Effects of Tropomyosin Internal Deletions on Thin Filament Function*

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Striated muscle tropomyosin spans seven actin monomers and contains seven quasi-repeating regions with loose sequence similarity. Each region contains a hypothesized actin binding motif. To examine the functions of these regions, full-length tropomyosin was compared with tropomyosin internal deletion mutants spanning either five or four actins. Actin-troponin-tropomyosin filaments lacking tropomyosin regions 2–3 exhibited calcium-sensitive regulation in in vitro motility and myosin S1 ATP hydrolysis experiments, similar to filaments with full-length tropomyosin. In contrast, filaments lacking tropomyosin regions 3–4 were inhibitory to these myosin functions. Deletion of regions 2–4, 3–5, or 4–6 had little effect on tropomyosin binding to actin in the presence of troponin or troponin-Ca2+, or in the absence of troponin. However, all of these mutants inhibited myosin cycling. Deletion of the quasi-repeating regions diminished the prominent effect of myosin S1 on tropomyosin-actin binding. Interruption of this cooperative, myosin-tropomyosin interaction was least severe for the mutant lacking regions 2–3 and therefore correlated with inhibition of myosin cycling. Regions 3, 4, and 5 each contributed about 1.5 kcal/mol to this process, whereas regions 2 and 6 contributed much less. We suggest that a myosin-induced conformational change in actin facilitates the azimuthal repositioning of tropomyosin which is an essential part of regulation.

Examination of tropomyosin mutants has been helpful in understanding the regulatory function of the thin filament. In particular, rat a-Tm(Δ47–165) is a recombinant tropomyosin that is missing 119 internal residues, which spans four instead of the usual seven actins, and which inhibits both in vitro motility and solution myosin S1 MgATPase activity (8). This tropomyosin (here redesignated Δ234Tm because it lacks the second, third, and fourth of tropomyosin’s seven quasi-repeats) bound much more weakly to myosin S1-decorated actin than did control tropomyosin. Therefore, it was suggested that its inhibitory properties were caused by destabilization of the myosin-induced conformation of the thin filament, the conformation corresponding to full azimuthal movement of tropomyosin. This conclusion has since been supported by three-dimensional reconstructions of negatively stained thin filament electron micrographs, which demonstrate that the tropomyosin deletion does not alter the large, calcium-induced azimuthal movement of tropomyosin.2 The results suggested that the calcium-induced movement of tropomyosin is insufficient to permit cross-bridge function. Rather, full tropomyosin movement is required.

These previous data suggest that the deleted portion of tropomyosin is somehow important for thin filament activation, but they do not identify the specific structural changes in Δ234Tm which are responsible for its altered function. The mutation’s dual effects of impaired tropomyosin binding to actin-S1 and inhibition of myosin cycling could be coincidental properties of one mutant or could suggest more general features that are necessary for proper thin filament regulation. Also unclear is whether the most significant feature of Δ234Tm is its shortened length, or whether its behavior is caused by deletion of a specific region. To explore these issues, we now report the properties of a series of tropomyosin internal deletion mutants. The results indicate that it is not the length of tropomyosin that is critical for proper regulatory function, but rather it is the specific regions of tropomyosin which are present or missing. Also, a broad internal region of tropomyosin is important for tropomyosin binding to myosin S1-decorated actin, and the strength of this binding process correlates with retention of physiological tropomin-tropomyosin-mediated regulation.

