak and Smillie, 1981; Dabrowska et al., 1983; Heeley et al., 1987; Cho et al., 1990; Cho and Hitchcock-DeGregori, 1991; Willadsen et al., 1992; Butters et al., 1993; Novy et al., 1993a,b; Urbancikova and Hitchcock-DeGregori, 1994; Hammell and Hitchcock-DeGregori, 1996; Pittenger et al., 1995; Moraczewska et al., 1999; Sano et al., 2000; Cho, 2000). Of particular importance for striated muscle tropomyosin is N-acetylation of the initial methionine, a modification that stabilizes the coiled coil α-helix and allows the N-terminus to be coiled coil to the end (Greenfield et al., 1994; Brown et al., 2001). In the absence of troponin, recombinant striated muscle tropomyosin expressed in *Escherichia. coli*, which is not acetylated, binds only weakly to actin. In the presence of troponin it binds well and can regulate the actomyosin ATPase. A fusion protein in which the N-terminal acetyl group is replaced by an 80-residue polypeptide binds well to actin, but the binding of troponin is Ca^{2+} sensitive and it does not confer effective calcium sensitivity on the actomyosin ATPase (Hitchcock-DeGregori and Heald, 1987; Heald and Hitchcock-DeGregori, 1988). The dipeptide AlaSer fused to the N-terminus of tropomyosin seems sufficient to replace the function of the N-terminal acetyl group in that it restores actin binding, head-to-tail polymerization, and the ability to inhibit the ATPase (Monteiro et al., 1994; Maytum et al., 2000).

The exon 9-specified C-terminus is also a major functional determinant. Replacement of the striated muscle specific exon 9a encoded C-terminus with that encoded by exon 9d, expressed in smooth and nonmuscle cells, allows unacetylated muscle tropomyosin to bind well to actin (Cho and Hitchcock-DeGregori, 1991). The higher affinity is attributable to the C-terminal nine amino acids encoded by exon 9d (Hammell and Hitchcock-DeGregori, 1996), in particular Gln276 and Thr 277 (Cho, 2000). However, the striated muscle specific exon 9a is required for binding to troponin T and for troponin to promote the binding of tropomyosin to

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Address reprint requests to Thomas Palm, Tel.: 732-235-4528; Fax: 732-235-4029; E-mail: palmth@umdnj.edu.

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actin (Cho and Hitchcock-DeGregori, 1991; Hammell and Hitchcock-DeGregori, 1996, 1997).

Our aim in the present work was to understand the isoform and structural specificity of the end-to-end association of tropomyosin molecules to form an overlap complex and to form a ternary complex with troponin T. For these studies we have used a model system that allows us to study the interaction of tropomyosin ends directly using N- and C-terminal model peptides, and a soluble troponin T fragment that binds to the C-terminus of tropomyosin (Palm et al., 2001). Model peptides have proven useful to study the structure and function of tropomyosin (Greenfield et al., 1994; Holtzer et al., 1995; Greenfield et al., 1998; Emerson Holtzer et al., 2001; Greenfield et al., 2001; Greenfield and Fowler, 2002). Here, with analysis of model peptides using circular dichroism we have been able to measure the affinity of the N-terminus for the C-terminus in forming an overlap complex, the conformational change that takes place upon binding, and the effect of N-terminal and C-terminal modifications on complex formation. Furthermore, the specificity for binding to troponin T to form a ternary overlap complex has been investigated, and correlated with the ability of troponin and the troponin T to promote binding of full-length tropomyosin to actin. The results give insight into the structural requirements and sequence specificity for formation of functional complexes.

MATERIALS AND METHODS

Preparation of TM9a$_{251–284}$

The synthetic gene for TM9a$_{251–284}$ (Table 1) was designed to encode a histidine purification tag and a TEV protease recognition site for removal of the His-tag after peptide purification. As previously described for GlyTM1bZip (Greenfield et al., 2001), we selected the best expressed codons in *E. coli* using the software DNA* (DNASTAR, Inc., Madison WI). The DNA was cloned into pET11-HTb (Greenfield et al., 2002) between NdeI and KpnI, and expressed in *E. coli* BL21(DE3) (Studier et al., 1990). TM9a$_{251–284}$ was isolated and oxidized as previously described (Greenfield et al., 2002). The typical yield of oxidized lyophilized peptide was ~0.5 mg/l of culture medium.

Synthetic tropomyosin peptides

Synthetic C-terminal peptides (TM9a$_{246–284}$N279K: last 39 residues of rat α-TM with the sequence encoded by exon 9a with a Lys replacing the native Asn at position 279; TM9d$_{246–284}$: last 39 residues of rat α-TM with the sequence encoded by exon 9d) and N-terminal peptides (N-acetylated AcTM1aZip, AlaSerTM1aZip, unacetylated TM1aZip, and AcTM1bZip, Table 1) of tropomyosin were commercially synthesized, purified to >95% purity by reverse phase HPLC and analyzed by mass spectrometry (SynPep, Dublin, CA).

Recombinant tropomyosin and troponin T peptides

The N-terminal TM model peptide GlyTM1aZip was prepared as published earlier (Greenfield et al., 2002). The human cardiac troponin T fragment hcTnT$_{70–170}$ was expressed in *E. coli* and purified as previously described (Palm et al., 2001).

Recombinant tropomyosin isoforms and chicken skeletal α-tropomyosin

DNA constructs and protein expression for the rat α-tropomyosin isoforms 1a9a, 1a9d, 1b9a, and 1b9d have been previously described (Cho and Hitchcock-DeGregori, 1991; Hammell and Hitchcock-DeGregori, 1996; Moraczewska et al., 1999). The cDNA for AlaSer-tropomyosin was made from rat striated TM cDNA in a pET11d vector using the oligonucleotide primers 5′-CATATGGCTTCTATGGACGCCATCAAGAAGAAGCGCC-3′ and 5′-TATATCTCCTTCTTAAAGTTAAACAAAATTATTTC-3′ and standard PCR techniques. The product contained a novel NdeI site to facilitate selection of positive clones. AlaSer-TM was expressed in *E. coli* BL21(DE3). All recombinant tropomyosin isoforms were purified as described previously (Hitchcock-DeGregori and Heald, 1987; Hammell and Hitchcock-DeGregori, 1996; Moraczewska et al., 1999). The N-termini are unacetylated. Acetylated striated muscle α-tropomyosin was purified from the isoelectric precipitate from a troponin preparation from chicken pectoral muscle (Hitchcock-DeGregori et al., 1985).

