The Troponin Tail Domain Promotes a Conformational State of the Thin Filament That Suppresses Myosin Activity*

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Larry S. Tobacman‡‡, Mahta Nihli‡, Carol Butters‡, Mark Heller‡, Victoria Hatch‡, Roger Craig‡§, William Lehman†, and Earl Homsher†

From the Departments of Internal Medicine and Biochemistry, The University of Iowa, Iowa City, Iowa 52242, the Department of Physiology, School of Medicine, The University of California, Los Angeles, California 90024, the Department of Physiology and Biophysics, Boston University School of Medicine, Boston, Massachusetts 02118, and the Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

In cardiac and skeletal muscles tropomyosin binds to the actin outer domain in the absence of Ca$^{2+}$, and in this position tropomyosin inhibits muscle contraction by interfering sterically with myosin-actin binding. The globular domain of troponin is believed to produce this B-state of the thin filament (Lehman, W., Hatch, V., Korman, V. L., Rosol, M., Thomas, L. T., Maytum, R., Geeves, M. A., Van Eyk, J. E., Tobaeman, L. S., and Craig, R. (2000) J. Biol. Chem. 372, 593–606) via tropinin I-actin interactions that constrain the tropomyosin. The present study shows that the B-state can be promoted independently by the elongated tail region of troponin (the NH$_2$ terminus (TnT-(1–153)) of cardiac troponin T). In the absence of the tropinin globular domain, TnT-(1–153) markedly inhibited both myosin S1-actin-tropomyosin MgATPase activity and (at low S1 concentrations) myosin S1-ADP binding to the thin filament. Similarly, TnT-(1–153) increased the concentration of heavy meromyosin required to support in vitro sliding of thin filaments. Electron microscopy and three-dimensional reconstruction of thin filaments containing TnT-(1–153) and either cardiac or skeletal muscle tropomyosin showed that troponin was in the B-state in the complete absence of troponin I. All of these results indicate that portions of the tropinin tail domain, and not only tropinin I, contribute to the positioning of tropomyosin on the actin outer domain, thereby inhibiting muscle contraction in the absence of Ca$^{2+}$.

Calcium binding to the thin filament protein tropinin is required for cardiac and skeletal muscles to contract, and several studies indicate that this regulation involves shifting the tropomyosin position on the actin filament. When the regulatory sites of troponin do not have bound Ca$^{2+}$, tropomyosin is located on the actin outer domain. In this position tropomyosin sterically interferes with much more of the myosin-binding site than it does in the presence of Ca$^{2+}$, and therefore contraction is inhibited at low Ca$^{2+}$ concentrations. This regulatory scheme is supported by three-dimensional helical reconstructions of thin filaments examined by electron microscopy with negative staining (1, 2) or unstained in vitreous ice (3), and by modeling of x-ray diffraction patterns of muscle (4). Furthermore, it is consistent with the solution kinetics of myosin S1-thin filament binding in the absence of ATP (5). However, it is unclear how troponin affects the position of tropomyosin on actin, and more generally the inhibitory action of troponin is not well understood at a structural level, as opposed to better understandings of tropomyosin.

Troponin consists of a relatively globular domain (including TnI and TnC) and an elongated tail region, the NH$_2$ terminus of TnI (6, 7). The inhibitory actions of troponin have long been attributed to the TnI subunit. Skeletal muscle TnI inhibits the actin-myosin ATPase rate in the absence of the other troponin subunits TnC and TnT (8–10), and this effect requires lower TnI concentrations in the presence rather than in the absence of tropomyosin (11). Cardiac TnI has similar properties, although the inhibition is less effective (12–14). The inhibitory effects of skeletal muscle TnI can be mimicked by the so-called inhibitory peptide, residues 96–116 (10, 15), or identically by the corresponding cardiac peptide (14). The reversal of inhibition is related to Ca$^{2+}$-dependent TnI-TnC interactions, elucidated in part at the atomic level (16). An additional TnI region, approximately 130–150 residues, has also been implicated in inhibition (17–19). These and other data are consistent with an inhibitory mechanism consisting primarily of a TnI-actin interaction that is reversed by Ca$^{2+}$, i.e. a localized actin-troponin interaction tethers the much longer tropomyosin on the actin outer domain in the absence, but not in the presence, of Ca$^{2+}$. Indeed, our own electron microscope results show that Ca$^{2+}$ causes a decrease in troponin density in contact with actin (20). However, no high resolution data exists for these interactions, or for the assembled thin filament, and it remains possible that other mechanisms are also important for regulation and for determining the shifting positions of tropomyosin on the actin surface.