MATERIALS AND METHODS

Preparation of Tropomyosins Containing Internal Deletions—Rat striated/cardiac muscle α-tropomyosin cDNA (19) was altered using the ExSite mutagenesis kit (Strategene). This approach uses polymerase

1 The abbreviations used are: myosin S1, myosin subfragment 1; Δ23Tm, rat Ala-Ser α-tropomyosinΔ(47–123); Δ234Tm, rat Ala-Ser α-tropomyosinΔ(47–165); Δ34Tm, rat Ala-Ser α-tropomyosinΔ(80–165); Δ345Tm, rat Ala-Ser α-tropomyosinΔ(89–207); Δ456Tm, rat Ala-Ser α-tropomyosinΔ(124–242); ΔASTm, rat Ala-Ser α-tropomyosin. Δ23Tm, Gln3 through Ser167; Δ34Tm, Asn27 through Val165; Δ456Tm, Asn27 through Leu165; Δ567Tm, Glu24 through Ala242; TnT, tropinin T; MOPS, 4-morpholinepropanesulfonic acid.

2 M. Rosol, W. Lehman, R. Craig, C. Landis, and L. S. Tobacman, submitted for publication.

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chain reaction and contiguous oppositely directed primers to generate circular plasmids missing a region that is a gap in one of the primers. The starting DNA encoded Met-Ala-Ser-tropomyosin in pET3d (8). All mutant coding sequences were confirmed by automated sequencing at the University of Iowa DNA Core Facility. The mutant constructs included deletions of either 231 base pairs (Δ23Tm and Δ34Tm) or 357 base pairs (Δ345Tm and Δ456Tm). Numbering according to the rat sequence, the deletions were: Gln47 through Ser123 (Δ23Tm), Asn39 through Val165 (Δ34Tm), Asn89 through Leu207 (Δ345Tm), and Glu234 through Ala447 (Δ456Tm). Using this same designation pattern, previously described Δ234Tm lacks residues Gin47 through Val165 (38). The previous notation Δ23Tm (Δ49–167) took the beginning of the fusion dipeptide Ala-Ser as residue 1.

**Protein Purification**—Recombinant tropomyosins were purified according to Willadsen et al. (20). Rabbit fast skeletal muscle actin (21) and myosin subfragment 1 (22) and bovine cardiac troponin (23) were obtained using published procedures. Prior to motility assays, rabbit skeletal muscle heavy meromyosin was mixed with actin in the presence of ATP, and inappropriately strong binding molecules were removed by centrifugation (24). Cardiac troponin subunits were purified as reported by Tobacman and Lee (25), and the TnT subunit was stoichiometrically labeled on Cys39 with [3H]iodoacetic acid (26). The labeled TnT was then mixed in a 1:1:1 ratio with the other troponin subunits under denaturing, high ionic strength conditions, and the tropomyosin complex was purified as described (25). Tropomyosin was labeled on Cys190 under denaturing conditions with [3H]iodoacetic acid (27).

**Assays**—The binding of regulatory proteins to actin was measured by cosedimentation in a TLA100 rotor (Beckman) for 30 min at 35,000 rpm after a 30-min incubation at 25 °C. Conditions were: 10 mM Na2HPO4 (pH 7.0), 5 mM MgCl₂, 1 mM EGTA, 4.5 or 5 μM actin, either 0 or 5 mM myosin S1, and between 60 and 300 mM KCl (see figure legends). Total and supernatant radioactivity were compared to calculate the bound protein. When troponin as well as tropomyosin was present, either tropomyosin was added in a 0.1 μM excess over troponin when the label was on the troponin, or troponin was added in the same excess when the label was on the tropomyosin. Binding data were fit initially with MATLAB (MathWorks), using an equation for the binding of a long ligand to a linear lattice (27–29). Different data sets were then normalized based upon the MATLAB results, similar experiments combined, and the program Scientist (MicroMath) used for refinement and for calculation of errors. Competitive binding experiments were done under the same conditions as above, except an identical saturating concentration (see legends) of [3H]Δ234Tm was included in all samples. The competition was analyzed as reported previously for competitive binding of troponin to the thin filament (30). Curvefitting with Scientist was used to determine the ratio of the actin or actin-S1 affinities of the competing unlabeled tropomyosin versus the labeled tropomyosin.