Circular dichroism studies

Circular dichroism (CD) data were collected on an Aviv model 62D spectropolarimeter (Aviv, Lakewood, NJ) equipped with a five-sample thermal-equilibration chamber as previously described (Greenfield and Hitchcock-DeGregori, 1995). N-terminal TM-peptides were measured either alone, or in equimolar mixtures with C-terminal TM model peptides (overlap complex), or with C-terminal TM model peptides and hcTnT$_{70–170}$ (ternary complex).

Data were collected at a concentration of 10 μM for each peptide in 10 mM potassium phosphate, pH = 6.5. Circular dichroism spectra were measured at 0°C and the α-helical content of the TnT peptides was calculated from ellipticity data using the neural network program, CDNN (Böhm et al., 1992). Thermal stability measurements were performed by following the ellipticity at 222 nm as a function of temperature between 0°C and 70°C. Unless mentioned otherwise, the midpoints of the thermal transitions (T_m) were determined from fits of the melting curves to the Gibbs-Helmholtz equation for a single transition. The enthalpy (ΔH) and entropy (ΔS) of folding for the tropomyosin and troponin T peptides were determined from the change in ellipticity as previously described (Greenfield et al., 1998). To estimate ΔH and ΔS for the binary tropomyosin overlap complexes, the melting curves were fit to the Gibbs-Helmholtz equation for a single transition, assuming that one mole of the complex dissociates into one mole of each of the components. For the ternary peptide complexes of troponin T and the tropomyosin overlap, the enthalpy and entropy of folding were

TABLE 1 Peptide sequences

TM9a$_{251–284}$	GCGKSIDDLEDELYAQKLKYKAISEELDHALNDMTSI
TM9a$_{246–284}$N279K	VTKLEKSIDDLEDELYAQKLKYKAISEELDHALKDMTSI
TM9d$_{246–284}$	VTKLEKSIDDLEEKVAHAKEENLSMHQMLDQTLLELNNM
TM1aZip	MDAIKKKMQMLKLDNYHLENEVARLKKLVGER
TM1bZip	AGSSSLEAVRRKIRSLQEQNYHLENEVARLKKLVGER

Homologous sequences in the C-terminal and the N-terminal peptides are aligned.

determined by fitting the CD of the complex as a function of temperature to the linear van't Hoff equation. Apparent binding constants were estimated from the free energy of folding (ΔG) of the binary and ternary peptide complexes at 20°C. The free energy of folding was calculated from ΔH and ΔS using the Gibbs equation, $\Delta G = \Delta H - T\Delta S$, in which T is the absolute temperature and ΔH and ΔS were assumed to be independent of temperature. Using the assumption that the entire difference in ΔG between the complexes and their components ($\Delta\Delta G$) was due to binding, the apparent dissociation constant was estimated using the equation $K_d(app) = 1/\exp(-\Delta\Delta G/nRT)$. These estimates depend on the fact that the complexes unfolded in two-state reversible cooperative transitions. This was the case for all complexes that formed except the ternary complex that involved unacetylated TM1aZip. The apparent dissociation constants reported here for the ternary complexes are the dissociation constants of the troponin T peptide for the tropomyosin overlap. The apparent dissociation constant for the whole ternary complex would be the product of the dissociation constant of the TM overlap complex and the dissociation constant of the troponin T peptide for the tropomyosin overlap complex.

Native gel electrophoresis

Samples of ternary complexes of N-terminal TM model peptides with $TM9a_{251–284}$ and $hcTnT_{70–170}$ for native gel electrophoresis were prepared at a concentration of 10 μM in 10 mM potassium phosphate, pH = 6.5, 10% glycerol. Ten μl samples were run at 4°C on 10% acrylamide, 10% glycerol gels in 20 mM Tris, pH = 8.8, 120 mM glycine (Katayama and Nozaki, 1982). Gel bands were quantitated using a Molecular Dynamics 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Tropomyosin binding to F-actin

The effect of $hcTnT_{70–170}$ on the affinity of TM isoforms for actin was measured by co-sedimentation. Tropomyosin was combined with $hcTnT_{70–170}$ in a 1:1.2 molar ratio in 100 or 300 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 10 mM Tris, pH 7.5 and centrifuged at 60,000 rpm, 20°C, for 25 min in a Beckman TLA-100 rotor to remove precipitated protein. F-actin (5 μM final concentration) was combined with the TM and $hcTnT_{70–170}$ mixture (0–6 μM final concentration) and centrifuged as before. The actin and TM were quantitated in SDS polyacrylamide gels of the pellets and supernatants using a Molecular Dynamics 300A computing densitometer. The data were fit to the Hill equation to determine the affinity of TM for actin:

$$\nu = n[\mathrm{TM}]^H / \left((K_{dapp})^H + [\mathrm{TM}]^H\right) \quad (1)$$

where [TM] is the concentration of free tropomyosin in the sample, ν is the observed TM/actin density ratio at [TM], n is the maximal TM/actin density ratio, K_{dapp} is the apparent equilibrium constant at which 50% of TM is bound to actin, and H is the Hill coefficient.

General methods

Skeletal muscle actin was prepared from chicken pectoral muscle acetone powder (Hitchcock-DeGregori et al., 1982). SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Unless mentioned otherwise, the concentration of proteins and peptides was determined with the micro biuret method (Goa, 1953).

RESULTS

Design of tropomyosin and troponin T model peptides

We used a functional peptide model system to investigate the isoform and structural specificity of the tropomyosin ends for formation of a binary overlap complex between the N- and C-termini, and for formation of a ternary complex with the N-terminus of troponin T, independent of the rest of the tropomyosin molecule. We originally developed the model to study the effects of hypertrophic cardiomyopathy-causing mutations in TnT on interactions with tropomyosin and actin (Fig. 1 and Palm et al., 2001). The tropomyosin C-terminus is a 37-residue peptide, $TM9a_{251–284}$, consisting of residues 251–284 of rat striated α-tropomyosin with the N-terminal extension Gly-Cys-Gly to allow oxidative cross-linking via disulfide bond formation (Table 1). The recombinant peptide includes the entire region encoded by striated muscle specific exon 9a (residues 258–284) plus seven amino acids encoded by the constitutively expressed exon 8. To compare the function of the striated muscle-specific exon 9a C-terminus with the smooth/nonmuscle tropomyosin exon 9d-encoded C-terminus we used synthetic model peptides with the last 39 amino acids ($TM9a_{246–284}$N279K, $TM9d_{246–284}$). The $TM9a_{246–284}$N279K peptide contains a functionally neutral Asn279Lys mutation that increases the stability of the peptide (Palm et al., 2001).