This report describes new and unexpected attributes of the troponin tail, i.e. the NH$_2$ terminus of TnT. In the absence of all other portions of troponin, including TnI, cardiac TnI-(1–153) inhibited the interaction of myosin with actin-tropomyosin filaments. Helical reconstruction of filaments indicated that the

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‡ To whom correspondence should be addressed: Depts. of Internal Medicine and Biochemistry, 200 Hawkins Dr., SE-610 GH, The University of Iowa, Iowa City, IA 52242. Tel.: 319-356-3703; Fax: 319-356-3086; E-mail: larry-tobacman@uiowa.edu.

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1 The abbreviations used are: TnI, troponin I; TnC, troponin C; TnT, troponin T; TnT-(1–153), bovine cardiac TnT fragment 1–153; RhP-actin, actin-labeled on Cys-374 with rhodamine-phalloidin; myosin S1, myosin subfragment 1; HMM, heavy meromyosin; DTT, dithiothreitol; Ap$_3$A, 5'-adenosine 3'-pentaphosphate; Pipes, 1,4-piperazine-dithanesulfonic acid; MOPS, 4-morpholinoepanesulfonic acid; dibromo-BAPTA, 1,2-bis-(2-amino-5-bromophenoxo)ethane-N,N,N',N'-tetraacetic acid.
isolated tropinin tail caused troponin to bind preferentially to the actin outer domain, a position in which tropomyosin sterically interferes with strong myosin binding to actin. The results indicate that the tropinin tail contributes directly to the inhibition of muscle contraction that occurs in the absence of calcium.

MATERIALS AND METHODS

Protein Purification—Bovine cardiac troponin (21), tropomyosin (21), and TriT-(1–153) (22), and rabbit fast skeletal muscle actin (23), myosin S1 (24), and heavy meromyosin (25) were separately purified to homogeneity as previously described. Radiolabeled troponin was prepared by stoichiometrical reaction of TnT Cys-40 with [3H]iodoacetate acid (26), followed by reconstitution of tropinin from its three subunits (27). Cys-374-labeled pyrene actin was prepared as in an earlier report (28).

Bnding of TriT-(1–153) to Actin-Tropomyosin—Troponin or TriT-(1–153) bind very tightly to the thin filament, making their affinities difficult to measure. Therefore, the relative affinity of TriT-(1–153) for actin-tropomyosin was measured by its ability to displace [3H]-labeled whole tropinin from the thin filament. Bound and free [3H]-tropinin were separated by ultracentrifugation as previously described (26). By analyzing the data to Equation 1 in Ref. 22, the affinity of TriT-(1–153) (or unlabeled whole tropinin in control experiments) for actin-tropomyosin was calculated. The conditions used were: 25 °C, 7 μM actin, 3 μM tropinin. 3H-labeled tropinin, 10 nm Tris (pH 7.5), 300 mM KCl, 3 mM MgCl2, 0.2 mM DTT, 0.3 mg/ml bovine serum albumin, and 0.1 mM CaCl2 (Fig. 1), 0.5 mM EGTA, or 7 μM myosin S1. CaCl2 was also present in experiments including myosin S1, but had no effect on tropinin-thin filament binding under these conditions (29).

Actin-activated MgATPase Assays—The thin filament-activated myosin S1 MgATPase rate was measured by serial determinations of [32P]P, released from [γ-32P]ATP (30). Rates were linear with time during the initial 10 min used for the assay. Unless whole tropinin was present, the Ca2+ concentration had no effect in the results. In the absence of actin, the myosin S1 MgATPase rate was negligible. The conditions used were: 25 °C, 5 mM Tris-HCl (pH 7.5), 1 mM DTT, 3 mM MgCl2, 1 mM ATP, 0.5 mM dihydro-ATP, 0.3 μM myosin S1, and actin between 0 and 74 μM. Regulatory proteins were included at sufficient concentrations to ensure saturation of the actin: [tropomyosin] and [actin] relationships.

