Importance of Internal Regions and the Overall Length of Tropomyosin for Actin Binding and Regulatory Function†

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ABSTRACT: Tropomyosin (Tm) binds along actin filaments, one molecule spanning four to seven actin monomers, depending on the isoform. Periodic repeats in the sequence have been proposed to correspond to actin binding sites. To learn the functional importance of length and the internal periods we made a series of progressively shorter Tms, deleting from two up to six of the internal periods from rat striated α-TM (dAc2−3, dAc2−4, dAc3−5, dAc2−5, dAc2−6, dAc1.5−6.5). Recombinant Tms (unacetylated) were expressed in *Escherichia coli*. Tropomyosins that are four or more periods long (dAc2−3, dAc2−4, and dAc3−5) bound well to F-actin with troponin (Tn). dAc2−5 bound weakly (with EGTA) and binding of shorter mutants was undetectable in any condition. Myosin S1-induced binding of Tm to actin in the tight Tm-binding "open" state did not correlate with actin binding. dAc3−5 and dAc2−5 did not bind to actin even when the filament was saturated with S1. In contrast, dAc2−3 and dAc2−4 did, like wild-type-Tm, requiring about 3 mol of S1/mol of Tm for half-maximal binding. The results show the critical importance of period 5 (residues 166−207) for myosin S1-induced binding. The Tms that bound to actin (dAc2−3, dAc2−4, and dAc3−5) all fully inhibited the actomyosin ATPase (+Tn) in EGTA. In the presence of Ca^{2+}, relief of inhibition by these Tms was incomplete. We conclude (1) four or more actin periods are required for Tm to bind to actin with reasonable affinity and (2) that the structural requirements of Tm for the transition of the regulated filament from the blocked-to-closed/open (relief of inhibition by Ca^{2+}) and the closed-to-open states (strong Tm binding to actin-S1) are different.

Tropomyosin (Tm)[1] is a parallel coiled coil that binds along the length of the actin filament in the long pitch grooves of the helix (*1*−*4*). The sequence contains periodic patterns of amino acids that relate to its structure and its function. The heptapeptide repeat of hydrophobic amino acids is fundamental for formation of the stable interface between the α-helical chains that allows for formation of a coiled coil (*5*−*8*). Early sequence analysis of striated α-Tm revealed a second pattern, a poorly conserved 7-fold periodic repeat of amino acids that is sufficiently regular to represent actin binding sites (*8*−*10*). The lengths of naturally occurring Tms correspond to an integral number of periods and actin monomers in the filament: seven in 284-residue Tms found in muscle and certain nonmuscle isoforms, six in 247-residue nonmuscle isoforms, and five or four in yeast Tms (reviewed in refs *11* and *12*).

Tropomyosins form a family of highly conserved proteins that are found in virtually all eukaryotic and nonmuscle cells. Functions common to Tms include cooperative binding to F-actin, the ability to stabilize and stiffen the actin filament, and cooperative activation of the actin filament by myosin (e.g., refs *13*−*20*, reviewed in refs *12* and *21*). To understand the structural requirements for these conserved functions we have made mutants of striated muscle α-Tm. In addition to carrying out common Tm functions, striated muscle Tm also binds to Tn and is required for Ca^{2+}-dependent regulation of contraction in striated muscles (reviewed in refs *21* and *22*). We have shown that the ends of Tm, a continuous coiled coil, and an integral number of periods or half periods are primary determinants of high affinity, cooperative actin binding (*23*−*32*). A full complement of periods is less important. Indeed, deletions of one, two, and even three actin periods generally have small effects on actin affinity (*27*, *30*, *33*, *34*). While this again emphasizes the importance of the ends for actin binding, these larger deletions are deficient in regulatory function.

Thin filament regulatory function has long been recognized to be cooperative and allosteric, depending on both Ca^{2+} binding to TnC and myosin binding to actin (e.g., refs *14*, *16*, *17*, *35*, and *36*; recently reviewed in ref *22*). Geeves, Lehrer, and their colleagues have proposed three regulatory states (*37*−*39*) that Lehman and co-workers have related to thin filament structures (*40*, *41*). The current models have evolved from earlier two-state models (*42*−*45*), though the issue is not completely resolved (*46*). In the "blocked" state in the absence of Ca^{2+}, myosin cannot bind to actin presumably because of steric blocking of the myosin binding site on actin by Tm (with Tn). In the "open" state, there is strong binding of myosin to actin with accompanying force development. The switch from the blocked to the open state

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[1] Abbreviations: Ac, actin; Tm, tropomyosin; Tn, troponin.

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is cooperative and allosteric involving the binding of myosin to actin-Tm and, when Tn is present, binding of Ca^{2+} to TnC. The open state is thought to correspond to a structure in which Tm is located medial to the myosin binding site located on the outer domain of the actin helix. In the blocked state, in the absence of Ca^{2+}, Tm moves azimuthally toward the outer domain. The "closed" state is kinetically and structurally intermediate where myosin can bind weakly to actin and Tm partially occludes the myosin binding site on actin. The states have been described in terms of the position of Tm on the actin filament, and the affinity of myosin for actin, but there is evidence that changes in the actin subunit must also take place (*47*−*50*, and references therein).

