Functions of Tropomyosin's Periodic Repeats[†]

Sarah E. Hitchcock-DeGregori,*[,‡] Yuhua Song,[§] and Norma J. Greenfield[‡]

Department of Neuroscience and Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, and Bioarray Solutions, Warren, New Jersey 07060

Received July 26, 2002; Revised Manuscript Received October 7, 2002

ABSTRACT: Tropomyosin binds along actin filaments and regulates actin−myosin interaction in muscle and nonmuscle cells. Seven periodic amino acid repeats are proposed to correspond to actin binding sites, and the middle periods are important for cooperative activation of actin by myosin. The functional contributions of individual periods were studied in mutants in which periods 2−6 were individually deleted from rat striated muscle αα-tropomyosin or replaced with a leucine zipper sequence. Unacetylated recombinant tropomyosins were assayed for actin binding, regulation of the actomyosin ATPase with troponin, cooperative myosin S1-induced binding to actin, and thermal stability. Tropomyosin function is relatively insensitive to deletion of period 2, but loss increases as the deletion is shifted toward the C-terminus. Retention of function upon deletion of the periodic repeats is in the order of $2 > 3 \sim 4 \sim 6 \gg 5$. Internal periods are important for specific functions and are not quasiequivalent. Deletion of period 5 (residues 166−207), and especially deletion or replacement of residues 166−188, a constitutively expressed region encoded by exon 5, had severe consequences on actin affinity and cooperative myosin S1-induced binding to actin. Period 6, residues 208−242, part of the troponin binding site, is required for full inhibition of the actomyosin ATPase in the absence of calcium. The effect of the deletion can depend on its context, suggesting that sequence alone is not the only factor important for function. We propose that the local structure and stability, and consequent flexibility, of the coiled coil are major determinants of actin affinity.

Actin filament dynamics and function in muscle and nonmuscle cells are modulated by tropomyosin (TM[1]), a parallel, two-chained coiled coil that binds along the length of the actin filament (*1*−*4*), reviewed by (*5*). Tropomyosins are a family of highly conserved proteins expressed in muscle and nonmuscle cells that stiffen and stabilize actin filaments and regulate myosin-dependent motile function. Cooperative regulation of actin filament function in striated muscle by myosin and troponin (Tn) depends on tropomyosin [reviewed by (*5*)]. Tropomyosin regulates actin filament dynamics in multiple ways. Tropomyosin alone, and with tropomodulin, stabilizes the "pointed" end of the filament (*6*−*10*), protects filaments from severing and depolymerization by proteins such as cofilin and DNase I (*11*, *12*), and inhibits the Arp2/3 complex-nucleated actin polymerization and branch formation (*13*).

The highly conserved tropomyosin sequence contains periodic patterns of amino acids that relate to its structure and its function. In addition to the characteristic heptapeptide repeat of hydrophobic amino acids characteristic of coiled coils, there is a poorly conserved 7-fold periodic repeat proposed to represent quasiequivalent actin binding sites (*14*−*16*). The lengths of naturally occurring tropomyosins correspond to an integral number of periods and actin monomers in the filament: seven in 284-residue muscle and nonmuscle forms and six in 247-residue nonmuscle isoforms. In addition, yeast has tropomyosins that span the length of four and five actin monomers (*17*, *18*).

The contributions of individual periods to tropomyosin function have been evaluated by deleting or replacing one or more repeat. Deletion analysis of striated muscle α-tropomyosin showed that the presence of an integral number of periodic repeats and an uninterrupted coiled coil are required for actin binding (*19*, *20*) but that regions of the molecule contribute differentially to regulatory function (*21*−*23*). Two and three period deletions within the molecule have small effects on actin affinity but big effects on activation of the thin filament by Ca^{2+}, as measured using actomyosin ATPase and motility assays (*20*−*23*). Deletion of certain internal regions impair myosin S1-induced binding of tropomyosin to actin (*23*), and tropomyosin binding to actomyosin S1 (*22*), leading to the suggestion that the allosteric activation of the actin filament by tropomyosin is promoted more strongly by the middle of the molecule than the ends (*24*). We have inferred that period 5 is of particular significance for myosin S1 promotion of tropomyosin binding to actin in the "open" conformation required for full thin filament activation (*23*).

To define the specific regions of tropomyosin important for thin filament regulation, we probed the contribution of specific periods to tropomyosin functions by deleting individual periods or replacing portions of periods with leucine zipper sequences. Using this approach we show that the

[†] Supported by National Institutes of Health Grants GM36326, GM63257, and HL35726 to SEHD and NJG.

* Corresponding author. Address: Department of Neuroscience and Cell Biology, 675 Hoes Lane, Piscataway, NJ 08854. Tel: 732-235-5236. Fax: 732-235-4029. E-mail: hitchcoc@umdnj.edu.

[‡] UMDNJ-Robert Wood Johnson Medical School.

[§] Bioarray Solutions.

[1] Abbreviations: TM, tropomyosin; Tn, troponin.

10.1021/bi026519k CCC: $22.00
Published on Web 11/15/2002

individual periods have specific functions. The deletion mutants are widely different in actin affinity and myosin-induced tropomyosin binding to actin. The periods also differ in their importance for Ca^{2+}-dependent regulation by troponin.

EXPERIMENTAL PROCEDURES

DNA Constructions, Protein Expression, and Purification. A rat striated muscle α-tropomyosin cDNA clone, a gift of Dr. B. Nadal-Ginard (*25*), was used for expression of wild-type tropomyosin and for construction of all mutants reported for the first time here. The cDNA was previously cloned into pET11d (*26*) at NcoI and BamH1 sites for expression in *Escherichia coli*. The dAc5, dAc4/5, dAc5, dAc5−N, dAc5−C, dAc6, and TM−5Nzip mutants created for the present study were made using oligonucleotide-directed mutagenesis of double stranded DNA (in pET11d) using a Stratagene ExSite or Quick-change PCR-based site-directed mutagenesis kits (La Jolla, CA) with Taq or Pfu DNA polymerase. Two synthetic oligonucleotides served as primers for the PCR for each mutation. One was complementary to the region of the cDNA adjacent to the first codon to be deleted at the 5′ end of the cDNA. The second oligonucleotide was complementary to the noncoding stand starting at the last codon to be deleted, extending toward the 3′ end of the cDNA. All oligonucleotides were synthesized by the UMDNJ DNA Synthesis and Sequencing Facility, Robert Wood Johnson Medical School, Piscataway, NJ.