Binding of Myosin S1 to the Thin Filament—Myosin S1-ADP binding to the thin filament was measured as described (33) using pyrene-labeled actin (32), monitoring the decrease in fluorescence intensity as increasing S1 was added. The conditions used were: 1 μM actin, 0.4 μM tropinin, 0.4 μM control or mutant tropinin, 20 mM imidazole (pH 7.5), 125 mM KCl, 3 mM MgCl2, 2 mM ADP, 0.2 mg/ml bovine serum albumin, 25 units of hexokinase, 1 mM glucose, 20 mM DTT, and photobleaching protective agents (35): 14 mM glucose, 240 units of glucose oxidase/ml, and 9 × 103 units of catalase/ml (both enzymes were from Sigma). The intrinsic (50 mM) and pH of the activating solution were calculated as previously described (36). If the actin contained either tropinin or the tropinin-thin filament fragment, it was added to the motility solution to 5 μM.

Quantification of the thin filament sliding speed was performed using a Motion Analysis System (Santa Rosa, CA). Data was acquired and analyzed as previously described (25) and were expressed as the mean ± S.E. In these analyses filaments not moving at a uniform sliding speed were rejected. However, the results were qualitatively the same if, instead, all filaments were averaged, including those moving at non-uniform speeds.

Electron Microscopy—Bovine cardiac or rabbit skeletal muscle tropomin (4.6 μM) were combined with TriT-(1–153) (4.5 μM) and then with F-actin (25 μM) in a solution of 100 mM NaCl, 0.2 mM EGTA, 3 mM MgCl2, 1 mM NaN3, 1 mM Na3-ATP, 0.15% methyl cellulose, and 20 mM DTT, and photobleaching protective agents (35): 14 mM glucose, 240 units of glucose oxidase/ml, and 9 × 103 units of catalase/ml (both enzymes were from Sigma). The intrinsic (50 mM) and pH of the activating solution were calculated as previously described (36). If the actin contained either tropomin or the tropomin-thin filament fragment, it was added to the motility solution to 5 μM.

RESULTS

The Tropomin Tail Region Binds Tightly to the Thin Filament in Both the Presence and Absence of Myosin S1—The significance of the isolated tropomin tail for myosin-thin filament interactions can be assessed most directly by studying a tail construct that binds tightly and specifically to the thin filament. As shown in a recent report (22), cardiac TriT-(1–153) promotes the binding of troponin to actin (as also does TriT-(70–170) (47)) almost as strongly as does whole tropomin. Furthermore, representative data in Fig. 1 show that this same TriT fragment binds to actin-tropominin tightly enough to displace whole tropominin from the thin filament. Because whole tropominin binds to actin-tropominin with an affinity of 3 × 108 M−1 in the presence of Ca2+ (48) (the Fig. 1 conditions), these competitive binding data imply that TriT-(1–153) binds to the thin filament with affinity of 5 × 107 M−1. This quantitative result is consistent with the previous, more qualitative conclusion that the tightly bound tropominin binds to the thin filament involves an interaction between the tropominin COOH terminus and the NH2-terminal half of TroT (reviewed in Ref. 49); in the current experiments, the TroT NH2-terminus is the only portion of tropominin that is present, and it binds tightly to actin-tropominin. In part, for this basis is revealed in a recent crystallographic study (50), in which COOH-
In contrast to the effects of tropomyosin plus troponin, cardiac troponin alone decreased not only the $K_{\text{app}}$ but also the $V_{\text{max}}$ with an 8-fold effect. This alteration in $V_{\text{max}}$ agrees with previous data employing skeletal muscle tropomyosin, which decreases the $V_{\text{max}}$ by 5-fold (56). The troponin tail fragment enhanced this inhibitory effect of the tropomyosin, decreasing the $V_{\text{max}}$ by another 60%, i.e. from 2.6 to 1.0 s$^{-1}$, which is less than 5% of the $V_{\text{max}}$ observed for either unregulated actin or actin-tropomyosin-troponin-Ca$^{2+}$. This inhibitory effect of tropomyosin-TnT-(1–153) is similar to that produced by adding EGTA to fully regulated thin filaments: a drop in $V_{\text{max}}$ by 96%, with little effect on $K_{\text{app}}$ (21). These data imply a significant inhibitory effect of the troponin tail region on myosin ATPase activity, in the absence of any other portion of troponin. The cardiac troponin tail enhances the inhibitory actions of cardiac tropomyosin.