*In vitro* sliding of filaments over a heavy meromyosin-coated coverslip was examined by epifluorescence microscopy and digital analysis of movement (31). Rhodamine phalloidin-labeled actin was bound to the motility surface in the absence of ATP, and then 100 nM tropomin was added, along with 100–150 mM of one of the various forms of tropomyosin. When ATP was added movement was assessed both in the presence (pCa 5) and in the absence (pCa 9) of calcium and at ionic strengths of 50 and 100 mM.

MgATPase assays were performed as described previously (32), under conditions of low myosin S1:actin ratio and saturation, and linearly with myosin S1 concentration: 10 mM Na2HPO4 (pH 7.0), 5 mM MgCl₂, 1 mM γ-[32P]ATP, 4 μM actin, 1 μM myosin S1, and either 0.5 mM EGTA or 0.1 mM CaCl₂. Tropomin and tropomyosin concentrations are indicated in the figure legends.

**RESULTS**

**Design of a Series of Tropomyosin Internal Deletion Mutants**—Fig. 1 schematically shows the α-striated muscle tropomyosins examined in the present report. The tropomyosin amino acid sequence has seven quasi-repeating regions with periodicity corresponding to the spacing between adjacent actin residues on the filament (33). The first residues of the regions are indicated for the present study as indicated beneath the full-length molecule in Fig. 1. Paramount in these definitions was the constraint that the number of residues deleted should be a multiple of seven, to preserve the heptad repeat of the coiled-coil. Also, the boundaries used in previous studies (8, 34, 35) of regions 2, 3, and 4 were retained. Mutant tropomyosins either spanned five acts as a result of the deletion of 77 residues (two regions), or spanned four acts as a result of the deletion of 119 residues (three regions). ASTm and Δ234Tm are the same molecules studied previously (8). All forms include an Ala-Ser NH₂-terminal dipeptide that corrects the poor polymerizability of unacetylated tropomyosin expressed in bacteria (36).

Tropomin binds to tropomyosin most tightly by interactions between the NH₂-terminal portion of TnT and the COOH terminus of tropomyosin (region 7) (30, 37–42). There is a weak site of tropomin binding near tropomyosin residues 150–190 in repeating regions 4 and 5 (38, 40, 43–46), and the two proteins may be in contact for an extended region involving the COOH-terminal third of tropomyosin (47). Therefore, constructs lacking regions 4, 5, and/or 6 may have altered interactions with tropomin. However, the strongest site of interaction (region 7) is retained in all mutants, as are sequences involved in normal end-to-end tropomyosin-tropomyosin contacts.

**Effect of Internal Tropomyosin Mutations on Calcium-mediated Regulation**—The effects of the mutations on the unloaded sliding of heavy meromyosin-propelled actin-tropomin-tropomyosin thin filaments were examined in an *in vitro* motility assay (31). In the presence of calcium 90% of the thin filaments containing full-length cardiac tropomyosin moved at a fast uniform sliding speed (right half of Table I). Removal of calcium inhibited the sliding of 97% of these control filaments, and those that continued to move smoothly did so at <7% of the speed observed in the presence of calcium. Filaments containing bovine cardiac tropomyosin or recombinant full-length ASTm behaved indistinguishably (8). Δ23Tm also caused calcium-sensitive movement, although the regulation was not as complete: 67% of the filaments moved continuously when calcium was added, at 73% of the speed of the control filaments. Δ234Tm is the same length as Δ23Tm but has very different effects: few filaments moved in the presence or in the absence of calcium, and those that moved continuously did so with <7% of the speed of the control filaments. Δ345Tm and Δ456Tm filaments were also poorly regulated, with movement greatly depressed in the presence of calcium as well as in its absence. Comparable results were obtained when the ionic strength was
increased from 100 mM to 50 mM (data not shown). All of the tropo- nin-tropomyosin complexes suppressed movement in the absence of calcium, and calcium relieved the inhibition only for control filaments and for Δ23Tm filaments.