The sequences of the mutagenic oligonucleotides were as follows:

dAc4, 5′ end:
3′-CCGACTCTTCCGACGTCTACTCTCA-5′

dAc4, 3′ end:
5′-GCCCGTAAGCTGGTCATCATCGAGAG-3′

dAc4/5, 5′ end:
3′-CTTCTTTTCTACCTCTAAGTCCTCTAG-5′

dAc4/5, 3′ end:
5′-AAATGTGCGGAGCTTGAAGAAGAGT-3′

dAc5, 5′ end:
3′-CTTCTACGACTGGCTTTCATACTTCTCCAC-5′

dAc5, 3′ end:
5′-GAGGCTCAGGCTGAGAAGTACTCTC-3′

dAc5−N, 5′ end: same as dAc5

dAc5−N, 3′ end:
5′-GAAGGCAAATGTGCGGAGCTTGAAG-3′

dAc5−C, 5′ end:
3′-GCCTCCTCTCCCGACTCGAGAGC-5′

dAc5−C, 3′ end: same as dAc5

dAc6, 5′ end:
3′-CTTTTGCCACTGCTTGTTGAACTTCAGTGAC-5′

dAc6, 3′ end: 5′-GAGAGATCAGTAACC
AAATTGGAGAAAAGC-3′

TM−5Nzip, 5′end:
3′TCTACGACTGGCTTTCATACTTCTC*GAACTT*
CTATTTCAACTTCTTGAAGACAGATTTTTGATG
GTAGAATTTTTGCTTCAACGAGCAGAATTTCTG-5′

TM−5Nzip, 3′ end: same as dAc4/5

Following ligation and cloning in DH5α cells, the DNA from a single colony was purified. The presence of the mutation was confirmed by sequence determination of one strand of the cDNA at the UMDNJ DNA Synthesis and Sequencing Facility at Robert Wood Johnson Medical School, Piscataway, NJ. For protein expression, the pET11d vector carrying the wild-type or tropomyosin cDNA was transformed into *E. coli* strains BL21(DE3) or BL21(DE3) pLysS (*26*) for expression and purification as previously reported (*23*, *27*, *28*) by ammonium sulfate fractionation, chromatography on DE52 cellulose, and hydrolylapatite.

Other Proteins. Actin was isolated from White Leghorn chicken pectoral muscle acetone powder (*29*), 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 (*30*). Troponin was purified from chicken pectoral muscle (a gift of Dr. J. Fagan, Rutgers University, New Brunswick, NJ) according to the method of Potter (*31*) with modifications described in (*32*).

The concentrations of actin, myosin, myosin S1, and troponin were spectrophometrically determined using the extinction coefficients (0.1%) of 1.1, 0.53, 0.83, and 0.45, respectively. Concentrations of recombinant tropomyosin were determined by differential absorption spectra of tyrosine as previously described (*28*, *33*, *34*).

Circular Dichroism Measurements. CD measurements were carried out and analyzed using an Aviv model 62 DS spectropolarimeter as previously described (*35*). The helical content was estimated from the mean residue ellipticity (deg cm^2 $dmol^{-1}$) 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 tropomyosins and −43 000 is the maximal ellipticity observed at 0 °C.

Actin Binding Assays. Tropomyosin binding to F-actin was directly measured using a cosedimentation assay as previously described (*36*) with modifications (*28*). The amounts of bound and free tropomyosin in the pellets and supernatants, respectively, were quantitated by densitometry of SDS−polyacrylamide gels (12%) stained in Coomassie Blue (*37*) using a Molecular Dynamics model 300A computing densitometer (Sunnyvale, CA). Despite the high concentrations of tropomyosin used for some mutants, trapping and/or nonspecific binding was less than 10% for most mutants. Where the trapping exceeded these levels, the data from the binding isotherm were corrected for the trapped tropomyosin. The free tropomyosin in the supernatants was calculated from standard curves for wild-type tropomyosin, or dAc4TM.

The apparent K_a (K_{app}) of tropomyosin for F-actin and the Hill coefficient (nH) were determined by fitting the experimental data to the following equation using SigmaPlot (SPSS Science, Chicago, IL):

$$\upsilon = (n[\text{TM}]^{nH}K_{app}{}^{nH})/(1 + [\text{TM}]^{nH}K_{app}{}^{nH})$$

where υ = fraction maximal tropomyosin binding to actin, n = maximal tropomyosin bound, and [TM] = $[\text{TM}]_{free}$.

The TM:actin ratio was normalized to 1.0 by dividing the TM:actin ratio obtained from densitometry by the observed TM:actin ratio for saturating, stoichiometric binding. We have previously shown that the density ratio at saturation reflects stoichiometric binding of tropomyosin to actin (*38*).

Myosin S1-Induced Tropomyosin Binding to Actin. Actin (3 μM) and tropomyosin (3 μM) in 30 mM NaCl, 0.5 mM $MgCl_2$, 1 mM 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 (*37*). 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 versus the initial S1:actin molar ratio. The data were normalized to the value for stoichiometric binding at saturation.

Actomyosin MgATPase Assay. The actomyosin ATPase activity was measured as a function of tropomyosin concentration using 2.4 μM actin, 0.6 μM myosin, 1 μM troponin, and 0−10 μM tropomyosin depending on the actin affinity, typically 1−1.6 μM, 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 (*39*). The plates were read in a Molecular Devices ThermoMax plate reader with a 650 nm filter (Menlo Park, CA).

RESULTS

Mutant Design. The mutations were deletions of single periodic repeats proposed to represent quasiequivalent actin binding sites (Figure 1A): 35 amino acids (periods 3 and 6) or 42 amino acids (periods 2, 4, and 5) following the Phillips' proposal (*16*). The designation of the periods takes into account the helical (azimuthal) position of the α-helix as well as the linear (supercoil) position of each amino acid, with an average repeat of $39^1/_3$ amino acids. Also, the deletions, being 35 or 42 amino acids, are multiples of seven to retain the heptapeptide repeat of hydrophobic residues important for coiled coil function (*40*, *41*). We also made two half-period deletions (21 amino acids) of period 5 based on our earlier work showing that a half-period deletions of period 2 had little effect on function (*19*). Mutations replacing tropomyosin sequence with GCN4 leucine zipper sequence maintained the length of wild-type tropomyosin (284 amino acids, Figure 1B). Figure 1C shows the GCN4 leucine zipper sequences compared to the tropomyosin sequences. Work on some of these mutants is published (*19*, *20*, *42*).