The tropomyosin N-terminal model peptides were synthetic chimeric peptides with known structures (Table 1; Greenfield et al., 1998, 2001). In both, the C-terminal 18 residues are the last 18 C-terminal residues of the GCN4 transcription factor leucine zipper (Landschulz et al., 1988), included to promote and stabilize formation of a parallel two-chained coiled coil. For the N-terminus found in long TMs (all muscle tropomyosins as well as many nonmuscle isoforms), the first 14 amino acids are residues 1–14 encoded by rat α-tropomyosin exon 1a (TM1aZip). Modifications of the N-terminal methionine will be discussed below. The alternative N-terminal peptide for short tropomyosin isoforms, TM1bZip, includes residues 1–19 encoded by rat α-tropomyosin exon 1b, where the N-terminal Ala is acetylated (in a synthetic peptide, AcTM1bZip). This peptide contains 14 residues of exon 1b that are homologous to exon 1a plus a native five amino acid N-terminal extension (Table 1; Lewis et al., 1983; Greenfield et al., 2001). The $hcTnT_{70–170}$ is a recombinant fragment of human cardiac TnT that binds well to the C-terminus of striated muscle α-tropomyosin and

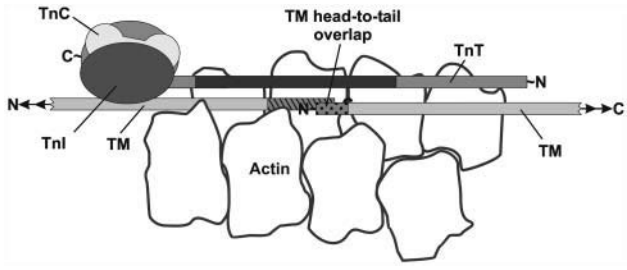

FIGURE 1 Model of the troponin-tropomyosin complex on actin (modified from Heeley et al., 1987). The dark shaded area represents residues 70–170 of TnT. The dotted area is the part of tropomyosin that corresponds to the N-terminal model peptides used in this study and the striped area corresponds to the C-terminal model peptides.

stabilizes the overlap complex with the N-terminus (Palm et al., 2001).

Isoform specificity of formation of binary and ternary overlap complexes

Our work with full-length recombinant tropomyosins established the critical importance for the alternatively expressed N- and C-terminal sequences in determining actin affinity and binding to troponin T (Cho and Hitchcock-DeGregori, 1991; Hammell and Hitchcock-DeGregori, 1996, 1997; Moraczewska et al., 1999). To study the specificity of the N- and C-terminal overlap formation we used the 39-residue, uncross-linked model peptides, $TM9a_{246–284}N279K$ and $TM9d_{246–284}$.

Circular dichroism in 10 mM phosphate was used to determine the secondary structure and thermal stability of tropomyosin model peptides. Initial experiments were also performed at physiological salt concentrations. These experiments showed essentially the same results as the experiments without salt but the total effect was smaller. Therefore, only the experiments without salt are discussed here. The increase in α-helix and helical stability, as well as an increase in the cooperativity of unfolding when the peptides were combined, were used as measures of complex formation. Apparent dissociation constants of peptide complexes were estimated from van't Hoff enthalpies. Recently several papers have shown that binding constants determined from van't Hoff enthalpies of folding are equivalent to those determined from direct measurements of the enthalpies of binding determined by isothermal calorimetry (Horn et al., 2001, 2002). Also, in a similar study of tropomyosin binding to tropomodulin fragments we found that the apparent dissociation constants determined from the denaturation curves are within experimental error to those determined by direct titration (Greenfield and Fowler, 2002).

Even though the 39-residue C-terminal peptides are less helical and less stable than the cross-linked peptide $TM9a_{251–284}$ (see below), they served to illustrate isoform specific complex formation (Table 2). Both C-terminal peptides formed more stable complexes with AcTM1bZip (the N-acetylated peptide, or with GlyTM1bZip where Gly replaces the acetyl group, unpublished results) than with AcTM1aZip. However, $hcTnT_{70–170}$ formed a convincingly stable ternary complex only with AcTM1aZip and $TM9a_{246–284}N279K$, resulting in a small increase in T_m, a marked increase in negative ellipticity (Table 2), and an increase in the enthalpy and entropy of unfolding, as a function of temperature as reflected by the increased cooperativity (i.e., steepness) of the melting curves (data not shown).

Specificity of the N-terminal structure for complex formation with the C-terminus and troponin T

N-terminal modifications of tropomyosin influence actin affinity and the cooperativity of thin filament activation (for example Heald and Hitchcock-DeGregori, 1988; Urbancikova and Hitchcock-DeGregori, 1994; Monteiro et al., 1994; Maytum et al., 2000). Here we investigated the structural requirements of the tropomyosin N-terminus to form binary and ternary overlap complexes. For these studies we used a shorter C-terminal peptide, $TM9a_{251–284}$, that contains a cysteine. The increased stability of the cross-linked $TM9a_{251–284}$ peptide allowed detection of stable binary and ternary complexes, that was difficult with the uncross-linked, $TM9a_{246–284}N279K$ peptide (Table 2).

Conformational stability of binary and ternary overlap complexes measured using circular dichroism

AcTM1aZip and $TM9a_{251–284}$ formed a stable binary overlap complex, as reflected in greater cooperativity of unfolding as well as a slight increase of α-helix and an increase in T_m of 9.4°C (ΔT_m, the midpoint of the thermal transition of the mixture versus the sum of components, Fig. 2, Table 3). Addition of an equimolar amount of $hcTnT_{70–170}$ to the binary overlap complex resulted in a further ΔT_m of 7.4°C, indicating the formation of a stable ternary complex of the striated muscle overlap with the N-terminal part of TnT (Table 4).

To understand the specificity of the local structure of the N-terminus, both in terms of function and the structures (Greenfield et al., 1998, 2001; Brown et al., 2001), we made three N-terminal modifications of AcTM1aZip, the wild-type form: unacetylated (with a free amino group on Met1), Gly preceding Met1, and AlaSer preceding Met1. Both unacetylated and GlyTM1aZip are unstable compared to AcTM1aZip with T_m temperatures of less than 10°C, whereas AlaSerT-

TABLE 2 Properties of C-terminal TM model peptides and their complexes with different N-termini and $hcTnT_{70–170}$

C-terminal model peptide	% α-helix	T_m peptide (°C)	N-terminal model peptide	T_m binary complex (°C)	ΔT_m (°C)	Δ Ellipticity at 0°C (mdeg.)	T_m ternary complex (°C)	ΔT_m (°C)	Δ Ellipticity at 0°C (mdeg.)
$TM9a_{246–284}$ N279K	29 ± 1 (2)	<0	AcTM1aZip	20.5 (1)	0.3 (1)	−2.6 (1)	29.2 (1)	1.5 (1)	−20.8 (1)
			AcTM1bZip	44.1 (1)	2.2 (1)	−4.9 (1)	25.9 and 45.0 (1)	0.5 (1)	−7.7 (1)
$TM9d_{246–284}$	20 ± 1 (2)	<0	AcTM1aZip	21.0 ± 1.6 (2)*	−0.2 ± 0.4 (2)	−1.1 ± 0.4 (2)	28.8 (1)	0.3 (1)	−3.3 (1)
			AcTM1bZip	44.0 (1)	2.2 (1)	−4.3 (1)	22.5 and 44.6 (1)	0.3 (1)	−3.9 (1)

Data were collected at a peptide concentration of 10 μM in 10 mM potassium phosphate, pH = 6.5.
*Numbers in parenthesis give the number of independent experiments and errors are standard deviations.