Analysis of Tms lacking internal periods gives us insight into the involvement of specific regions in regulatory function, in addition to actin binding per se. The shortest Tms studied to date span the length of four actins along the filament (*33*, *34*), as does the shortest reported naturally occurring Tm found in yeast (*51*). Is this the minimum length required for cooperative actin binding and regulatory function? To address this question, we have made a series of progressively shorter Tms, as short as one actin long. The deletions are of internal regions of sequence, leaving the ends, known to be crucial for function of Tm function, intact (*23*−*26*, *28*, *29*, *31*, *52*−*65*). We report here that Tms four or more actins long bind to F-actin with reasonable affinity. A Tm that is three actins long (from deletion of four of the seven periods) results in a major reduction of actin affinity. No binding could be measured with even shorter forms. The Tms that span four and five actin subunits are different from wild-type Tm in regulatory function inferring the involvement of specific actin periods in particular thin filament states. Tropomyosins four or more periods long can regulate the actomyosin ATPase in the absence of Ca^{2+}, but relief of inhibition in the presence of Ca^{2+} was severely impaired, generally supporting the findings of Landis et al. (*33*, *34*). Myosin S1-induced binding of Tm to actin was lost in all mutants lacking period 5. Portions of this work were reported in abstract form (*66*, *67*).

EXPERIMENTAL PROCEDURES

DNA Constructions, Protein Expression, and Purification. A rat striated α-Tm cDNA clone [(gift of Dr. B. Nadal-Ginard (*68*)] was used for expression of wild-type Tm and construction of all mutants except dAc2−3, which was prepared in chicken striated α-Tm (*30*). The cDNA was previously cloned into pET11d for expression in *Escherichia coli* (*28*, *30*, *69*). The dAc2−4, dAc3−5, and dAc2−5 mutants created for the present study were made using oligonucleotide-directed mutagenesis of double-stranded DNA (in pET11d) using a Stratgene ExSite or Quick-Change PCR-based site-directed mutagenesis kits (La Jolla, CA) with Taq DNA polymerase.

Sequences of mutagenic oligonucleotides complementary to the coding sequence on the 5′-end of the mutations:

dAc2−4, dAc2−5:
3′-CGACCTTCTACTCGACCACAGTGAC-5′

dAc3−5:
3′-CTACGACTTCGACTGCATCGTAGAGAC-5′

Sequences of mutagenic oligonucleotides of the coding sequence extending from the 3′-end of the mutation:

dAc2−4:
5′-GCCCGTAAGCTGGTCATCATCGAGAG-3′

dAc3−5, dAc2−5:
5′-GAGGCTCAGGCTGAGAAGTACTCTC-3′

The dAc2−6 and dAc1.5−6.5 mutants were made in the rat striated α-Tm cDNA cloned in pUC119 using oligonucleotide-directed mutagenesis of single-stranded DNA employing a Bio-Rad Muta-Gene T7 mutagenesis kit (based on ref *70*). The nucleotides encoding 196 residues in dAc2−6 (amino acids 39−234) and 238 residues in dAc1.5−6.5 (amino acids 21−258) were deleted using the following oligonucleotides (to the coding sequence) where the slash indicates the position of the deletion. In the dAc1.5−6.5 mutant, Lys15 was mutated to a Cys (codon in italics) to allow interchain cross-linking for increased coiled coil stability.

dAc2−6:
5′-GACCGGAGCAAGCAG/GCTGAGACCCGG-3′

dAc1.5−6.5:
5′-GATGCTGAAGCTCGAC*TGC*GAGAACG
CCTTGGAT/GAGCTGTATGCTCAG-3′

The oligonucleotides used for mutagenesis were synthesized on Applied Biosystems DNA synthesizers, purified using NENSORB cartridges (UMDNJ-DNA Synthesis and Sequencing Facility, Piscataway, NJ), and phosphorylated enzymatically using phage T4 polynucleotide kinase. Following plaque purification (in the cases of dAc2−6 and dAc1.5−6.5) or single colony isolation for the other mutants, the sequence of one strand of the entire cDNA sequence was determined manually using the dideoxynucleotide chain termination method with α-^{35}S-dATP (*71*) or using an ABI Perkin-Elmer-Cetus 277 automated sequencer (UMDNJ-DNA Synthesis and Sequencing Facility at Robert Wood Johnson Medical School, Piscataway). Construction of chimeras of dAc2−6 and dAc1.5−6.5, in which the C-terminal nine amino acids are encoded by exon 9d, was carried out as previously described (*28*). Briefly, an *Ava*II−*Bam*HI fragment containing codons 276−284 (as well as part of the 3′ noncoding sequence and the linker region of pUC119) from a Tm cDNA containing exon 9d was exchanged for the comparable region of rat striated α-Tm which has the same restriction sites. The constructs were confirmed by restriction analysis, referred to as dAc2−6/9d and dAc1.5−6.5/9d.

For expression, the cDNAs were cloned into pET11d at the *Nco*I and *Bam*HI sites and transformed into BL21(DE3) or BL21(DE3)pLysS for expression (*69*). All the Tm cDNAs expressed well except dAc1.5−6.5/9d.

Protein Purification. The recombinant proteins were purified as previously described (*23*, *28*) except the $(NH_4)_2SO_4$ fractionation was 35−70% instead of 35−60%. Briefly, Tm was purified from the heat-stable fraction by chromatography on DE52 cellulose (Whatman) or a MonoQ column (Pharmacia) followed by chromatography on hydroxylapaptite (Bio-Rad). The dAc2−6 and dAc1.5−6.5 mutants were purified only on a DE52 column, omitting the hydroxylapatite column as they did not bind.