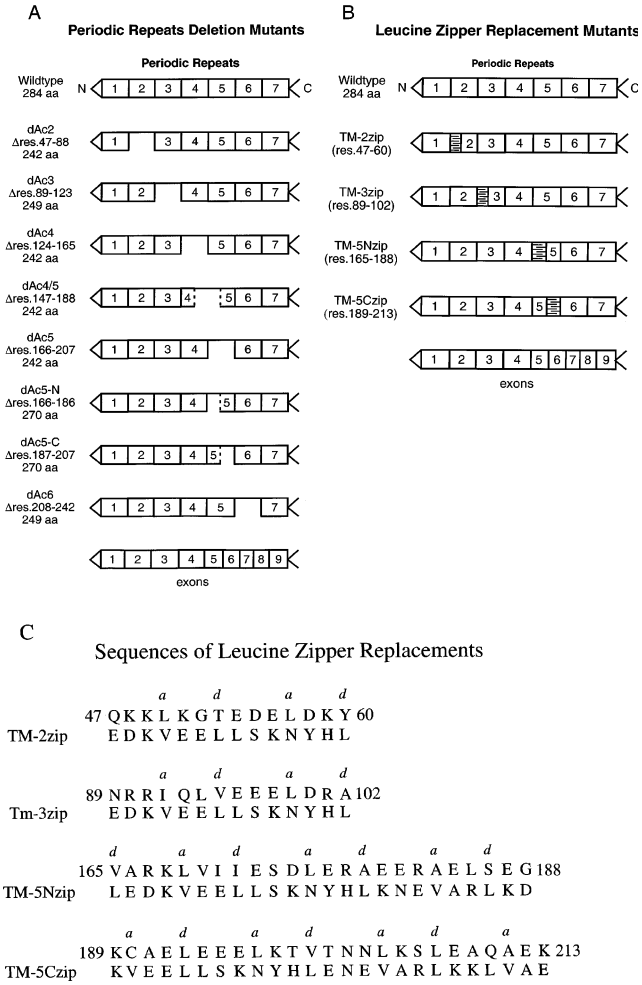

FIGURE 1: Design of the deletion mutants. The numbered blocks represent the seven periodic repeats in 284-residue tropomyosins. Each block (period) corresponds to the length of an actin monomer spanned by tropomyosin in the thin filament. The pointed (N-terminus) and barbed (C-terminus) ends reflect the orientation of tropomyosin on the actin filament. The designation "N" or "C" refers to the N-terminal or C-terminal region of the period. (A) The deletions are of individual periods or half periods. In the diagram the blocks represent the regions remaining in the mutants. Each mutant is named, the region deleted, and remaining number of amino acids listed. At the bottom of the figure, the coding exons are listed, aligned with the corresponding periods. (B) The zipper motifs show where GCN4 leucine zipper sequence replaces tropomyosin sequence and the residues that were replaced. All mutants are 284 residues in length. (C) The amino acid sequences of regions of tropomyosin (top) that were replaced with GCN4 leucine zipper sequence (*46*). The *a* and *d* refer to the interface positions in the coiled coil heptad repeat. The TM−5Nzip mutants contains two changes from the GCN4 sequence: 180K (instead of E) and 188D (instead of K).

Contributions of Individual Periodic Repeats to Actin Affinity. A universal tropomyosin function is the ability to bind cooperatively to F-actin. The actin affinity was measured by cosedimentation with F-actin in the presence of troponin, with Ca^{2+} (*36*, *43*). Recombinant striated muscle α-tropomyosin expressed in *E. coli* is unacetylated at the N-terminal Met and requires troponin for reasonable actin affinity (*36*). The deletions all reduced the affinity of tropomyosin for actin, but removal of period 5 had the most severe effect (Figure 2A, Table 1). Removal of period 2 reduced actin affinity less than 10-fold (*19*, *20*). Deletion of periods 4 and 6 caused 10−30-fold reductions in affinity,

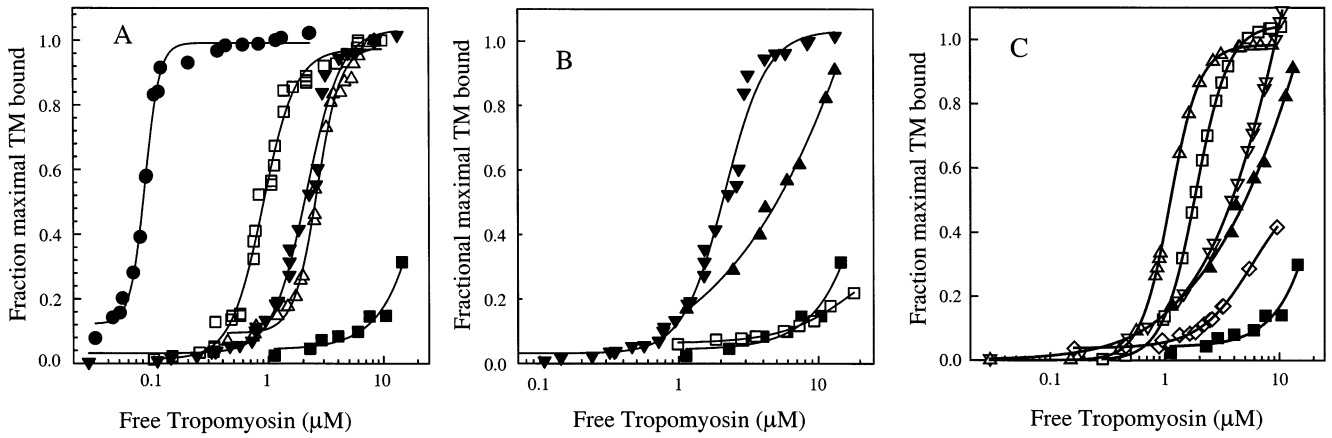

FIGURE 2: Binding of tropomyosin deletion mutants to actin with troponin. Tropomyosin at concentrations ranging from 0 to 20 μM, depending on the mutant, was cosedimented with 5 μM actin in 150 mM NaCl, 10 mM Tris−HCl, pH 7.5, 2 mM $MgCl_2$, 0.5 mM dithiothreitol with either 0.2 mM $CaCl_2$ (A, B) or 0.2 mM EGTA (C). The troponin concentration was at a 1.2-fold molar excess over tropomyosin. The curves were fit to the data using the Hill equation. The K_as are reported in Table 1. The Hill coefficients were typically 3−4. (A) Binding of single period deletion mutants to actin with troponin, $+Ca^{2+}$. Symbols: ●, wild-type; □, dAc4; ▼, dAc4/5; ■, dAc5; △, dAc6. (B) Binding of period 5 mutants to actin with troponin, $+Ca^{2+}$. Symbols: ▼, dAc4/5 (same data as in panel A); ■, dAc5 (same data as in panel A); ▲, dAc5−C; □, TM−5Nzip. dAc5−N does not bind. (C) Binding of period 5 mutants to actin with troponin, +EGTA. Symbols: □, dAc5, +EGTA; ■, dAc5, $+Ca^{2+}$; ▽, dAc5−N, EGTA; △, dAc5−C, EGTA; ▲, dAc5−C, Ca^{2+}; ◇, TM−5Nzip, EGTA.

Table 1: Summary of Measured Parameters of Tropomyosin Mutants

protein	actin affinity +Tn, Ca^{2+} K_{app} ($M^{-1} \times 10^{-6}$)	S1/Actin for 1/2 max TM binding	S1/TM 1/2 max TM binding	T_M unfolding, °C overall[a]	T_M unfolding, °C major dissociating transition[b]
wild-type	12.8 ± 0.3	0.2 ± 0.02	1.4	42.6 ± 0.5	45.0 ± 1.0
dAc2[c]	1.4 ± 0.1	0.24 ± 0.04	1.4	43.4	45.9
dAc3[c]	0.5 ± 0.1	0.57 ± 0.09	3.4	36.4	37.9
dAc4	1.1 ± 0.2	0.65 ± 0.02	3.9	50.3	50.8
dAc4/5	0.48 ± 0.18	0.40 ± 0.05	2.4	43.1	45.7
dAc5	≪0.1	>1.0	not saturated	41.3	44.2
dAc5−N	≪0.1	no binding	not saturated	42.9	48.5
dAc5−C	0.25	0.76	4.9	35.2	48.2
d191−211[d]		0.17 ± 0.01	1.12	43.1	44.9
dAc6	0.39 ± 0.01	∼0.7	∼4.2	44.4	47.3
TM−2zip[c]	1.7 ± 0.1	−	−	43.8	45
TM−3zip[c]	1.3 ± 0.1	0.68 ± 0.04	4.8	43.9	45.4
TM−5Nzip	≪0.1	no binding	not saturated	56.4	68.3
TM−5Czip[d]	17 ± 0.1	0.21 ± 0.01	1.5	36.9	50
D121N	7.6	−	−	43.4	45.9
D175N[e]	7.1 ± 0.3	−	−	43.8	45.6
E180G[e]	3.7 ± 0.1	−	−	40.5	47.9