The effects of the mutations were also examined in myosin S1 MgATPase assays, and the results (Fig. 2) paralleled the motility data. Δ23Tm filaments (∆, EGTA; *, Ca2+) displayed calcium-sensitive ATPase rate regulation, similar to data reported for ASTm (8). In contrast, actin-troponin-tropomyosin filaments containing Δ234Tm, Δ345Tm, and Δ456Tm, and Δ34Tm were all inhibitory to the ATPase rate, both in the presence (filled symbols) and in the absence (open symbols) of calcium (Fig. 2). Filaments containing Δ456Tm (triangles) demonstrated slightly less inhibition than the others, similar to the motility data (Table I).

Effect of Tropomyosin Mutations on Binding to Myosin-free Actin and to Myosin S1-decorated Actin—To determine whether the inhibitory properties of the tropomyosins were related to destabilization of the myosin-activated conformation of the thin filament, their affinities for actin were determined in the absence and in the presence of myosin S1. Deletion of quasi-repeating regions 2, 3, and 4 has little effect on the affinity of tropomyosin for myosin-free actin (8), suggesting that these regions have little interaction with actin under such conditions. This issue was explored with the new mutants, with representative results shown in Fig. 3A and a data summary presented in the right side of Table II. Both Δ345Tm and Δ456Tm were able to displace Δ234Tm from actin (Fig. 3A), indicating that the affinities of all three tropomyosins were similar. More precisely, the actin affinity of Δ456Tm (crosses) relative to Δ234 was 0.75 ± 0.08, and the corresponding affinity ratio for Δ345Tm (squares) was 0.43 ± 0.07. These data were obtained by competitive displacement assay (30) because Δ345Tm and Δ456Tm lack the Cys190 labeling site. Control experiments using unlabeled Δ234Tm showed it to have a relative affinity compared with labeled Δ234Tm of 0.9 ± 0.2 (data not shown). In other experiments, Δ23Tm and Δ34Tm were labeled on Cys190, and the binding of each to actin was measured directly. Their actin affinities, 1.53 ± 0.03 and 2.02 ± 0.02 × 10^6 M^-1, were each similar that of full-length ASTm, 1.6 × 10^6 M^-1. Table II shows that, in the absence of myosin and of tropo- nin, full-length tropomyosin binds to actin with at most a 4-fold higher affinity than any of the deletion mutants. This strongly suggests that regions 2, 3, 4, 5, and 6 contribute little to actin binding under these conditions. This is consistent with other evidence that it is the NH2 and COOH termini of tropomyosin which are particularly important for this process (20, 39, 48–51).

In contrast to the above results, the deletions had large and sequence-specific effects on tropomyosin binding to myosin S1-decorated actin. The affinities of Δ345Tm and Δ456Tm for actin-myosin S1 were examined by competition with labeled Δ234Tm (Fig. 3B). Because Δ234Tm binds to actin-myosin S1 at least 2 orders of magnitude more weakly than does full-length ASTm (8), these experiments required ionic conditions (60 mM KCl) that were favorable for binding. As shown in Fig. 3B, Δ456Tm (crosses) displaced Δ234Tm from actin-myosin S1, with a relative affinity of 0.83 ± 0.06. Both tropomyosins bind poorly, but approximately equally. In contrast, Δ345Tm (squares) was not even able to displace the weakly binding Δ234Tm, with an affinity of 0.014 ± 0.04 relative to the binding affinity of Δ234Tm. Poor binding of Δ345Tm to actin-myosin S1 was also confirmed by SDS-polyacrylamide gel electrophoresis (data not shown).