Confirmation of Recombinant Products by Mass Spectrometry. The molecular weights of the larger Tms that bound

to actin were determined by electrospray mass spectrometry at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). The observed masses corresponded well to the calculated values: wild-type Tm, calculated 32 698, measured 32 700 ± 6; dAc2−4, calculated 19 013, observed 19 020 ± 4; dAc3−5, calculated 18 906, observed 18 908 and 18 890 ± 4 (possibly a dehydration product); dAc2−5, calculated 14 272, observed 14 280 ± 3 (possibly reflecting an extra bound water).

Other Proteins. Actin was isolated from White Leghorn chicken pectoral muscle acetone powder (*72*), except that actin was polymerized by addition of KCl and $MgCl_2$ to 20 and 0.7 mM, respectively, and incubated at 37 °C for 10 min before polymerization at room temperature. Myosin was prepared from chicken pectoral muscle; myosin S1 was prepared by papain digestion of myosin (*73*). Troponin was purified from chicken pectoral muscle (gift of Dr. J. Fagan, Rutgers University, New Brunswick, NJ) according to the method of Potter (*74*) with modifications described in ref *31*.

The concentrations of actin, myosin, and Tn were spectrophotometrically determined using the extinction coefficients at 280 (0.1%) of 1.1, 0.53, and 0.45, respectively. Concentrations of recombinant Tm were determined by differential absorption spectra of tyrosine as previously described (*28*, *75*, *76*).

Circular Dichroism Measurements. CD measurements were carried out and analyzed using an Aviv model 62 DS spectropolarimeter as previously described (*77*). The helical content was estimated from the mean residue ellipticity (deg·cm^2/dmol) at 222 nm using the following formula:

$$\% \text{ helix} = ([\theta]_{222} + 5000)/(-43\,000 + 5000)$$

where −5000 is the ellipticity of all the fully unfolded Tms and −43 000 is the maximal ellipticity observed at 0 °C.

Actin Binding Assays. Tm binding to F-actin was directly measured using a cosedimentation assay as previously described (*24*) with modifications (*29*). The amount of bound and free Tm in the pellets and supernatants, respectively, were quantified by densitometry of SDS−polyacrylamide gels (*78*) using a Molecular Dynamics model 300A computing densitometer (Sunnyvale, CA). The electrophoresis was optimized for each Tm variant: for wild-type Tm, 12% polyacrylamide; d2−4, 15% polyacrylamide; dAc3−5, and the shorter mutants, 15% polyacrylamide, or 14.5% polyacrylamide containing 2 M urea. Since dAc3−5 comigrated with TnI, binding of TnI and Tm to actin was measured; TnI does not bind in the absence of Tm. Despite the high concentrations of Tm, trapping and/or nonspecific binding was less than 10%. In one experiment, there was 25% trapping at the higher concentrations; the data from that binding isotherm were corrected without altering the K_a. The free Tm in the supernatants was calculated from standard curves for each Tm mutant as the staining coefficients for Coomassie blue differed.

The apparent K_a of Tm for F-actin and Hill coefficient (α^H) were determined by fitting the experimental data to the following equation using SigmaPlot (Jandel Scientific, San Rafael, CA):

$$\upsilon = n[\text{Tm}]^{n\text{H}}K_{\text{app}}^{n\text{H}}/1 + [\text{Tm}]^{n\text{H}}K_{\text{app}}^{n\text{H}}$$

where υ = fraction maximal Tm binding to actin, n = maximal Tm bound, $[\text{Tm}] = [\text{Tm}]_{\text{free}}$. The Tm:actin ratio was normalized to 1.0 by dividing the Tm:actin ratio obtained from densitometry by the observed Tm:actin maximal ratio at saturation. We have previously shown that the density ratio at saturation reflects stoichiometric binding of Tm to actin (*26*). We have normalized the data because the intensity of the staining is somewhat variable from experiment to experiment.

Myosin S1-Induced Tropomyosin Binding to Actin. Actin (3 μM) and Tm (1 or 3 μM) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1mM DTT, 10 mM imidazole, pH 7.0, were mixed with myosin S1 (0−4.2 μM). The mixture was incubated at room temperature for 30 min to ensure hydrolysis of residual ATP from F-actin and then centrifuged in a TLA-100 rotor for 25 min, at 60 000 rpm, 20 °C, in a Beckman TL-100 ultracentrifuge (Fullerton, CA). The pellets were washed with assay buffer and then solubilized in actin extraction buffer (5 mM imidazole, pH 7.0, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.1 mM ATP) by sonication in an ultrasonic cleaner. Pellets were electrophoresed on 12% SDS−PAGE gels (*78*). Proteins were visualized with Coomassie brilliant blue. The composition of proteins sedimented in pellets was analyzed by densitometry. The results were plotted as the Tm:actin and S1:actin ratio obtained from intensities of protein bands on the gel vs the initial S1:actin molar ratio. In the case of dAc2−4, which comigrated with a myosin light chain, the observed intensity was corrected for light chain by subtracting the intensity of myosin S1 light chain bound to actin in the absence of Tm. The data were normalized to the maximal, saturating value.