[a] This is the temperature where the protein is 50% folded, assuming that it is completely folded at 0 °C and the temperature of complete unfolding was experimentally determined. [b] This is the transition with the highest T_M, assumed to accompany chain dissociation. [c] These mutations were in chicken striated α-tropomyosin. The description and analysis of these mutants have been previously published (*20*). The K_{app} for wild-type is 9.6 ± 0.5 × 10^6 M^{-1}. [d] The description and analysis of these mutants have been published (*42*). The tropomyosins differ from those in the present study in that residues 39−80 are encoded by exon 2a, whereas those in the present study are encoded by exon 2b. The values are compared to a wild-type in which there are 2a- and 6a-encoded regions (vs 2b and 6b in the present wild-type): K_{app} for actin, 70 × 10^6 M^{-1}; overall T_m, 35.2 °C; S1/actin ratio for half-maximal binding, 0.16. [e] The description and analysis of these hypertrophic cardiomyopathy-causing mutations in human striated muscle α-tropomyosin were previously published in (*60*, *61*). K_{app} of wild-type TM for actin, 10.3 ± 0.3 × 10^6 M^{-1}; overall T_m, 44.5 °C.

similar to that previously reported for period 3 (*20*). Surprisingly, however, deletion of period 5 resulted in loss of measurable affinity for actin with troponin, $+Ca^{2+}$, though it did bind weakly in the absence of Ca^{2+} (K_{app} = 0.54 ± 10^6 M^{-1}, at least 200-fold weaker than wild-type tropomyosin). The results suggested that period 5, residues 166−207, is more important for actin binding than the other four internal periods.

Period 5 (residues 166−207) is encoded by most of exon 5 (residues 165−188), a constitutively expressed exon, and most of exon 6 (residues 189−213), an alternatively spliced exon, with exon 6b expressed in muscle tropomyosins (*17*, *18*). To determine which region of exon 5 is important for function, we made three additional mutants (Figure 1A). One (dAc4/5) deleted residues 147−188, corresponding to exon 5 and part of exon 4, totaling 42 residues. Two were of half-period deletions of the N- and C-terminal halves of period 5: residues 166−186 in dAc5−N and residues 187−207 in dAc5−C, corresponding approximately to exons 5 and 6, respectively. The half-period deletions were based on the McLachlan and Stewart proposal that there are fourteen quasiequivalent actin binding sites in tropomyosin (*15*). We previously reported that deletion of half (21 residues) or all (42 residues) of period 2 had similar mild effects on function, but nonintegral deletions (14 or 28 residues) resulted in loss of function (*44*, *45*). Deletion of dAc4/5 and dAc5−C weakened the actin affinity of tropomyosin with troponin below that of dAc4 (Figure 2A,B, Table 1). The dAc5−C

deletion did not saturate, and the binding was noncooperative, but only dAc5−N mimicked the dAc5 mutation in exhibiting loss of measurable affinity for F-actin with troponin, $+Ca^{2+}$ (Table 1). In the absence of Ca^{2+} with troponin, dAc5−C bound with higher affinity than dAc5, and both exhibited cooperative, saturable binding (Figure 2C). The binding of dAc5−N did not saturate, indicating the actin affinity is at least 1000-fold weaker than that of wild-type tropomyosin (Figure 2C), similar to the effect of deleting the N or C terminus.

To begin to understand the specificity of the sequence encoded by exon 5 for actin affinity, we replaced it with a GCN4 leucine zipper sequence (*46*). The consequences were as deleterious as for the deletion itself in that TM−5Nzip had unmeasurable affinity for actin with troponin in the presence as well as in the absence of Ca^{2+} (Figure 2B,C). In contrast, substitution of regions of period 2 (TM−2zip), 3 (TM−3zip), or the C-terminal half of period 5 (exon 6, TM−5Czip) with leucine zipper sequence (Figure 1C, Table 1) reduced actin affinity less than 10-fold (*20*, *42*). On face value the results suggest that the sequence itself, not the deletion of the period, per se, is significant for function (See Discussion).

Effect of Deletions and Leucine Zipper Replacement Mutants on Myosin S1-Induced Binding to F-Actin. Cooperative activation of the thin filament by myosin depends on tropomyosin. Tropomyosin alone is thought to be in equilibrium between the closed and open states, in terms of the Geeves and Lehrer model (*47*−*49*). Myosin binding shifts the equilibrium of actin−tropomyosin to favor the open, force-producing state in which both tropomyosin and myosin bind with higher affinity. The functional states have been associated with different positions of tropomyosin on the actin filament (*50*). Deletion of period 2 had little effect even though it reduced actin affinity 7-fold (Figure 3, Table 1). In contrast, dAc5 and dAc5−N were unable to bind, even at saturating myosin S1. The others were also impaired, but did bind to F-actin in the presence of saturating myosin S1. The dAc6 mutant maximally bound the actin filaments at 1 myosin S1:1 actin, but binding was less than stoichiometric. The low values for the myosin S1-induced binding of dAc5−C and dAc6 may be influenced by their low actin affinity. Considering the cooperativity of myosin-induced binding of tropomyosin in terms of the length of the tropomyosin molecule (6 or 6.5 vs 7 actins, rather than the occupancy of actin by myosin S1), the significance of periods 3−6 becomes even more apparent (Table 1). The results are consistent with previous studies where myosin S1 failed to promote the actin binding of tropomyosins with multiperiod deletions lacking period 5, even one that bound well to F-actin (*23*).

The amount of S1 required for binding of these internal deletion mutants was greater than for tropomyosins with mutations at the N- and C-terminal ends that cause similar reductions in actin affinity (*32*). To have saturable binding of dAc3, dAc4, and dAc5−C, higher concentrations of tropomyosin were used (3 vs 1 μM in earlier studies, with 3 μM F-actin). The results suggest that internal regions of tropomyosin are required in a specific fashion for myosin promotion of tropomyosin binding to actin in the open, force producing conformation. However, the binding positions of these tropomyosins on the actin filament are unknown.