Full-length ASTm binds to actin-myosin S1 with an affinity too tight to measure reliably by sedimentation with actin; its affinity is at least 10^5 M^-1 even in the presence of 300 mM KCl (8). Under these same high ionic strength conditions both Δ23Tm (×, Fig. 3C) and Δ34Tm (∆, Fig. 3D) bound much more weakly, particularly Δ34Tm, with K_app < 8 × 10^8 M^-1. Decreasing the KCl concentration to 150 mM enhanced the binding of both of these tropomyosins to actin-myosin S1 (+, Fig. 3C and D). Comparisons between the two panels show that Δ34Tm, which is inhibitory, bound to actin-myosin S1 an order of magnitude more weakly than did Δ23Tm, regardless of the KCl concentration.

Effect of the Deletions on Tropolin-Tropomyosin Binding to Actin—To understand the inhibitory properties of the mutants, it was important to determine the mutations’ effects on tropo- nin-tropomyosin binding to actin. Two of the mutant tropomyosins lack Cys190, so a novel method for monitoring binding of tropolin-tropomyosin to F-actin was applied, using radiolabeled tropolin instead of radiolabeled tropomyosin. In support of the method’s validity, the stoichiometry of binding depended...
FIG. 3. Large and specific effects of internal deletions on tropomyosin binding to actin-myosin S1 and small effects on binding to actin. Panel A, in the absence of myosin, binding of Δ345Tm (squares and dashed line) or Δ456Tm (crosses and solid line) to actin was measured by competitive displacement of 3H-labeled Δ234Tm. Increasing concentrations of unlabeled competing tropomyosin were added to otherwise identical samples, with the results fit to derive the affinities of competing tropomyosins relative to that of Δ234Tm: 0.75 ± 0.08 for Δ456Tm and 0.43 ± 0.07 for Δ345Tm. The affinity of Δ234Tm for actin under these conditions (60 mM KCl) is 9 × 10⁶ M⁻¹ (not shown), and full-length tropomyosin binds similarly with an affinity of 1.8 × 10⁶ M⁻¹. None of these deletions makes a critical difference in the binding of tropomyosin to actin. Panel B, same as panel A except for the addition of saturating concentrations of myosin S1. The affinity of Δ345Tm for actin-myosin S1 relative to that of Δ234Tm was 0.014 ± 0.004. For Δ456Tm the relative affinity was 0.83 ± 0.06. Under the conditions of the experiment (60 mM KCl), Δ234Tm binds to actin-myosin S1 with an affinity of 5.3 × 10⁶ M⁻¹ (8), and full-length tropomyosin has an affinity that is too tight to measure even if the KCl concentration is increased to 300 mM. Panel C, binding of radiolabeled Δ23Tm to actin-myosin S1 was measured directly in the presence of either 150 mM KCl (+; Kapp = 8 × 10⁶ M⁻¹) or 300 mM KCl (×; Kapp = 9.7 × 10⁶ M⁻¹) and y = 15 ± 2). Panel D, for each of these KCl concentrations weaker binding to actin-myosin S1 was observed for Δ34Tm, 150 mM KCl (+); Kapp = 1.65 ± 0.04 × 10⁶ M⁻¹ and y = 12 ± 1, 300 mM KCl (×); Kapp < 8 × 10⁴ M⁻¹. Competition assay samples contained 5 μM actin, 3.3 μM 3H-labeled Δ234Tm, 60 mM KCl, 10 mM NaH₂PO₄ (pH 7.0), 5 mM MgCl₂, and 1 mM EGTA. The data were fit to Equation 1 in Ref. 30, producing the calculated curves shown in panels A and B.

Fig. 4, A and B, presents normalized troponin-tropomyosin binding isotherms for the new deletion mutants, and Table II summarizes these results and those for control ASTm and Δ234Tm. For troponin-Δ23Tm, troponin-Δ34Tm, troponin-Δ345Tm, and troponin-Δ456Tm, binding to actin occurred cooperatively, with similar affinity, and was weakened modestly by calcium. Affinities in the absence of calcium ranged from a low value of 4.0 × 10⁶ M⁻¹ to a high value 8.3 × 10⁶ M⁻¹ for Δ345Tm and Δ23Tm, respectively. The ratios of the Kapp values in the absence versus the presence of calcium were: 1.6, 1.84, 1.23, 1.23, 1.44, 1.56 for ASTm, Δ234Tm, Δ345Tm, Δ456Tm, Δ23Tm, and Δ34Tm, respectively. Although Fig. 4 and Table II show modest differences among the various tropomyosins, the most prominent feature is that none of the deletions had a major effect on troponin-tropomyosin binding to actin, regardless of whether or not Ca²⁺ was present.