Actomyosin MgATPase Assay. The actomyosin ATPase activity was measured as a function of Tm concentration using 2.4 μM actin, 0.6 μM myosin, 1 μM Tn, and 0 to 1 μM Tm in 40 mM NaCl, 5 mM $MgCl_2$, 5 mM imidazole, pH 7.0, 0.5 mM DTT, and either 0.2 mM $CaCl_2$ or 0.2 mM EGTA. Assays were carried out in 96-well microtiter plates at 28 °C in a thermoequilibrated Molecular Devices ThermoMax microtiter reader (Menlo Park, CA). The reaction was initiated by adding MgATP to final concentration 5 mM and terminated after 15 min by adding SDS and EDTA to final concentration 3.3% and 30 mM, respectively. The amount of inorganic phosphate released was determined colorimetically (*79*). The plates were read in a Molecular Devices ThermoMax plate reader with a 650 nm filter (Menlo Park, CA). Specific activity was expressed as moles Pi/sec/mole myosin.

RESULTS AND DISCUSSION

Mutant Design. To define the minimum length of Tm that retains function we made a series of progressively longer internal deletions, keeping the functionally critical ends intact. We used Phillip's model (*80*) for the periodic actin binding sites to design the mutants. His postulate considers the helical (azimuthal) position on the α-helix as well as the linear (supercoil) position of each amino acid. The periods are 35 or 42 amino acids, depending on the repeat, preserving the heptapeptide repeat of hydrophobic amino acids. Consequently, the deletions do not perfectly correspond to the average $39^1/_3$ amino acid repeat observed by McLachlan and Stewart (*9*). The differences may influence the match of the

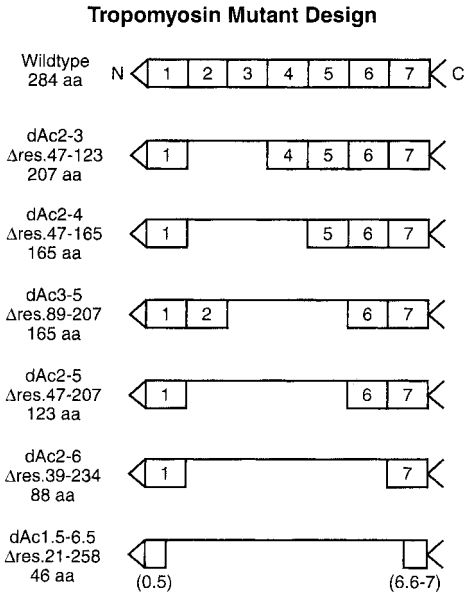

FIGURE 1: Design of the deletion mutants. The numbered blocks represent the seven periodic repeats in 284-residue Tms. Each block (period) corresponds to the length of an actin monomer spanned by Tm in the thin filament. The pointed (N-terminus) and barbed ends (C-terminus) reflect the orientation of Tm on the actin filament. The deletions are of integral numbers of periods from the internal regions of Tm, leaving the ends intact. In the diagram the blocks represent the regions remaining in the mutants. For example, dAc2−4 has periods 1, 5, 6, and 7 but lacks periods 2, 3, and 4. The lengths of the Tm mutants range from five actin monomers (dAc2−3) to one monomer (dAc1.5−6.5) in the filament.

remaining periods to the actin monomers as well as the orientation of the ends relative to each other on the filament. The deletions become progressively larger, starting with the second period, moving toward the C-terminus (Figure 1). The resulting Tms are one to five actin periods long compared to wild-type Tm which has seven periods and spans the length of seven actin monomers in the filament. In our earlier work we showed that deletion of periods 2 and 3 reduced the Tm affinity for regulated actin (actin with Tn) only 2-fold (*30*), recently confirmed by Landis et al. (*34*) who studied a fusion Tm carrying the same deletions that could bind to actin in the absence of Tn. The present study extends the analysis to deletions of three and more periods. The deletions were made in striated muscle α-Tm: chicken for the previously published dAc2−3 (*30*), and rat for the rest.

Conformational Analysis. The Tm mutants were expressed in *E. coli*; the N-terminal Met is unacetylated. The conformation and folding of the Tm variants were analyzed using circular dichroism spectrophotometry. All Tms except dAc2−6 and dAc1.5−6.5 formed α-helical coiled coils. The folding following thermal denaturation was reversible and complete, though they differed in stability (Table 1). Deletion of up to three periods resulted in small increases or decreases of the overall T_M, depending on the deletion. The stability is not related to length, dAc2−3 being less stable than dAc2−4. Differences were expected since the stability of the coiled coil varies along the length of the molecule (*77*). Deletions of more than three periods caused larger reductions in T_M, that of dAc2−5 being 10 °C lower than wild-type. The shortest forms (only 88 and 46 amino acids long), not surprisingly, folded very poorly, requiring much higher concentrations to fold, and even then with low T_Ms (<20 °C) and low α-helical content (≤50% at 0 °C).

Table 1: Conformational Analysis of Tropomyosin Mutants Using Circular Dichroism[a]

tropomyosin	T_M (°C)[b]	% α-helix (20 °C)[c]	% folded (20 °C)
wild-type	42.7	90	90.5
dAc2−3	37.0	77	89.5
dAc2−4	44.2	92	92.7
dAc3−5	39.0	90	93.3
dAc2−5	33.0	89	92.7
dAc2−6	12	3	13
dAc1.5−6.5	<0	6	14

[a] The ellipticity was measured as a function of temperature in 500 mM NaCl, 10 mM sodium phosphate, pH 6.5, 1 mM EDTA, 1 mM DTT, 0.1−0.2 mg/mL tropomyosin. [b] The T_m is midpoint of the transition between the fully folded and fully unfolded conformations. [c] The percent α-helix was calculated assuming a mean residue ellipticity of −43 800 deg·cm^2/dmol represents 100% α-helix.