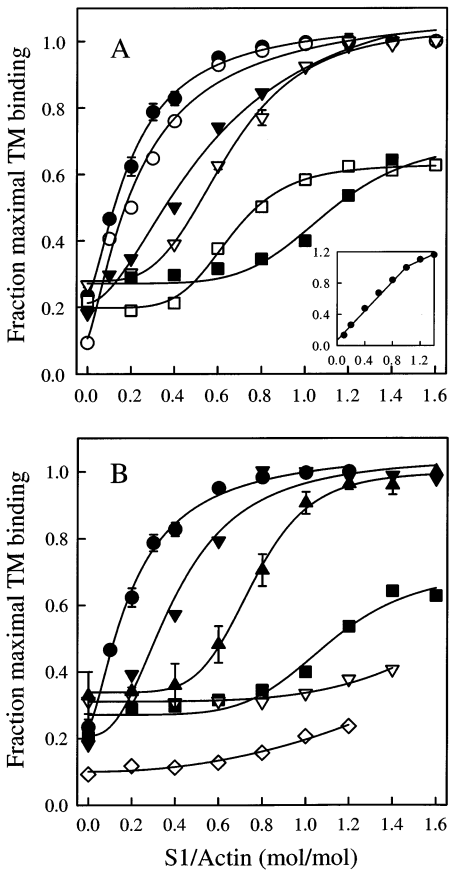

FIGURE 3: Myosin S1-induced binding of tropomyosin mutants to actin. Binding of tropomyosin (3 μM) and myosin S1 to actin (3 μM) were measured as a function of myosin S1 concentration (0−4.8 μM) in 30 mM NaCl, 10 mM imidazole, pH 7.0, 0.5 mM $MgCl_2$, 1 mM dithiothreitol as described in Experimental Procedures. The conditions were selected where tropomyosin binding in the absence of myosin S1 is minimal, and the binding of most mutants is stoichiometric at saturating amounts of S1. The points are averaged from 2−5 independent experiments. Standard deviations are shown for wild-type and for one, arbitrarily chosen mutant. The tropomyosin:actin ratio of the pellets were normalized to the ratio obtained with wild-type tropomyosin at saturation. (A) Myosin S1-induced binding of single period deletion mutants. Symbols: ●, wild-type; ○, dAc2; ▼, dAc3; ▽, dAc4; ■, dAc5; □, dAc6. (B) Binding of period 5 mutants to actin with myosin S1. Symbols: ●, wild-type; ▼ dAc4/5; ■, dAc5; ▽, dAc5−N; ▲, dAc5−C; ◇, TM−5N zip. The inserted graph shows that binding of myosin S1 to actin is linear from 0 to 1.0 S1:actin molar ratio.

To address the specificity of internal periods for S1-induced tropomyosin binding, we assayed the leucine zipper mutants. Replacement of exon 5 (residues 165−188) with a leucine zipper sequence (TM−5Nzip) resulted in loss of the ability to bind to actin, even in the presence of myosin (Figure 3B). In contrast, TM−5Czip, with leucine zipper in the C-terminal half of period 5, encoded by exon 6 (residues 189−213), had normal myosin S1-induced binding, as did a variant with an exon 6a (vs exon 6b)-encoded sequence (unpublished results). TM−3zip did bind to actin with myosin S1 but, like dAc3, required stoichiometric binding of myosin S1 to actin illustrating the specificity period 3 for this function (Table 1).

Certain anomalies are, by now, apparent to the reader. The impact of the deletion or substitution depends on the context. The effect of deleting residues 166−207 was severe when deleted alone (dAc5−N), or with the rest of period 5 (dAc5),

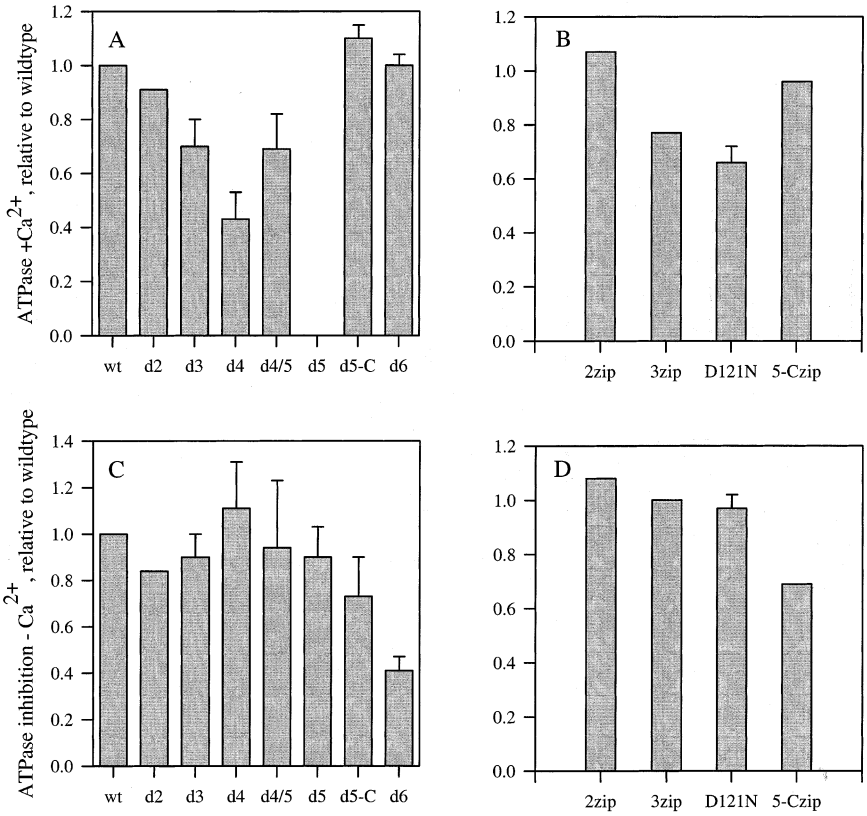

FIGURE 4: Regulation of the actomyosin ATPase by tropomyosin mutants with troponin. The ATPase activity was assayed as a function of tropomyosin concentration. The ATPase relative to wild-type was calculated for the tropomyosin concentration at which the effect on the ATPase was saturated to account for differences in affinity among the different tropomyosin mutants. The ATPase of actin (2.4 μM), myosin (0.6 μM), 1 μM troponin, and as a function of tropomyosin concentration, 0−10 μM, typically 0−1.6 μM, depending on the actin affinity, was measured at 28 °C in 40 mM NaCl, 5 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and either 0.2 mM $CaCl_2$ or 0.2 mM EGTA. The error bars are included for 2−4 independent experiments. (A and B) The values for maximal ATPase (Ca^{2+}) relative to that with wild-type tropomyosin (set at 1.0) at a tropomyosin concentration at which the effect of the mutant was saturated. The results for dAc5 (d5) were not included because the mutant does not bind to actin. Values less than 1.0 reflect a combination of incomplete relief of inhibition by troponin and incomplete activation of the regulated thin filament in the presence of Ca^{2+}. (C and D) Relative inhibition in the absence of Ca^{2+}. A value of 1.0 is the maximal inhibition of the actomyosin ATPase by tropomyosin−troponin in the absence of Ca^{2+}, typically 18−20% of the actomyosin ATPase in the absence of tropomyosin. This 80% inhibition is set at 1.0. A lower relative value represents poorer inhibition in the absence of Ca^{2+}. The relative values for the mutants were taken at tropomyosin concentrations, at which the effect of the mutant was saturated. In TM−5Czip, residues 39−80 are encoded by exon 2a, not 2b as in the other tropomyosins. The ATPase data were normalized to allow comparison of data collected over a period of years.

but relatively mild when deleted with part of period 4 (dAc4/5). It is also surprising that the consequence of deleting of residues 187−207 (dAc5−C) was much greater than that with a deletion shifted just three residues toward the C terminus (d191−211). However, d191−211 (and also TM−5Czip) differ from the other tropomyosins in the study in that residues 39−80 are encoded by exon 2a, a smooth muscle-specific exon, rather than exon 2b. Another illustration of the long-range importance of context is in that tropomyosins with an exon 9d-encoded C-terminus (found in smooth and nonmuscle isoforms), the residue 191−211 deletion or TM−5Czip replacement both lost measurable actin affinity (*42*).

Ca^{2+}-Dependent Regulation of the Actomyosin ATPase. Previously published work has emphasized the importance of internal regions of tropomyosin for stabilization of the open, force-producing state of the thin filament (*21*−*23*, *51*). Study of the mutants generated for the present study shows that residues 81−186 are required for relief of inhibition of the actomyosin ATPase by troponin in the presence of Ca^{2+}, and residues 208−242 for full inhibition in the absence of Ca^{2+}.