DISCUSSION

Fig. 5 summarizes the very unfavorable effects of the deletions on the free energy (ΔΔG) of tropomyosin binding to actin-myosin S1 (dashes), and, by contrast, the small effects on binding in the absence of myosin (circles, squares, diamonds). All of the mutants had impaired binding to actin-myosin S1 (positive ΔΔG), but the smallest defect was for Δ23Tm, which is the only form that permitted calcium-sensitive activation of myosin cycling. This suggests that the ability of tropomyosin to bind tightly to actin-myosin is a requirement for activation. However, it is also true that Δ23Tm is the only mutant that still retains repeat region 4. Therefore, one possibility is that a specific interaction between troponin and repeat region 4 could be the crucial factor distinguishing this tropomyosin from the other, inhibitory forms.

upon the length of the tropomyosin. Grouping the tropomyosins by length and averaging, saturating amounts of bound tropo-
nin-tropomyosin in the presence of 4.5 μM actin were 0.64 ±
0.04 μM for full-length ASTm, 0.93 ± 0.01 μM for tropomyosins
spanning five actins, and 1.01 ± 0.02 μM for tropomyosins
spanning four actins (data not shown).

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Control and mutant tropomyosin binding to actin was determined in the presence and absence of troponin. Tropomyosin-troponin data are from Fig. 4 for δT3Tm, δT4Tm, δT35Tm, and δT45Tm. Corresponding data for δT3Tm and δT4Tm were obtained similarly, using labeled troponin. Data in the absence of troponin were from Fig. 3A, experiments described in the text, and Ref. 8. Conditions in the presence of troponin were 10 mM NaH2PO4 (pH 7.0), 5 mM MgCl2, 150 mM KCl, 4.5 μM actin, and either 0.5 mM EGTA or 0.1 mM CaCl2. To strengthen tropomyosin-actin binding, the KCl concentration was decreased to 60 mM for the experiments performed in the absence of troponin. The apparent affinity constants (Kapp) include contributions (y) from cooperative tropomyosin-troponin interactions, as well as from actin-binding per se (27, 28). Tn-Tm refers to the tropomyosin-tropinin complex. ND means not determined.

### TABLE II

| Tropomyosin-troponin (EGTA) | Tropomyosin-troponin-Ca2+ | Tropomyosin only |
|-----------------------------|---------------------------|-----------------|
| Kapp (μM)                  | Kapp (μM)                 | Kapp (μM)       |
| y                           | y                          | y               |
Thus, all of the tropomyosin binding curves are cooperative, so this is not likely to be a major factor. It is unclear why there are some differences in the degree of cooperativity, even for tropomyosins of the same length (Table II and Ref. 35).