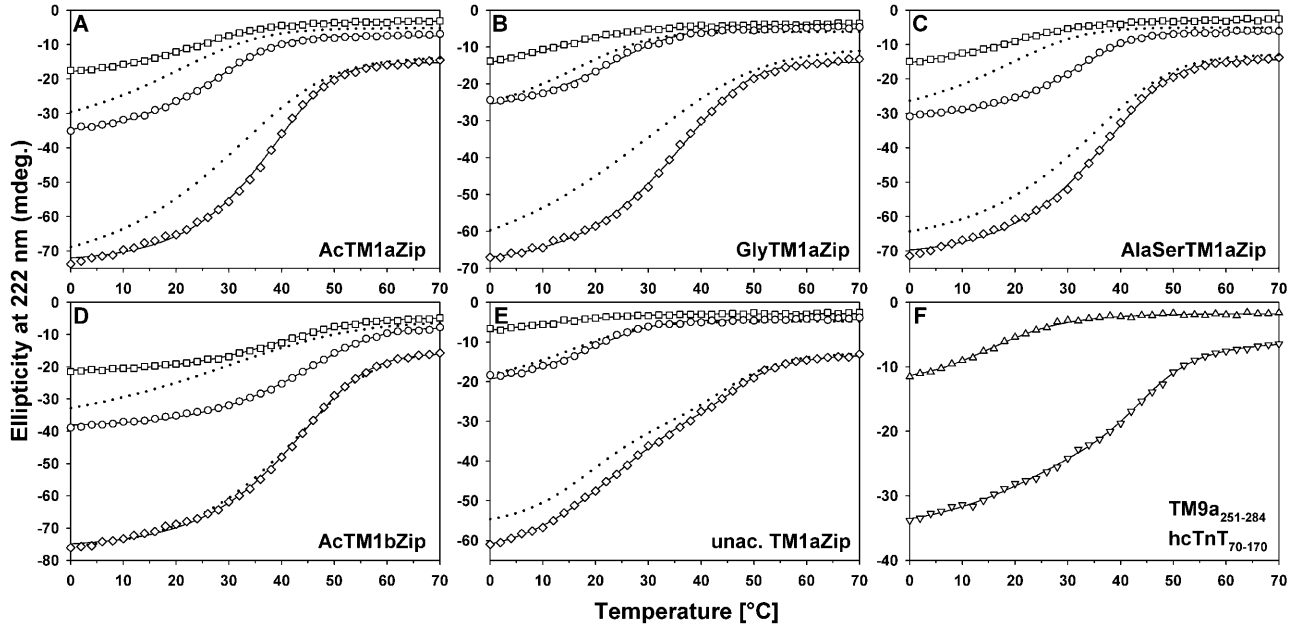

FIGURE 2 Thermal transitions of the N-terminal tropomyosin model peptides AcTM1aZip (*A*), GlyTM1aZip (*B*), AlaSerTM1aZip (*C*), AcTM1bZip (*D*), and unacetylated TM1aZip (*E*) alone (□), in complex with $TM9a_{251–284}$ (○), and in complex with $TM9a_{251–284}$ and $hcTnT_{70–170}$ (◇). Solid lines represent fits to the mixtures, and dotted lines represent fits to the sum of the components. Thermal transitions of $TM9a_{251–284}$ (△) and $hcTnT_{70–170}$ (▽) are shown in panel *F*.

M1aZip is intermediate (Table 3). Modification of Met1 also affects complex formation with the C-terminus, as measured by an increase in T_m and cooperativity of thermal denaturation. The Gly modification is almost as effective as N-acetylation in binary complex formation, whereas AlaSerTM1aZip and AcTM1bZip form even stronger complexes (Fig. 2, Table 3). However, the stability and cooperativity of unfolding of the mixture of unacetylated TM1aZip with-$TM9a_{251–284}$ are only marginally greater than the sum of the components.

The N-terminal structure also has a major influence on the formation of a ternary complex with $hcTnT_{70–170}$ (Fig. 2, Table 4). The ΔT_m for the ternary complex with GlyTM1aZip complex was close to that of AcTM1aZip, accompanied by an increased enthalpy and entropy of unfolding as reflected by the increased cooperativity (i.e., steepness) of the melting curves (Fig. 4). The ΔT_m of the ternary complex with unacetylated TM1aZip complex was smaller, and the analysis was complicated by the presence of two transitions indicating that the ternary complex dissociates before the TnT fragment unfolds. In contrast, the stable binary complex formed with AlaSerTM1aZip was considerably less effective in forming a ternary complex with $hcTnT_{70–170}$ than AcTM1aZip or GlyTM1aZip, having a ΔT_m for the ternary complex of only ~2°C. No obvious change was seen with AcTM1bZip.

Native gel electrophoresis

Gel electrophoresis of the peptides under nondenaturing conditions directly showed complex formation between N- and C-terminal tropomyosin peptides with $hcTnT_{70–170}$ (Fig. 3). Equimolar amounts of the peptides were electro-

TABLE 3 Effect of TM N-terminus on the formation of overlap complexes with the striated muscle C-terminal peptide $TM9a_{251–284}$

Model peptide	N-terminal modification	% α-helix at 0°C	T_m peptide(°C)	T_m binary complex (°C)	Δ ellipticity at 0°C (mdeg.)	ΔT_m (°C)	K_d (app) (μM, 20°C)
TM1aZip	Acetylated	91 ± 2 (5)*	21.7 ± 0.2 (4)	25.6 ± 0.1 (2)	−4.2 ± 2.6 (2)	9.4 ± 0.4 (2)	0.83 ± 0.01 (2)
	Unacetylated	40 ± 5 (2)	8.0 ± 2.1 (2)	16.5 ± 0.2 (2)	−0.1 ± 0.1 (2)	3.1 ± 1.6 (2)	1.06 ± 0.32 (2)
	Gly	72 ± 2 (2)	9.7 ± 1.8 (2)	22.8 ± 3.4 (2)	−0.1 ± 0.2 (2)	8.9 ± 2.3 (2)	0.34 ± 0.23 (2)
	AlaSer	78 ± 3 (3)	16.6 ± 0.7 (3)	30.0 ± 0.6 (2)	−4.2 ± 0.2 (2)	16.0 ± 2.7 (2)	0.09 ± 0.02 (2)
TM1bZip	Acetylated	88 ± 2 (2)	38.9 ± 0.9 (2)	42.5 ± 0.3 (2)	−3.8 ± 3.3 (2)	16.2 ± 1.1 (2)	0.36 ± 0.07 (2)
$TM9a_{251–284}$		54 ± 5 (3)	16.3 ± 1.0 (2)				
$hcTnT_{70–170}$		94 ± 1 (7)	T_m1: 30.5 ± 2.4 (6) T_m2: 45.8 ± 0.9 (6)				

Data were collected at a peptide concentration of 10 μM in 10 mM potassium phosphate, pH = 6.5.
*Numbers in parenthesis give the number of independent experiments and errors are standard deviations.