Tropomyosin lacking periods 3 or 4 did not fully relieve inhibition (or activate) the thin filament in the presence of Ca^{2+} (Figure 4A). Replacement of the N-terminal region of period 3 with a leucine zipper sequence (TM−3zip) also impaired the ATPase, $+Ca^{2+}$, while the activity was normal when leucine zipper sequence was introduced into period 2 (TM−2zip) or the C-terminal part of period 5 (TM−5Czip, Figure 4B). The dAc5−N and TM−5Nzip mutants could not be evaluated because they do not bind to actin. However, the C-terminus of period 5 is not crucial since dAc5−C exhibited normal ATPase in the presence of Ca^{2+}, as did dAc6 and the residue191−211 deletion mutant (*42*).

With the exception of dAc6, and possibly dAc5−C and the leucine zipper replacement in this region (TM−5C zip), all tropomyosins inhibited the actomyosin ATPase in the absence of Ca^{2+} as well as wild-type tropomyosin, even dAc5, which bound with low affinity in the absence of Ca^{2+} (Figure 4C,D). dAc5−N and TM−5N zip were not tested since they do not bind to actin with troponin, even in the absence of Ca^{2+}.

Conformational Analysis Using Circular Dichroism Spectrometry. The functional assays of the tropomyosin mutants

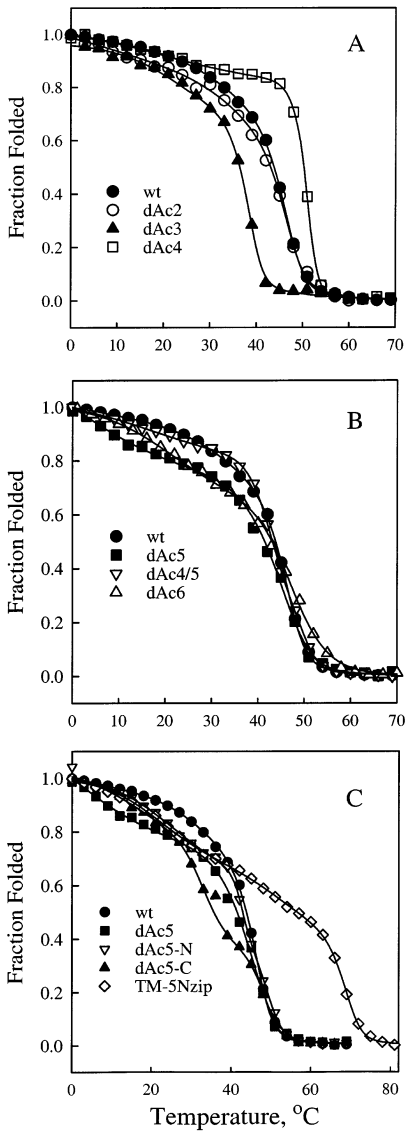

FIGURE 5: Temperature dependence of the unfolding of tropomyosin mutants using circular dichroism at 222 nm, a measure of α-helix. Conditions: 0.1 mg/mL (1.5 μM for wild-type tropomyosin) in 500 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5. (A) Symbols: ●, wild-type; ○, dAc2; ▲, dAc3; □, dAc4. (B) Symbols: ●, wild-type; ■, dAc5; ▽,dAc4/5; △,dAc6. (C) ●, wild-type; ■, dAc5; ▽, dAc5−N; ▲,dAc5−C; ◇, TM−5N zip.

show that the periodic sequence repeats are not functionally equivalent. Analysis of the secondary structure showed that most mutants had α-helical contents >90% of the wild-type value at 20 °C, except for dAc5 and dAc6. However, they varied widely in the temperature of the major unfolding transition associated with chain dissociation and in the number and cooperativity of the transitions (Figure 5, Table 1). The effects of the deletions and leucine zipper replacements are influenced by the context, as is the function. The mutants with unfolding isotherms most similar to wild-type were dAc2 (Figure 5A) and dAc4/5 (Figure 5B). The dAc3 mutant was less stable, dAc4 more stable. The overall T_ms of dAc5 and dAc6 were similar to that of wild-type, but the unfolding was less cooperative with a significant fraction unfolding below physiological temperature (Figure 5B). Both half period deletions in period 5, dAc5−N and especially dAc5−C, destabilized a significant fraction of the molecule (Figure 5C). Introduction of leucine zipper sequence at every location resulted in multiple transitions, with a portion of the molecule unfolding at a higher temperature and with lower cooperativity than the main transition of wild-type tropomyosin (Table 1) (*35*). Typically the change was 1−5 °C, but for TM−5N zip, the T_m of the major transition increased by >20 °C, and its unfolding was cooperative (Figure 5C). From these results we suggest that regional stability is an important determinant of tropomyosin function. Tropomyosin function may be altered by stabilization or destabilization of a particular region.

DISCUSSION

The present analysis of the individual internal periodic repeats in striated muscle α-tropomyosin shows that there are regions central to specific tropomyosin functions, and that the periods are not functionally quasiequivalent. We illustrate the importance of individual periods for function in Figure 6, where the function relative to wild-type is represented by saturation of a gray scale: white equals full saturation and full, wild-type function, while black equals absence of color and loss of detectable function.

All the functional assays we used depend on actin affinity. Through analysis of sequential periodic deletions we defined period 5 (residues 166−207), and especially the N-terminal half of the period (residues 166−186), to be the most important for binding to filamentous actin, with or without myosin S1. Replacement of residues 165−188, the region encoded by the constitutively expressed exon 5, with a leucine zipper sequence to maintain the wild-type molecular length similarly results in loss of function. Two mutations that cause hypertrophic cardiomyopathy are in this region [D175N, E180G (*51*)].

Period 5 is the most highly conserved period of tropomyosin, with 31% of the residues identical throughout the animal kingdom, compared to an average of 17% for the other six periods (15% for the internal periods 2−6). Residues 166−186 are even more highly conserved, 38%. In all periods the conserved residues are distributed throughout the seven positions of the heptapeptide repeat suggesting they are involved in universal tropomyosin functions, beyond maintenance of the coiled coil structure.

The consequence of deleting period 5 on actin affinity depends in a complicated fashion on the context. While deleting period 5 alone devastates actin affinity, larger deletions that include period 4 retain the ability to bind cooperatively to actin. Tropomyosins lacking periods 3−5 or periods 4−6 bind actin and inhibit the regulated actomyosin ATPase in the absence of Ca^{2+} (*22*, *23*). Context also affects the N- and C-terminal halves of period 5. A mutant lacking residues 166−186 together with the C-terminus of period 5 (dAc4/5) binds to regulated actin, though with lower affinity than that with deletion of period 4, and of all the mutants studied its thermal stability is closest to that of wild-type. A deletion mutation of the C-terminal part of period 5 that differs by only four residues, residues 191−211, has much higher actin affinity and a T_m closer to that wild-type (*42*) than the dAc5−C mutation reported here with deletion of residues 187−207. In the d191−211 mutant, residues 39−80 are encoded by exon 2a, a smooth muscle α-tropomyosin-encoded exon, versus exon 2b in all the other tropomyosins in this study. Furthermore, deletions and