The present study suggests the importance of a largely unnoticed feature of thin filament behavior: the extremely tight binding of tropomyosin to myosin-decorated actin (10, 53, 54). Tropomyosin in the Ca2+ state sterically blocks part of the myosin binding site on actin (5). Additional, myosin-induced movement of tropomyosin beyond this position cannot easily be explained by steric blocking because myosin S1 produces much tighter (rather than weaker) association of tropomyosin with the thin filament. This greater Kapp could be caused by direct myosin binding to tropomyosin. However, because myosin binding causes spectroscopically detectable changes in F-actin (55–57), it is more likely that myosin allosterically enhances tropomyosin binding via a conformational change in actin. The major regulatory significance of such a conformational change is apparent from the magnitude of the relevant myosin-tropomyosin cooperative interaction. The measured effect of myosin on tropomyosin binding to actin is >100-fold (10), and deletional analysis implies differences as large as 7,000-fold (δT35Tm versus δT3Tm). These large values are consistent with estimates of the same process that can be made from published data on the energetically linked process of myosin S1 binding to actin. There is a 4–7-fold increase in binding of myosin S1 to actin-tropomyosin or actin-tropomyosin-Ca2+ when either is compared with binding to actin alone (12, 58, 59). Equilibrium linkage calculation using the most conservative of these data (58) translates to an 8,000-fold effect of myosin S1 on the binding of the regulatory proteins to actin (δT35Tm versus δT3Tm). We suggest that myosin binding causes a conformational change in actin, producing a distinct, highly favorable site for tropomyosin binding. The deletion mutants weaken this movement and conformational change, perhaps via binding of fewer tropomyosin α-sites to actin-myosin, particularly sites 3, 4, and 5 that are predominantly responsible for the effect. In this proposal tropomyosin has both steric and allosteric roles. It sterically blocks myosin binding (to a greater or lesser extent depending upon Ca2+ binding to tropinin) but allosterically promotes an actin conformational change that occurs when myosin binds to actin. Myosin binding promotes tropomyosin movement, and repositioned tropomyosin enhances myosin binding by promoting the actin conformational change.

Although the above mechanism is sufficient to explain the energetics of the azimuthal movement of tropomyosin in the presence of myosin, the mechanism lacks kinetic detail. One

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|-----------------------------|---------------------------|-----------------|
| Kapp (μM)                  | Kapp (μM)                 | Kapp (μM)       |
| y                           | y                          | y               |

Fig. 5 allows estimation of the relative importance of the repeating regions of tropomyosin on its affinity for myosin S1-decorated actin. There is a notable staircase pattern to the data. The free energy scales with how many of regions 3, 4, and 5 are missing; deletion of one, two, or three of these regions produces serially greater loss of binding. Region 4 appears more significant than region 2 because δT4Tm is more defective than is δT3Tm. Region 2 appears to contribute little, because δT3Tm and δT4Tm behave similarly. Region 5 contributes significantly, because δT5 is much more defective than is δT4 and because δT5 is the most impaired of all of the mutants. Region 6 contributes much less than region 3 (δT6 versus δT5) and apparently no more than does region 2. Regardless of whether some of the details of this additive analysis are faulty because of unknown aspects of the protein structures, the overall pattern is that a broad internal portion of tropomyosin contributes to the molecule’s tight binding to myosin S1-decorated actin. This is expected if the repeating tropomyosin α-sites are actin-binding motifs that participate in the myosin-induced, active state of the thin filament. In contrast, our data provide evidence against the thesis that these sites are actin binding motifs in the absence of myosin. Under these conditions the deletions produce much smaller effects on the binding free energy.

Hitchcock-DeGregori and colleagues found that deletion of regions 2 or 3 does not interrupt regulation and also that these regions contribute modestly if at all to tropinin-tropomyosin binding to actin (34, 35). They further showed that exon 6 (residues 189–213 in repeats 5–6) has little effect on TnT binding to tropomyosin or on tropinin-tropomyosin binding to actin. Deletion of half-regions does not affect regulation (34, 52), despite in one case (deletion of residues 191–211; see Ref. 52) removal of part of the α-site of tropomyosin repeat 6 (defined as residues 205–224 in Ref. 33). In unpublished work, an unacyetylated tropomyosin similar to δT3Tm was inhibitory in the presence and in the absence of Ca2+, and one similar to δT3Tm allowed partial Ca2+-sensitive activation. None of these results conflicts with the current data. However, when internal deletions are made in unacyetylated tropomyosin there may be larger effects on tropinin-tropomyosin binding to actin than found in the present work.