Affinity of Tropomyosin Mutants for F-Actin with Troponin. Despite being only ∼60% the length of wild-type, both dAc2−4 and dAc3−5 bound to and saturated actin, but with reduced affinity (with Tn, +Ca^{2+}; Figure 2A, Table 2). The actin affinity of dAc3−5 was half that of dAc2−4 and may reflect particular significance of period 5 for actin binding with Tn. The cooperativity of binding, as estimated by the Hill coefficient, was similar for mutants and wild-type (Table 2). Our results are in general agreement with Landis et al. (*33*, *34*) who also measured binding of a set of deletion mutants of the same design, both with and without Tn. We cannot directly compare our observed K_{app}s. The Tms in the present study have a native N-terminal sequence, but the N-terminal Met is unacetylated, requiring the presence of Tn for high affinity (*23*, *24*). In contrast, the Landis et al. Tms have a two amino acid N-terminal extension, AlaSer, to increase actin affinity in the absence of Tn (*64*). In addition, the methods and conditions of actin binding differ. Nevertheless, both sets of results show that Tms the length of four actins in a filament bind to actin, as does a naturally occurring form found in yeast (*51*, *55*).

The next larger deletion (dAc2−5), resulting in a Tm that is only three actins long, reduced actin affinity to a level too weak to measure with Tn in the presence of Ca^{2+} (Figure 2A). However, it did bind in the absence of Ca^{2+} with an affinity of $\sim 2 \times 10^5$ M^{-1}, measurable but at least 1000-fold weaker than wild-type. The affinity of wild-type is too high to measure in the conditions of the assay. Since Ca^{2+} typically weakens the affinity of Tm-Tn for actin 2−10-fold, depending on the conditions, the affinity in the presence of Ca^{2+} would be too weak to measure in our assay. The binding in the absence of Ca^{2+} nevertheless is specific since Tn alone did not bind to actin. The experiment shown in Figure 2B was carried out at 5 °C to promote coiled coil formation of dAc2−5, which has a lower T_M than wild-type. The stability of Tm is lower at 150 mM NaCl than at 500 mM NaCl used in the CD analysis reported in Table 1. The assay could not be carried out at lower ionic strength (e.g., 100 mM), where the Tm affinity is greater, because Tn alone binds to actin, in the absence of Tm (*81*).

We were unable to detect binding of the two shortest Tm variants. At high concentrations (15−20 μM) neither dAc2−6 (with either the exon 9a or exon 9d-encoded C-terminus)

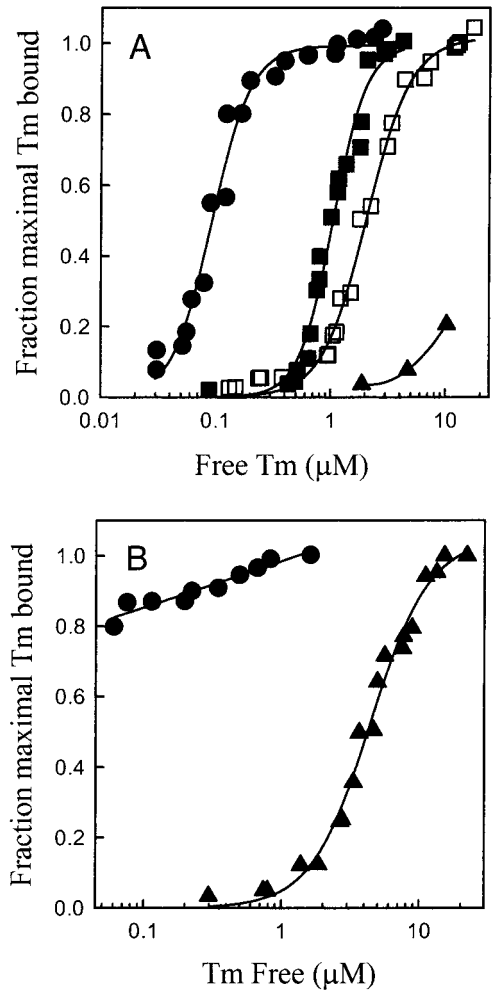

FIGURE 2: Binding of tropomyosin deletion mutants to actin with troponin. Tropomyosin at concentrations ranging from 0 to 20 μM, depending on the variant, was cosedimented with 5 μM actin in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.5 mM DTT with either 0.2 mM $CaCl_2$ (A) or 0.2 mM EGTA (B). The troponin concentration was at a 1.2-fold molar excess over TM. The curves were fit to the data using the Hill equation. The apparent K_a and Hill coefficient are reported in Table 2. (●) Wild-type Tm; (■) dAc2−4 Tm; (□) dAc3−5 Tm; (▲) dAc2−5 Tm.