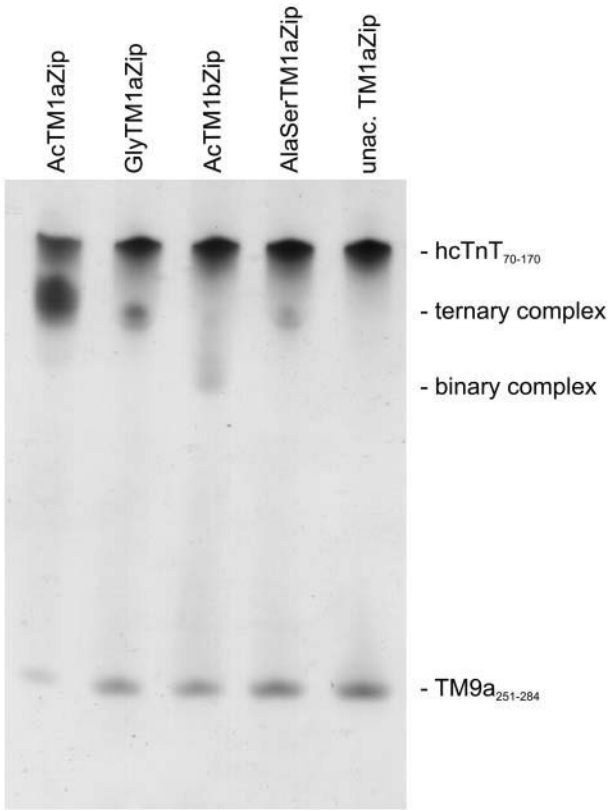

FIGURE 3 Ternary complexes of $hcTnT_{70–170}$, $TM9a_{251–284}$, and different N-terminal tropomyosin model peptides run at nondenaturing conditions on a 10% glycerol, 10% acrylamide gel at pH = 8.8, 4°C.

phoresed at 4°C, pH 8.8. The N-terminal tropomyosin model peptides have isoelectric points above nine and can not be seen on the gel as they move to the anode.

In general, the results agree with the CD results, even though the gel was run at a higher pH. Ternary complexes were observed for AcTM1aZip, GlyTM1aZip, and AlaSer-TM1aZip. Although the increase in thermal stability (ΔT_m) for the ternary complexes with AcTM1aZip or GlyTM1aZip was similar, and higher than with AlaSerTM1aZip, the overall stability of the AcTM1aZip ternary complex is higher than both in native gels. No ternary complexes were observed for AcTM1bZip and unacetylated TM1aZip indicating that these complexes either did not form in the conditions of the native gel, or were not sufficiently stable to remain intact during gel electrophoresis. The only binary complex stable enough to be seen on the native gel was that with AcTM1bZip. The binary complex with AlaSerTM1aZip is of similar stability according to the CD data but was not detected on the native gel, probably because of the difference in pH of the buffers used for the CD and electrophoresis studies. Although the sequences are homologous, the 1b peptide has a region where RRK replaces KKK in the 1a peptide. The 1b peptide might be expected to be less affected by the high pH of the electrophoresis buffer (pH 8.8) than the 1a peptide because the pK_a of arginine is higher than that of lysine.

Promotion of binding of N-terminal variants of tropomyosin to actin by $hcTnT_{70–170}$

N-terminal modifications such as N-acetylation, AlaSer, or the exon 1b N-terminus increase the end-to-end affinity of striated muscle tropomyosin compared to unacetylated TM (Heald and Hitchcock-DeGregori, 1988; Willadsen et al., 1992; Urbancikova and Hitchcock-DeGregori, 1994; Monteiro et al., 1994; Moraczewska et al., 1999; Maytum et al., 2000). To correlate the specificity of the N-terminal tropomyosin structure in formation of a ternary overlap complex with actin affinity we measured the ability of $hcTnT_{70–170}$ to promote the binding of four N-terminal tropomyosin variants with an exon 9a-encoded C-terminus, and one with an exon 9d-encoded C-terminus to filamentous actin (Fig. 4, Table 5). The sequences are identical except at the ends. The N-terminal and C-terminal sequences found in striated muscle tropomyosin are both required for TnT to form a strong ternary overlap complex and to increase the affinity of tropomyosin for actin. The $hcTnT_{70–170}$ fragment was most effective in increasing the affinity of both acetylated (Fig. 4 *A*) and unacetylated (Fig. 4 *B*) striated muscle tropomyosin (with exons 1a and 9a) for actin 10- to 15-fold (Table

TABLE 4 Effect of TM N-terminus on the binding of TnT to overlap complexes with the striated muscle C-terminal peptide $TM9a_{251–284}$

Model peptide	N-terminal modification	Δ Ellipticity at 0°C (mdeg.)	T_m ternary complex (°C)	ΔT_m (°C)	K_d (app) (μM, 20°C)
TM1aZip	Acetylated	−4.9 ± 1.6 (2)*	36.4 ± 0.3 (2)	7.4 ± 0.1 (2)	0.43 ± 0.01 (2)
	Unacetylated	−4.7 ± 1.5 (2)	T_m1: 25.6 ± 0.8 (2) T_m2: 47.1 ± 0.1 (2)	5.0 ± 0.4 (2)†	N.D.‡
	Gly	−10.7 ± 1.9 (2)	34.2 ± 0.6 (2)	6.5 ± 2.7 (2)	0.82 ± 0.59 (2)
	AlaSer	−6.3 ± 1.4 (2)	35.1 ± 0.3 (2)	2.3 ± 1.1 (2)	4.91 ± 1.72 (2)
TM1bZip	Acetylated	−2.1 ± 3.7 (2)	41.8 ± 0.8 (2)	1.5 ± 0.8 (2)	N.D.§

Data were collected at a peptide concentration of 10 μM in 10 mM potassium phosphate, pH = 6.5.
*Numbers in parenthesis give the number of independent experiments and errors are standard deviations.
†Only the lower T_m changed.
‡K_d can only be determined for melts with a single transition.
§Too weak to quantify.