Comparisons between tropomyosins of different lengths could reflect differences in the strength of end-to-end tropomyosin-tropomyosin contacts. This is because the lengths of the deletions (77 or 119 residues) give primacy to preservation of the heptad repeat and secondary importance to the number of coiled-coil residues (39 1/3) required to span one actin in the filament (33). However, all of the tropomyosin binding curves are cooperative, so this is not likely to be a major factor. It is unclear why there are some differences in the degree of cooperativity, even for tropomyosins of the same length (Table II and Ref. 35).

The present study suggests the importance of a largely unnoticed feature of thin filament behavior: the extremely tight binding of tropomyosin to myosin-decorated actin (10, 53, 54). Tropomyosin in the Ca2+ state sterically blocks part of the myosin binding site on actin (5). Additional, myosin-induced movement of tropomyosin beyond this position cannot easily be explained by steric blocking because myosin S1 produces much tighter (rather than weaker) association of tropomyosin with the thin filament. This greater Kapp could be caused by direct myosin binding to tropomyosin. However, because myosin binding causes spectroscopically detectable changes in F-actin (55–57), it is more likely that myosin allosterically enhances tropomyosin binding via a conformational change in actin. The major regulatory significance of such a conformational change is apparent from the magnitude of the relevant myosin-tropomyosin cooperative interaction. The measured effect of myosin on tropomyosin binding to actin is >100-fold (10), and deletional analysis implies differences as large as 7,000-fold (δT35Tm versus δT3Tm). These large values are consistent with estimates of the same process that can be made from published data on the energetically linked process of myosin S1 binding to actin. There is a 4–7-fold increase in binding of myosin S1 to actin-tropomyosin or actin-tropomyosin-Ca2+ when either is compared with binding to actin alone (12, 58, 59). Equilibrium linkage calculation using the most conservative of these data (58) translates to an 8,000-fold effect of myosin S1 on the binding of the regulatory proteins to actin (δT35Tm versus δT3Tm). We suggest that myosin binding causes a conformational change in actin, producing a distinct, highly favorable site for tropomyosin binding. The deletion mutants weaken this movement and conformational change, perhaps via binding of fewer tropomyosin α-sites to actin-myosin, particularly sites 3, 4, and 5 that are predominantly responsible for the effect. In this proposal tropomyosin has both steric and allosteric roles. It sterically blocks myosin binding (to a greater or lesser extent depending upon Ca2+ binding to tropinin) but allosterically promotes an actin conformational change that occurs when myosin binds to actin. Myosin binding promotes tropomyosin movement, and repositioned tropomyosin enhances myosin binding by promoting the actin conformational change.

Although the above mechanism is sufficient to explain the energetics of the azimuthal movement of tropomyosin in the presence of myosin, the mechanism lacks kinetic detail. One

3 S. Hitchcock-DeGregori, personal communication.
possibility is that when tropomyosin-tropomyosin is in the calcium position (but not when in the EGTA position), enough of the myosin binding site on actin is exposed so that myosin binds and initiates a change in the actin, which in turn produces the additional regulatory protein movement. The structural features of this initial myosin binding cannot be discerned from current atomic models of myosin and of the thin filament. The other possibility is that the final azimuthal change in tropomyosin position must occur first, that only subsequent to this movement does the myosin bind stereospecifically to actin, and that this myosin binding causes actin conformation to change sufficiently so that tropomyosin then interacts strongly in this final position.

The inhibitory tropomyosins of the present report have greatly decreased affinities for actin specifically under one condition, when myosin is bound. This common property suggests that the normal, tight tropomyosin binding to the fully activated (so-called open) state of the thin filament is required for myosin cycling. In this sense the proposed conformational change in actin appears to be an essential part of regulation; it critically facilitates the final tropomyosin-tropomyosin movement away from the myosin binding site on actin, beyond the calcium position. To understand these phenomena further, it may prove useful to explore the effects of the tropomyosin deletions on muscle cross-bridge function and on myosin-actin binding equilibria and kinetics.

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