Table 2: Tropomyosin Binding to Actin in the Presence of Troponin or Myosin S1

tropomyosin	Tm-actin, K_{app} (M^{-1})	Hill coefficient	S1/actin molar ratio[d]	mol of S1/Tm/actin[d]
wild-type	$(1.1 \pm 0.1) \times 10^{7}$ [a]	2.6 ± 0.3[a]	0.42	2.9
dAc2−3	$(4.4 \pm 0.3) \times 10^{6}$ [b]	5.3 ± 1.5[b]	0.64	3.2
dAc2−4	$(9.6 \pm 0.4) \times 10^{5}$ [a]	3.0 ± 0.3[a]	0.70	2.8
dAc3−5	$(4.9 \pm 0.2) \times 10^{5}$ [a]	2.3 ± 0.1[a]	no binding	no binding
dAc2−5	$(2.3 \pm 0.1) \times 10^{5}$ [c]	2.0 ± 0.2[c]	no binding	no binding
dAc2−6	no binding	no binding	no binding	no binding
dAc1.5−6.5	no binding	no binding	no binding	no binding

[a] Values are from the data in Figure 2A (+Ca^{2+}), shown with standard errors. [b] Values are from ref *30*, +Ca^{2+}, shown with standard errors. [c] Values are from the data in Figure 2B (+EGTA), shown with standard errors. [d] Values are for half-maximal saturation of F-actin with Tm, from the data in Figure 3.

nor dAc1.5−6.5 bound to F-actin in the absence of Tn, in conditions in which ionic strength and $MgCl_2$ concentration were varied. In the presence of Tn, dAc1.5−6.5 did not bind to F-actin. The dAc2−6 Tms formed complexes with Tn that sedimented to the same extent in the absence or presence of actin. While this prevented the measurement of actin binding, it does show that dAc2−6 retains the ability to bind to Tn.

From our results we conclude that a Tm must be able to span three actins to bind to F-actin, four or more to bind well. The shorter Tms may not bind for steric reasons, such as incorrect alignment with actin monomers, or because the coiled coil is sufficiently unstable to form.

We have analyzed the regulatory function of the three Tms that bind to actin (dAc2−3, dAc2−4, and dAc3−5) to understand the functional importance of the internal, constitutively expressed regions. To measure binding of the Tms to actin in the open, force-producing state, we have used myosin S1-induced binding of Tm to actin (without Tn). The other two states were evaluated using the regulated actomyosin ATPase: in the absence of Ca^{2+} for the blocked state and in the presence of Ca^{2+} for the equilibrium between the closed and open states.

Myosin S1-Induced Binding of Tropomyosin Mutants to F-Actin. In addition to the structural requirements of Tm for cooperative binding to actin, we have been investigating the parameters for cooperative activation of the thin filament by myosin. The cooperativity depends on Tm. Tropomyosin alone on actin is thought to be in equilibrium between the closed and open states, in terms of the Geeves and Lehrer model (*37*−*39*). The binding of myosin heads to actin shifts the equilibrium of the actin-Tm from the closed to the open, force-developing state in which both Tm and myosin bind to actin with higher affinity. One way this function is manifested is by an increase in the affinity of Tm for actin by myosin (*31*, *82*, *83*).

For each mutant we analyzed the ability of myosin S1 to promote strong binding of Tm to actin. For the Tms that bound, we determined the fraction of actin subunits that was required to be occupied by myosin heads in order to switch the actin filament from the weak-Tm-binding, predominantly closed state to the tight Tm-binding, open state. We have monitored the closed-to-open transition using a direct cosedimentation assay in conditions where Tm binds poorly in the absence of myosin S1 (2−13% saturation, depending on the variant). We could not use a fluorescence change in Tm to report the transition (*84*) because the recombinant Tms do not bind well to actin alone (in the absence of Tn). For the same reason, we could not measure the effect of the Tm mutants on the affinity of myosin S1-ADP for actin. Also, some of the Tm mutants do not contain the Cys190, the residue used for covalent modification with the reporter, pyrene iodoacetamide.

Striking findings emerge from these studies (Figure 3, Table 2). Our previous studies of N- and C-terminal Tm variants showed that the cooperativity with which myosin S1 induced Tm binding to actin generally correlates with actin affinity (*31*). Here, the S1-induced binding was not related to actin affinity. Myosin S1 induced the binding of dAc2−3 and dAc2−4 to actin, but only at much higher occupancy levels than wild-type (0.42 vs 0.64 and 0.70). However, considering the mutant Tms span only five and four actins, respectively, the cooperativity with which myosin S1-induced binding in terms of the Tm molecule is unchanged (Table 2). The number of myosin S1 molecules per *Tm molecule* for half-maximal binding was the same for wild-type and mutant Tms (~3 mol of myosin S1/Tm molecule). In these experiments the Tm molar concentration

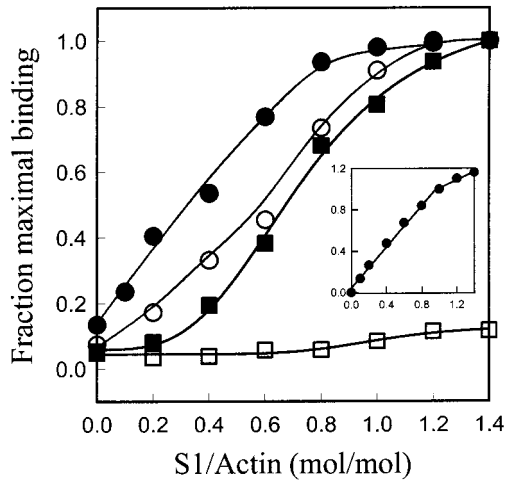

FIGURE 3: Myosin S1-induced binding of tropomyosin deletion mutants to actin. Binding of Tm (1 μM) and myosin S1 to actin (3 μM) were measured as a function of S1 concentration (0−4.2 μM) in 30 mM NaCl, 10 mM imidazole, pH 7.0, 0.5 mM $MgCl_2$, 1 mM DTT as described in Experimental Procedures. The points are the averaged data from two independent experiments. The inserted graph shows myosin S1 binding to actin. The line was fit by linear regression to the experimental points for S1:actin ration 0−1.0. The data are from four independent experiments (±SE). The error bars fall within the dimension of the symbols. The line at saturation was drawn manually. (●) Wild-type Tm; (○) dAc2−3 Tm; (■) dAc2−4 Tm; (□) dAc3−5 Tm.