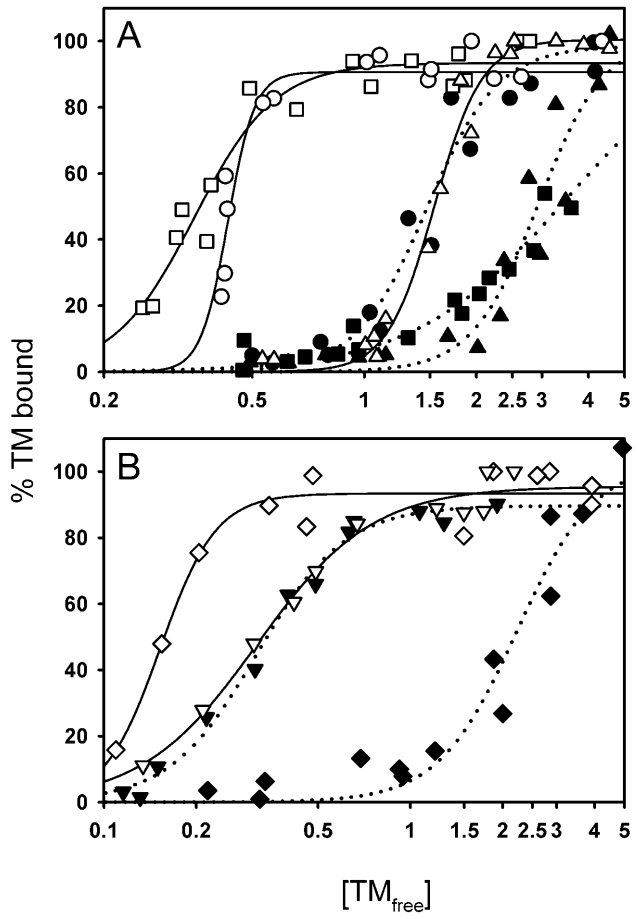

FIGURE 4 Effect of $hcTnT_{70–170}$ on the binding of the tropomyosin isoforms to actin measured at 300 mM NaCl (*A*) and 100 mM NaCl (*B*), respectively: AcTM1a9a (□), AlaSerTM1a9a (○), and TM1b9a (△), unacetylated TM1a9a (◇), and TM1a9d (▽) (*open symbols* and *solid line*, + $hcTnT_{70–170}$; *filled symbols* and *dotted line*, no TnT). Terminology: full length rat α-tropomyosins are designated by the codons that express their N- and C-terminal ends; i.e. TM1a9a is full length rat α-TM with an N-terminus encoded by exon 1a and a C-terminus encoded by exon 9a. Prefixes Ac and AlaSer stand for the respective N-terminal modifications. Concentrations are in μM.

5), in agreement with previously published results (Wegner and Walsh, 1981; Heald and Hitchcock-DeGregori, 1988; Hinkle et al., 1999). The binding of AlaSerTM (with exons 1a and 9a) was enhanced only threefold by $hcTnT_{70–170}$, less than the acetylated and unacetylated forms (Fig. 4A, Table 5). In these studies, the AlaSer1a9aTM was more similar to TM1b9a, consistent with the CD results using model peptides, even though it has both muscle tropomyosin exons. The $hcTnT_{70–170}$ fragment had the smallest influence on tropomyosins with exon 1b, or exon 9d (Fig. 4, *A* and *B*; Table 5), as in the CD and gel electrophoresis experiments reported above, and in experiments using native troponin complex (Cho and Hitchcock-DeGregori, 1991; Hammell and Hitchcock-DeGregori, 1996; Moraczewska et al., 1999).

TABLE 5 $hcTnT_{70–170}$ induced actin binding of tropomyosin isoforms

	K_d(app) of TM for actin (μM)	
TM ends	+ $hcTnT_{70–170}$	No TnT
100 mM NaCl*		
1a9a[†] (unacetylated)	0.21 ± 0.09[‡]	2.23 ± 0.41
1a9d (unacetylated)	0.35 ± 0.05	0.37 ± 0.08
300 mM NaCl		
Ac1a9a (acetylated)	0.35 ± 0.03	3.41 ± 0.18
AlaSer1a9a	0.43 ± 0.01	1.48 ± 0.16
1b9a (unacetylated)	1.56 ± 0.10	2.97 ± 0.48

*We used two different ionic strengths to allow evaluation of the effect of $hcTnT_{70–170}$ within the measurable range of the experiment. At the same ionic strength acetylated TM binds stronger than unacetylated TM (Heald and Hitchcock-DeGregori, 1988; Willadsen et al., 1992).
[†]Terminology, see legend to Fig. 4.
[‡]Errors are standard deviations of two independent experiments.

DISCUSSION

The work reported here defines certain structural requirements of the N-terminus of tropomyosin necessary to form a binary overlap complex with the C-terminus and to form a ternary complex with the N-terminus of troponin T. Formation of these complexes is required for tropomyosin to bind to actin with high affinity, and to cooperatively regulate the actin filament with troponin and myosin.

The mixtures of all of the N-terminal peptides with both the 9a and 9d C-terminal peptides, with the exception of unacetylated TM1aZip, have greatly increased stabilities (>8°C) compared to the sum of the unmixed components. The published structures of the N-terminus of tropomyosin give insight into the meaning of these results. In AcTM1aZip and AcTM1bZip, two peptides that form stable binary complexes, the regions proposed to participate in the overlap complex are α-helical coiled coils (residues 1–11 and the homologous region in TM1bZip, residues 6–16; Greenfield et al., 1998, 2001). When fully folded, AlaSerTM1aZip and GlyTM1aZip have the same ellipticity at 222 nm as AcTM1aZip (from extrapolation of unfolding curves or when measured at higher peptide concentrations, data not shown) showing that they form essentially the same structure as the acetylated peptide. In contrast, the first two residues of unacetylated 1a tropomyosins are not helical and are not part of the coiled coil domain (Brown et al., 2001). This is reflected in the ellipticity of the fully folded unacetylated peptide at 222 nm, which is ~30% lower than that of the acetylated peptide (data not shown). The unacetylated peptide is also less stable than the acetylated peptide. In summary, we suggest that the entire region homologous to residues 1–11 in AcTM1aZip must be able to form a fully folded coiled coil α-helix to form a strong overlap complex with the C-terminus.

It was impossible to determine the relative affinities of the N-terminal tropomyosin peptides for the $TM9a_{246–284}$N279K and $TM9d_{246–284}$ peptides because of the very low stability of

the 9d peptide. However, it is clear that all four complexes can be readily formed since in each case there are increases in the ellipticities of the mixtures compared to the sum of the ellipticities of the unmixed components.