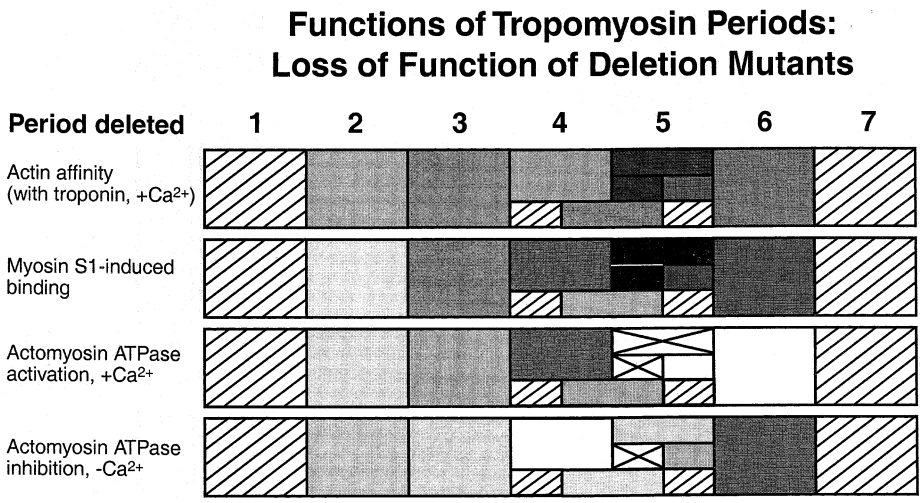

FIGURE 6: Summary of contributions of individual periodic repeats to tropomyosin function, based on the results with the deletion mutants. Each block represents an internal periodic repeat, 2−6. The functional importance of the ends (periods 1 and 7, crosshatched) is not included in this analysis. White represents saturation with color (full function), and black represents absence of color (no detectable function). The gray scale relates linearly to function except for actin affinity. Actin affinity: the log of the ratio of mutant to wild-type K_{app} was normalized to the wild-type; myosin S1-induced binding of tropomyosin to function. 1.0 = myosin S1:actin ratio for half-maximal saturation of actin with wild-type tropomyosin. ATPase, Ca^{2+}, from Figure 4A. Calcium sensitivity, from Figure 4C. Crosshatched regions: not included in the present analysis. X-ed regions: could not be studied because mutants do not bind to actin.

substitutions in this region are sensitive to the sequence of the C-terminal 27 amino acids (*42*), inferring long range influences.

While we are far from having a specific explanation for the apparent contradictions, we propose that the local sequence, structure and stability, and consequent flexibility, of the coiled coil together determine actin affinity. Just as the context influences actin affinity, it also affects thermal stability and the cooperative unfolding of extensive regions of the molecule. For example, deletion of residues 166−186 alone, or as part of period 5, destabilizes much of the molecule. When deleted with part of period 4 (in dAc4/5), the thermal stability is virtually unchanged from wild-type.

The structure of the residue 166−186 region may influence function. The region contains an alanine cluster (Ala/Ser at two *d* positions of the heptad, and an Ala at the intervening a position) with *a* sequence similar to residues 16−27 in the first period where in the crystal structure the α-helices pack closer and are bent relative to the coiled coil on either side (*52*). A pyrene probe at Cys190 has served as a conformational probe for period 5. In unfolding studies it is sensitive to the pretransition during which about 20% of the ellipticity at 222 nm is lost before cooperative unfolding and chain dissociation (*53*). Although the structure of the region and of the intermediate remain to be established, Lehrer's original work and subsequent studies have led to the appreciation that at physiological temperatures significant regions of tropomyosin are not fully coiled coil.

Observations of multiple unfolding transitions in tropomyosin detected using calorimetry (*54*, *55*), circular dichroism and the fluorescence of specific probes (*56*), and pressure denaturation (*57*) have led to the idea that there are multiple local unfolding domains where the stable coiled coil is interrupted by segments without stable secondary structure. The structures of such regions are unknown, and aside from the Cys190 region, the locations in the tropomyosin sequence are not established. It is attractive to speculate that the alanine clusters, noticed by Brown et al. (*52*) to occur periodically along tropomyosin and proposed to allow flexibility through bending, may be sites of local unfolding.

We suggest that, along with specific sequence requirements, the punctuation of tropomyosin's coiled coil with regions of instability or flexibility that form boundaries between stable coiled coil domains, are critical for actin binding. The less helical regions may be the binding sites themselves or may allow movement of tropomyosin on the filament surface to position itself for productive interaction. The results with the leucine zipper replacement mutants provide support for the idea that regions of local unfolding or stability are important for actin binding. Three of the mutants (TM−2zip, TM−3zip, and TM−5Czip) are in regions with typical coiled coil interface residues. While the highly stable leucine zipper sequence does increase the stability of part of the molecule (*20*, *35*), the overall effect is not great. These three mutants bind to actin with reasonable affinity. In contrast, the leucine zipper in TM−5Nzip replaces an alanine cluster resulting in a stretch of 10 heptads with stable interface residues, the most stable region of the molecule. The resulting tropomyosin is has a major transition >20 °C higher than that of wild-type and does not bind to actin. We suggest that the introduction of the leucine zipper has resulted in the loss of a region of local instability, and flexibility, that is required for tropomyosin to bind to actin. Previous work showed that increasing the stability of the C-terminus of tropomyosin impairs troponin T binding (*58*).

Just as too stable a coiled coil impairs actin binding, tropomyosins that have extensive unstable regions or that unfold noncooperatively have, in general, lower affinity. Replacement of a coiled coil sequence with a random coil sequence or creation of a skip by deleting a nonintegral heptad repeat (unpublished results) locally destabilizes the coiled coil (*35*) and reduces actin affinity (*20*).

Whereas actin affinity appears to depend on segmental differences in stability (flexibility), the regulatory functions of tropomyosin can be related to specific regions of tropomyosin. Deletion of periods 3, 4, or 5 individually,

replacement of sequence in periods 3 and 5 with leucine zipper sequence (this paper), or deletion of multiple internal periods (*21*−*23*) all impair production of the open, force-producing state of the actin filament, as measured by the actomyosin ATPase in the presence of Ca^{2+} and myosin S1-induced binding of tropomyosin to actin (Figure 6). A dominant lethal mutation in *Drosophila* tropomyosin has been mapped to a conserved residue toward the C-terminus of period 3 (*59*). Introduction of the mutation, Asp121Asn, into rat striated muscle α-tropomyosin had no effect on actin affinity with troponin, but did inhibit activation of the actomyosin ATPase in the presence of Ca^{2+} (Table 1, Figure 4B). Our results support the Tobacman proposal (*51*) that the midregion of tropomyosin is important for myosin to shift the thin filament to the open state.

Period 6 and possibly the C-terminal half of period 5 are required for full inhibition of the actomyosin ATPase with troponin in the absence of Ca^{2+}, even though they fully activate in the presence of Ca^{2+}. These regions of tropomyosin are part of the troponin binding site [reviewed in ref *5*]. Interestingly, dAc5 inhibits normally in the absence of Ca^{2+} where troponin promotes its binding to actin. Apparently the parameters important for actin binding and for myosin activation of the thin filament are not crucial for switching the actin filament into the blocked, inhibited state where tropomyosin occupies a different site on the filament (*50*).

ACKNOWLEDGMENT

We wish to thank Yongmi An and Yidong Liu for assistance in mutant construction, protein expression, purification, and preliminary analyses, and Dr. Bernardo Nadal-Ginard for the rat striated muscle α-tropomyosin cDNA clone.