was constant (1 μM); consequently the coiled coil concentration, in relation to actin, was variable. However, the relationship held even when the dAc2−4 molar concentration was 1.75 μM, the equivalent molecular length of 1 μM wild-type Tm. Still, an [S1]/actin ratio of 0.7 (2.8 mols S1/Tm) was required for half-maximal binding. Even though Tm molecules are aligned head-to-tail along the actin filament and recently modeled as a continuous cable (*85*), the myosin heads seem to be able to sense individual Tm molecules; that is, Tm is not isomorphous to myosin (and actin) along its length. To understand the mechanism will require the ability to analyze the spatial distribution of myosin S1 heads at below saturating concentrations in relation to individual Tm molecules. It is not known if the binding of Tm and myosin S1 are random or locally cooperative (discussed in refs *31* and *32*).

The second surprising result is that once period 5 (residues 166−207) was included in the deletion (dAc3−5 and any of the larger deletions), myosin S1 did not induce Tm binding even when the actin filament was fully occupied by myosin heads or at much higher Tm concentrations. The Tm concentration for the experiment in Figure 3 was 1 μM to minimize binding in the absence of myosin S1. Taking into account the lower actin affinity of dAc3−5 and shorter Tm mutants, we increased the Tm to 3 μM or higher. Even so, myosin S1 did not promote binding. The most obvious way to explain our results is to suggest that the conformational change upon myosin binding to actin leading to the open state of the thin filament alters the interaction with Tm in a way that specifically involves period 5. As long as period 5 is present, and the ends are intact, the cooperativity with which myosin S1 induces Tm binding in the open state depends on the number of molecules of Tm. Landis et al. (*34*) also found that a mutant comparable to dAc2−4 (with an AlaSer N-terminal extension) bound to actin saturated with myosin S1 whereas one comparable to dAc3−5 was not. Because of the importance of period 5 for myosin S1-induced strong binding of Tm, we could not define a minimum molecular length for this function.

Period 5 is encoded partly by exon 5 (residues 166−188) and partly by exon 6 (residues 189−207), an alternatively spliced exon (*86*). The exon 5-encoded portion is a highly conserved region of Tm: 8 of 23 residues are identical throughout phylogeny and 10 more are >80% identical or highly conserved (Asp/Glu; Ala/Ser). The highly conserved residues are distributed among interface and noninterface positions in the coiled coil. Mutations in two of these residues (D175N and E180G) cause familial hypertrophic cardiomyopathy (*87*). Period 5 is a stable region of the Tm coiled coil, as defined by the free energy of folding and frequency values for the interface *a* and *d* residues as well as the noninterface positions (*88*−*92*).

Ca^{2+}-Dependent Regulation of the Actomyosin ATPase. Tropomyosin, together with Tn, is required for Ca^{2+}-dependent activation of the actomyosin ATPase. In the actomyosin ATPase in vitro model for muscle regulation, full inhibition in the absence of Ca^{2+} (presence of EGTA) is taken to represent the blocked state in the three-state model. Full activation by myosin heads and Ca^{2+} (usually not fully observed in vitro) would represent the open, or fully active conformation. In actuality, the thin filament is in equilibrium between the closed and open states in the presence of Ca^{2+} and the ATPase activity can be used to compare the extent to which myosin can bind in the weak versus strong states to actin.

The Tm deletion mutants that bind well to actin clearly differ from wild-type in their ability to switch the regulated thin filament from one state to another in response to Ca^{2+}. Figure 4 shows that when the internal periods of Tm were deleted, Ca^{2+} did not fully relieve the inhibition observed with EGTA. While the activity with the mutant Tms in the presence of Ca^{2+} was higher than in EGTA (compare Figure 4A and 4B), all were markedly lower than that of wild-type. We have previously shown (and recently confirmed) that deletion of period 2 does not affect regulation of the actomyosin ATPase (*27*). Our interpretation is that the middle periodic repeats (periods 3−5) are required for Ca^{2+}-Tn to shift the equilibrium of the thin filament from the predominantly blocked state (in EGTA) to the predominantly open state (with Ca^{2+}). With the mutants the equilibrium of the thin filament appears to favor the closed state. The ATPase results do not parallel our myosin S1-induced binding experiments, which we used to evaluate the closed-to-open transition. We conclude that the structural requirements for the two transitions (blocked to closed/open; closed to open) are different. Our results are in general agreement with Landis et al.'s studies of similar mutants (*33*, *34*). One exception is that their dAc 2−3 mutant regulated rather normally whereas our unacetylated dAc2−3 had similar regulatory properties to dAc2−4 and dAc3−5. Although the relief of inhibition in the presence of Ca^{2+} was somewhat variable from experiment to experiment, the activity of dAc2−3 was always 50% or less that of wild-type. At this time the relationship to thin filament structure is unclear. The position of dAc2−3 (the AlaSer form) is the same as wild-type Tm in reconstructions of thin filaments reconstituted with Tm and Tn with Ca^{2+} (*93*).