Formation of a ternary complex with the N-terminus of troponin T, on the other hand, depends on the isoform specificity of both the N-terminal and C-terminal parts of the overlap complex. Troponin T is only expressed in striated muscle. The specificity is most clearly illustrated by the requirement for both striated tropomyosin exon 1a and exon 9a-encoded termini for strong ternary complex formation, as is required for troponin to promote binding of tropomyosin to actin (Cho and Hitchcock-DeGregori, 1991; Hammell and Hitchcock-DeGregori, 1996; Moraczewska et al., 1999). The overlap complex containing the N-terminus encoded by exon 1a has almost a 20-fold higher affinity for the TnT peptide than the complex containing the 1b N-terminus. Adding the AlaSer extension to the 1a N-terminus decreases the binding affinity of the overlap complex for the TnT peptide more than tenfold, suggesting that the AlaSer extension is mimicking the N-terminus of the 1b tropomyosins, rather than the acetyl group of native striated muscle TM1a. This suggestion is supported by the fact that the N-terminus of 1b tropomyosins is homologous to the N-terminus of 1a tropomyosins but has a native five amino acid N-terminal extension AlaGlySerSerSer which resembles AlaSer.

Although AlaSerTM1a and TM1b are impaired in binding the TnT peptide, the modifications increase actin affinity in the absence of TnT, as previously reported (Monteiro et al., 1994; Moraczewska et al., 1999). These results are consistent with results of Maytum et al. (2000; 2001) who find little difference in the actin affinities of yeast tropomyosin with various N-terminal extensions.

A full understanding of the structural requirements and specificity of the overlap complex and ternary complex with troponin T awaits atomic resolution structures. Toward that goal, we recently reported that binding of GlyTM1aZip to a related TM9a peptide, strongly perturbs the crosspeaks of residues 274–284 in its ^{1}H-^{15}N HSQC spectrum (Greenfield et al., 2002). In addition, the binding of the TM9a peptide to GlyTM1aZip perturbs the crosspeaks arising from residues 1–8 and 11–12 (unpublished results). Together the results suggest that the first and last 11 residues interact in the overlap complex. We have previously proposed models where the overlap complex of the TM1a and TM9a ends interact with residues 92 to 110 of troponin T to form a five-helix coiled coil or helix bundle. The five-residue nonhelical extension of the 1b tropomyosins and the AlaSer extension could inhibit binding of the TnT peptide by steric hindrance.

An alternate explanation is that the binary complexes of the TM9a peptide with the AlaSerTM1aZip and TM1bZip peptides, which are considerably more stable than the AcTM1aZip complex, are too stable for TnT to bind. A mutation, Q263L, that increases the stability of the TM9a peptide decreases the ability of its overlap complex to bind the $hcTnT_{70–170}$ fragment (Greenfield et al., 2002), suggesting that conformational flexibility is necessary for tropomyosin to bind TnT. Similarly, disease causing mutations that increase the α-helical stability of the TnT peptide reduce its affinity for tropomyosin and the overlap complex (Palm et al., 2001).

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REFERENCES

Böhm, G., R. Muhr, and R. Jaenicke. 1992. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng.* 5:191–195.

Brown, J. H., K. H. Kim, G. Jun, N. J. Greenfield, R. Dominguez, N. Volkmann, S. E. Hitchcock-DeGregori, and C. Cohen. 2001. Deciphering the design of the tropomyosin molecule. *Proc. Natl. Acad. Sci. USA.* 98:8496–8501.

Butters, C. A., K. A. Willadsen, and L. S. Tobacman. 1993. Cooperative interactions between adjacent troponin-tropomyosin complexes may be transmitted through the actin filament. *J. Biol. Chem.* 268:15565–15570.

Cho, Y. J. 2000. The Carboxyl Terminal Amino Acid Residues Glutamine276-Threonine277 Are Important for Actin Affinity of the Unacetylated Smooth α-Tropomyosin. *J. Biochem. Mol. Biol.* 33:531–536.

Cho, Y. J., and S. E. Hitchcock-DeGregori. 1991. Relationship between alternatively spliced exons and functional domains in tropomyosin. *Proc. Natl. Acad. Sci. USA.* 88:10153–10157.

Cho, Y. J., J. Liu, and S. E. Hitchcock-DeGregori. 1990. The amino terminus of muscle tropomyosin is a major determinant for function. *J. Biol. Chem.* 265:538–545.

Dabrowska, R., E. Nowak, and W. Drabikowski. 1983. Some functional properties of nonpolymerizable and polymerizable tropomyosin. *J. Muscle Res. Cell Motil.* 4:143–161.

Emerson Holtzer, M., L. Mints, R. Hogue Angeletti, D. A. d'Avignon, and A. Holtzer. 2001. CD and (13)C(alpha)-NMR studies of folding equilibria in a two-stranded coiled coil formed by residues 190–254 of alpha-tropomyosin. *Biopolymers.* 59:257–265.

Goa, J. 1953. A micro biuret method for protein determination. *Scand. J. Clin. Lab. Invest.* 5:218–222.

Greenfield, N., and V. Fowler. 2002. Tropomyosin requires an intact N-terminal coiled coil to interact with tropomodulin. *Biophys. J.* 82: 2580–2591.

Greenfield, N. J., and S. E. Hitchcock-DeGregori. 1995. The stability of tropomyosin, a two-stranded coiled-coil protein, is primarily a function of the hydrophobicity of residues at the helix- helix interface. *Biochemistry.* 34:16797–16805.

Greenfield, N. J., Y. J. Huang, T. Palm, G. V. Swapna, D. Monleon, G. T. Montelione, and S. E. Hitchcock-DeGregori. 2001. Solution NMR structure and folding dynamics of the N terminus of a rat non-muscle alpha-tropomyosin in an engineered chimeric protein. *J. Mol. Biol.* 312:833–847.

Greenfield, N. J., G. T. Montelione, R. S. Farid, and S. E. Hitchcock-DeGregori. 1998. The structure of the N-terminus of striated muscle alpha-tropomyosin in a chimeric peptide: nuclear magnetic resonance structure and circular dichroism studies. *Biochemistry.* 37:7834–7843.

Greenfield, N. J., T. Palm, and S. E. Hitchcock-DeGregori. 2002. Structure and Interactions of the Carboxyl Terminus of Striated Muscle α-Tropomyosin: It is Important to be Flexible. *Biophys. J.* 83:2754–2766.

Greenfield, N. J., W. F. Stafford, and S. E. Hitchcock-DeGregori. 1994. The effect of N-terminal acetylation on the structure of an N-terminal tropomyosin peptide and alpha alpha-tropomyosin. *Protein Sci.* 3:402–410.