REFERENCES

1. Hanson, E. J., and Lowy, J. (1963) *J. Mol. Biol. 6*, 48−60.
2. O'Brien, E. J., Bennett, P. M., and Hanson, J. (1971) *Philos. Trans. R. Soc. London, Ser. B 261*, 201−208.
3. Milligan, R. A., Whittaker, M., and Safer, D. (1990) *Nature 348*, 217−221.
4. Whitby, F. G., and Phillips, G. N., Jr. (2000) *Proteins 38*, 49−59.
5. Perry, S. V. (2001) *J. Muscle Res. Cell Motil. 22*, 5−49.
6. Wegner, A. (1982) *J. Mol. Biol. 161*, 217−227.
7. Broschat, K. O., Weber, A., and Burgess, D. R. (1989) *Biochemistry 28*, 8501−8506.
8. Broschat, K. O. (1990) *J. Biol. Chem. 265*, 21323−21329.
9. Weber, A., Pennise, C. R., Babcock, G. G., and Fowler, V. M. (1994) *J. Cell Biol. 127*, 1627−1635.
10. Weber, A., Pennise, C. R., and Fowler, V. M. (1999) *J. Biol. Chem. 274*, 34637−34645.
11. Hitchcock, S. E., Carisson, L., and Lindberg, U. (1976) *Cell 7*, 531−542.
12. Bernstein, B. W., and Bamburg, J. R. (1982) *Cell Motil. 2*, 1−8.
13. Blanchoin, L., Pollard, T. D., and Hitchcock-DeGregori, S. E. (2001) *Curr. Biol. 11*, 1300−1304.
14. Parry, D. A. (1975) *J. Mol. Biol. 98*, 519−535.
15. McLachlan, A. D., and Stewart, M. (1976) *J. Mol. Biol. 103*, 271−298.
16. Phillips, G. N., Jr. (1986) *J. Mol. Biol. 192*, 128−131.
17. Pittenger, M. F., Kazzaz, J. A., and Helfman, D. M. (1994) *Curr. Opin. Cell Biol. 6*, 96−104.
18. Lin, J. J., Warren, K. S., Wamboldt, D. D., Wang, T., and Lin, J. L. (1997) *Int. Rev. Cytol. 170*, 1−38.
19. Hitchcock-DeGregori, S. E., and Varnell, T. A. (1990) *J. Mol. Biol. 214*, 885−896.
20. Hitchcock-DeGregori, S. E., and An, Y. (1996) *J. Biol. Chem. 271*, 3600−363.
21. Landis, C. A., Bobkova, A., Homsher, E., and Tobacman, L. S. (1997) *J. Biol. Chem. 272*, 14051−14056.
22. Landis, C., Back, N., Homsher, E., and Tobacman, L. S. (1999) *J. Biol. Chem. 274*, 31279−85.
23. Hitchcock-DeGregori, S. E., Song, Y., and Moraczewska, J. (2001) *Biochemistry 40*, 2104−2412.
24. Tobacman, L. S. (1996) *Annu. Rev. Physiol. 58*, 447−481.
25. Ruiz-Opazo, N., and Nadal-Ginard, B. (1987) *J. Biol. Chem. 262*, 4755−65.
26. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
27. Hitchcock-DeGregori, S. E., and Heald, R. W. (1987) *J. Biol. Chem. 262*, 9730−9735.
28. Hammell, R. L., and Hitchcock-DeGregori, S. E. (1996) *J. Biol. Chem. 271*, 4236−4242.
29. Hitchcock-DeGregori, S. E., Mandala, S., and Sachs, G. A. (1982) *J. Biol. Chem. 257*, 12573−12580.
30. Margossian, S. S., and Lowey, S. (1982) *Methods Enzymol. 85*, 55−71.
31. Potter, J. D. (1982) *Methods Enzymol. 85*, 2412−63.
32. Moraczewska, J., Nicholson-Flynn, K., and Hitchcock-DeGregori, S. E. (1999) *Biochemistry 38*, 15885−15892.
33. Edelhoch, H. (1967) *Biochemistry 6*, 1948−1954.
34. Lehrer, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3377−3381.
35. Greenfield, N. J., and Hitchcock-DeGregori, S. E. (1995) *Biochemistry 34*, 16797−16805.
36. Heald, R. W., and Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem. 263*, 5254−5259.
37. Laemmli, U. K. (1970) *Nature 227*, 680−685.
38. Cho, Y. J., and Hitchcock-DeGregori, S. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10153−10157.
39. White, H. D. (1982) *Methods Enzymol. 85*, 698−708.
40. Crick, F. H. C. (1953) *Acta Crystallogr. 6*, 689−697.
41. McLachlan, A. D., Stewart, M., and Smillie, L. B. (1975) *J. Mol. Biol. 98*, 281−291.
42. Hammell, R. L., and Hitchcock-DeGregori, S. E. (1997) *J. Biol. Chem. 272*, 22409−22416.
43. Urbancikova, M., and Hitchcock-DeGregori, S. E. (1994) *J. Biol. Chem. 269*, 24310−24315.
44. Hitchcock-DeGregori, S. E. (1994) *Adv. Exp. Med. Biol. 358*, 85−96.
45. Hitchcock-DeGregori, S. E., and Liu, Y. (1998) *Biophys. J. 74*, A47.
46. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) *Science 240*, 1759−1764.
47. McKillop, D. F., and Geeves, M. A. (1993) *Biophys. J. 65*, 693−701.
48. Geeves, M. A., and Lehrer, S. S. (1994) *Biophys. J. 67*, 273−282.
49. Lehrer, S. S., and Geeves, M. A. (1998) *J. Mol. Biol. 277*, 1081−1089.
50. Xu, C., Craig, R., Tobacman, L., Horowitz, R., and Lehman, W. (1999) *Biophys. J. 77*, 985−992.
51. Tobacman, L. S., and Butters, C. A. (2000) *J. Biol. Chem. 275*, 27587−27593.
52. Brown, J. H., Kim, K. H., Jun, G., Greenfield, N. J., Dominguez, R., Volkmann, N., Hitchcock-DeGregori, S. E., and Cohen, C. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 8496−8501.
53. Lehrer, S. S. (1978) *J. Mol. Biol. 118*, 209−226.
54. Potekhin, S. A., and Privalov, P. L. (1982) *J. Mol. Biol. 159*, 519−535.
55. Sturtevant, J. M., Holtzer, M. E., and Holtzer, A. (1991) *Biopolymers 31*, 489−495.
56. Ishii, Y., Hitchcock-DeGregori, S., Mabuchi, K., and Lehrer, S. S. (1992) *Protein Sci. 1*, 1319−1325.
57. Suarez, M. C., Lehrer, S. S., and Silva, J. L. (2001) *Biochemistry 40*, 1300−1307.
58. Greenfield, N. J., Palm, T., and Hitchcock-DeGregori, S. E. (2002) *Biophys. J. 83*, 2754−2766.
59. Kreuz, A. J., Simcox, A., and Maughan, D. (1996) *J. Cell Biol. 135*, 673−87.
60. Golitsina, N., An, Y., Greenfield, N. J., Thierfelder, L., Iizuka, K., Seidman, J. G., Seidman, C. E., Lehrer, S. S., and Hitchcock-DeGregori, S. E. (1997) *Biochemistry 36*, 4637−42.
61. Golitsina, N., An, Y., Greenfield, N. J., Thierfelder, L., Iizuka, K., Seidman, J. G., Seidman, C. E., Lehrer, S. S., and Hitchcock-DeGregori, S. E. (1999) *Biochemistry 38*, 3850.

BI026519K