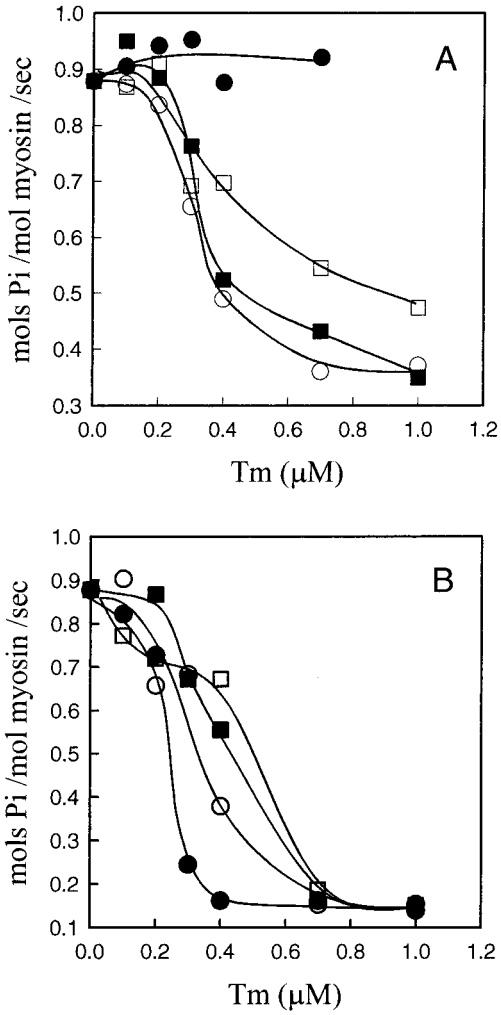

FIGURE 4: Actomyosin ATPase regulation by tropomyosin deletion mutants with troponin. The ATPase of actin (2.4 μM), myosin (0.6 μM), 1 μM Tn, and 0 to 1 μM Tm was measured at 28 °C in 40 mM NaCl, 5 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 0.5 mM DTT, and either 0.2 mM $CaCl_2$ (A) or 0.2 mM EGTA (B). The reaction was initiated by addition of MgATP to 5 mM. The results shown are of representative experiments. (●) Wild-type Tm; (○) dAc2−3 Tm; (■) dAc2−4 Tm; (□) dAc3−5 Tm.

One reason for incomplete relief of inhibition by Ca^{2+} binding to Tn may be that the deletions have altered or removed the Ca^{2+}-dependent binding site of Tn on Tm. Troponin is known to bind along the C-terminal one-third, or more, of Tm (*94*). Although Tn can be cross-linked to Cys 190 (in period 5) (*95*, *96*) and probes attached to Cys 190 respond to Tn and TnT binding to Tm (*97*−*100*), it is unknown how far Tn extends toward the N-terminus from Cys 190. Constitutively expressed exons of Tm may include the Ca^{2+}-dependent binding site for Tn. Support for this idea comes from a negative argument that when exon 6 (residues 189−213), an alternatively spliced exon, is deleted or replaced with another coiled coil sequence, the resulting Tm binds TnT and regulates the actomyosin ATPase (*101*). While this is a reasonable argument for the regulatory dysfunction of mutants lacking period 5, and even period 4, it would be hard to imagine that period 3 also directly binds to Tn.

Figure 4B shows that inhibition of the actomyosin ATPase in the absence of Ca^{2+} by the deletion mutants is comparable to wild-type Tm, except as it relates to actin affinity. The dAc2−5 mutant binds too weakly to actin, even in EGTA, to affect the ATPase. Clearly, the blocked state (as represented by the ATPase with EGTA) is insensitive to both Tm length and the presence of the internal periods. We have found in our research over the years that, in general, Tms that bind well to actin will inhibit the actomyosin ATPase (with Tn) in the absence of Ca^{2+}, even if they lack the regions of Tm that bind to Tn (e.g., refs *28* and *101* and unpublished results).

CONCLUSIONS

We have constructed a series of progressively shorter striated muscle Tms in which internal periodic repeats were deleted, leaving the ends intact. Analysis of these Tms has given us the following insights into Tm function.

The minimum length for cooperative binding to actin (with Tm) with reasonable affinity is a Tm that spans four subunits on the actin filament. A Tm the length of three actins will bind, but with extremely low affinity.

In the context of this series of multiple-period deletions period 5 (residues 166−207) is essential for myosin to promote strong binding of Tm to actin. When period 5 is deleted together with other regions, the Tm cannot bind to actin with myosin S1, even though the Tms bind to actin with Tn and inhibit the regulated actomyosin ATPase.

The middle region of Tm is required for normal activation of the regulated actomyosin ATPase in the presence of Ca^{2+}, but inhibition in the absence of Ca^{2+} is normal. We conclude that inhibition in the absence of Ca^{2+} is relatively nonspecific in terms of Tm structure, requiring only that it binds to actin and Tn. In contrast, activation by Ca^{2+} and myosin (representing the proposed closed-to-open transition of the thin filament) requires periods three to five, especially period 5, not merely actin binding.

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