Hammell, R. L., and S. E. Hitchcock-DeGregori. 1996. Mapping the functional domains within the carboxyl terminus of alpha-tropomyosin encoded by the alternatively spliced ninth exon. *J. Biol. Chem.* 271: 4236–4242.

Hammell, R. L., and S. E. Hitchcock-DeGregori. 1997. The sequence of the alternatively spliced sixth exon of alpha-tropomyosin is critical for cooperative actin binding but not for interaction with troponin. *J. Biol. Chem.* 272:22409–22416.

Heald, R. W., and S. E. Hitchcock-DeGregori. 1988. The structure of the amino terminus of tropomyosin is critical for binding to actin in the absence and presence of troponin. *J. Biol. Chem.* 263:5254–5259.

Heeley, D. H., K. Golosinska, and L. B. Smillie. 1987. The effects of troponin T fragments T1 and T2 on the binding of nonpolymerizable tropomyosin to F-actin in the presence and absence of troponin I and troponin C. *J. Biol. Chem.* 262:9971–9978.

Hinkle, A., A. Goranson, C. A. Butters, and L. S. Tobacman. 1999. Roles for the troponin tail domain in thin filament assembly and regulation. A deletional study of cardiac troponin T. *J. Biol. Chem.* 274:7157–7164.

Hitchcock-DeGregori, S. E., and R. W. Heald. 1987. Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in Escherichia coli. *J. Biol. Chem.* 262: 9730–9735.

Hitchcock-DeGregori, S. E., S. F. Lewis, and T. M. Chou. 1985. Tropomyosin lysine reactivities and relationship to coiled-coil structure. *Biochemistry.* 24:3305–3314.

Hitchcock-DeGregori, S. E., S. Mandala, and G. A. Sachs. 1982. Changes in actin lysine reactivities during polymerization detected using a competitive labeling method. *J. Biol. Chem.* 257:12573–12580.

Holtzer, M. E., D. L. Crimmins, and A. Holtzer. 1995. Structural stability of short subsequences of the tropomyosin chain. *Biopolymers.* 35:125–136.

Horn, J. R., J. F. Brandts, and K. P. Murphy. 2002. Van't Hoff and calorimetric enthalpies II: effects of linked equilibria. *Biochemistry.* 41:7501–7507.

Horn, J. R., D. Russell, E. A. Lewis, and K. P. Murphy. 2001. Van't Hoff and calorimetric enthalpies from isothermal titration calorimetry: are there significant discrepancies? *Biochemistry.* 40:1774–1778.

Katayama, E., and S. Nozaki. 1982. Ca^{2+}-dependent binding of synthetic peptides corresponding to some regions of troponin-I to troponin-C. *J. Biochem. (Tokyo).* 91:1449–1452.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science.* 240:1759–1764.

Lewis, W. G., G. P. Cote, A. S. Mak, and L. B. Smillie. 1983. Amino acid sequence of equine platelet tropomyosin. Correlation with interaction properties. *FEBS Lett.* 156:269–273.

Lin, J. J., K. S. Warren, D. D. Wamboldt, T. Wang, and J. L. Lin. 1997. Tropomyosin isoforms in nonmuscle cells. *Int. Rev. Cytol.* 170:1–38.

Mak, A. S., and L. B. Smillie. 1981. Non-polymerizable tropomyosin: preparation, some properties and F-actin binding. *Biochem. Biophys. Res. Commun.* 101:208–214.

Maytum, R., M. A. Geeves, and M. Konrad. 2000. Actomyosin regulatory properties of yeast tropomyosin are dependent upon N-terminal modification. *Biochemistry.* 39:11913–11920.

Maytum, R., M. Konrad, S. S. Lehrer, and M. A. Geeves. 2001. Regulatory properties of tropomyosin effects of length, isoform, and N-terminal sequence. *Biochemistry.* 40:7334–7341.

McLachlan, A. D., and M. Stewart. 1975. Tropomyosin coiled-coil interactions: evidence for an unstaggered structure. *J. Mol. Biol.* 98: 293–304.

Monteiro, P. B., R. C. Lataro, J. A. Ferro, and F. de Castro Reinach.1994. Functional alpha-tropomyosin produced in Escherichia coli. A dipeptide extension can substitute the amino-terminal acetyl group. *J. Biol. Chem.* 269:10461–10466.

Moraczewska, J., K. Nicholson-Flynn, and S. E. Hitchcock-DeGregori. 1999. The ends of tropomyosin are major determinants of actin affinity and myosin subfragment 1-induced binding to F-actin in the open state. *Biochemistry.* 38:15885–15892.

Novy, R. E., L. F. Liu, C. S. Lin, D. M. Helfman, and J. J. Lin. 1993a. Expression of smooth muscle and nonmuscle tropomyosins in Escherichia coli and characterization of bacterially produced tropomyosins. *Biochim. Biophys. Acta.* 1162:255–265.

Novy, R. E., J. R. Sellers, L. F. Liu, and J. J. Lin. 1993b. In vitro functional characterization of bacterially expressed human fibroblast tropomyosin isoforms and their chimeric mutants. *Cell Motil. Cytoskeleton.* 26:248–261.

Palm, T., S. Graboski, S. E. Hitchcock-DeGregori, and N. J. Greenfield. 2001. Disease-causing mutations in cardiac troponin T: identification of a critical tropomyosin-binding region. *Biophys. J.* 81:2827–2837.

Perry, S. V. 2001. Vertebrate tropomyosin: distribution, properties and function. *J. Muscle Res. Cell Motil.* 22:5–49.

Pittenger, M. F., A. Kistler, and D. M. Helfman. 1995. Alternatively spliced exons of the beta tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon. *J. Cell Sci.* 108:3253–3265.

Sano, K., K. Maeda, T. Oda, and Y. Maeda. 2000. The effect of single residue substitutions of serine-283 on the strength of head-to-tail interaction and actin binding properties of rabbit skeletal muscle alpha-tropomyosin. *J. Biochem.* 127:1095–1102.

Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.

Urbancikova, M., and S. E. Hitchcock-DeGregori. 1994. Requirement of amino-terminal modification for striated muscle alpha-tropomyosin function. *J. Biol. Chem.* 269:24310–24315.

Wegner, A., and T. P. Walsh. 1981. Interaction of tropomyosin-troponin with actin filaments. *Biochemistry.* 20:5633–5642.

Willadsen, K. A., C. A. Butters, L. E. Hill, and L. S. Tobacman. 1992. Effects of the amino-terminal regions of tropomyosin and troponin T on thin filament assembly. *J. Biol. Chem.* 267:23746–23752.