**Effect of the Troponin Tail Region on in Vitro Motility**—To determine whether the troponin tail region affects the mechanical function of myosin, as well as having the enzymatic effects shown above, *in vitro* motility experiments were performed (Fig. 2). In parallel with the myosin S1 ATPase results, TnT-(1–153) decreased the heavy meromyosin-propelled sliding speed of actin-tropomyosin filaments, and this inhibitory effect was much larger than the effect of tropomyosin alone. (The illustrated effects of tropomyosin alone resemble findings in an earlier report (31).) For all tested concentrations of heavy meromyosin that were attached to the sliding surface, the observed thin filament sliding speed was decreased when TnT-(1–153) was added to the acto-tropomyosin. Furthermore, both the threshold concentration of HMM required to induce movement, and the HMM concentration that produced 50% of maximal speed were increased when TnT-(1–153) was present. Qualitatively, these data suggest that the troponin tail weakens or inhibits the transition toward a myosin-thin filament attachment that is required for sliding to proceed. However, this inhibition is not as profound as that produced by whole troponin in the absence of Ca$^{2+}$, which prevents all filament sliding under conditions comparable with Fig. 2 (25, 36). This suggests that full inhibition requires TnI-actin interactions that are absent when the troponin tail is studied in isolation.

The Troponin Tail Region Increases the Cooperativity of Myosin S1-ADP Binding to the Thin Filament—Tropomyosin sterically interferes with strong binding of myosin to actin (i.e. binding observed in the absence of ATP), and TnT-(1–153) strengthens tropomyosin binding to actin. Therefore, the inhibitory properties of this TnT fragment could be because of tighter attachment of tropomyosin in a position that interferes with myosin. To test this, myosin S1-ADP binding to pyrene-labeled actin was examined in the presence of tropomyosin alone, or tropomyosin plus TnT-(1–153) (Fig. 3). Inclusion of the troponin tail region had little effect, with the notable exception of the initial portions of the S1-binding curves, at low myosin S1-ADP concentrations. From this region of the curves, it is apparent that myosin binding to the thin filament was more cooperative when the troponin tail was present. TnT-(1–153) inhibited the initial binding of myosin to myosin-free or nearly myosin-free filaments. This is consistent with an effect of the troponin tail region that consists of stabilizing tropomyosin in a position on actin that interferes with strong myosin binding. Once tropomyosin is displaced from this position by the binding of a relatively small number of myosin heads, the troponin tail has little effect on additional myosin binding. These conclusions are supported by the quantitative analysis of Fig. 3; from curve fitting using the model in Refs. 28 and 31, the equilibrium constant for tropomyosin to shift to the actin outer...
Troponin Tail Domain Suppresses Myosin Activity

The relative affinity of TnT-(1–153) for actin-tropomyosin, normalized to the affinity of radiolabeled troponin, was determined by competition as exemplified in Fig. 1. These data were converted to absolute affinity values (column 4), using published data for the thin filament affinity of troponins (column 1). Thin filament states B, C, or M (57) refer to the predominant azimuthal position of Tm on actin (A), as determined experimentally in the presence of either TnT-(1–153) or tropomin (Tn).

| Affinity of troponin | State of A-Tm-Tm filaments | Relative affinity of TnT-(1–153) | Affinity of TnT-(1–153) | State of A-Tm-TnT-(1–153) filaments |
|-----------------------|-----------------------------|-------------------------------|------------------------|-------------------------------------|
| EGTA/Mg2+ | 5 x 10^6 | B | 0.096 ± 0.047 | 5 x 10^7 | B |
| Ca2+ | 3 x 10^6 | C | 0.152 ± 0.009 | 5 x 10^7 | B' |
| Myosin S1 | 3 x 10^6 | M | 0.139 ± 0.017 | 4 x 10^6 | M' |

 TABLE II

Myosin S1-thin filament MgATPase rates

The actin-activated ATPase rate of myosin S1 was examined in the presence of various combinations of cardiac regulatory proteins, in each case as a function of thin filament concentration. Results were fitted to the Michaelis-Menten equation. Conditions were described under “Materials and Methods.”

| | Vmax | Actin Kapp |
|----------------|--------|------------|
| Actin | 22 ± 4 | 200 ± 46 |
| Actin-tropomyosin-troponin-Ca2+ | 26 ± 2 | 38 ± 5 |
| Actin-tropomyosin-TnT(1–153) | 1.0 ± 0.3 | 21 ± 13 |
| Actin-tropomyosin | 2.6 ± 0.1 | 9.4 ± 1.6 |

 FIG. 3. The troponin tail region increases the cooperativity of myosin S1-ADP binding to the thin filament. Increasing concentrations of myosin S1 were added to samples containing pyrene-labeled actin and tropomyosin, either with (asterisks) or without (circles) TnT-(1–153). Dashed lines are best-fit curves based on the thin filament model described in Ref. 28. For tropomyosin alone, Kp = 2.54 ± 0.05 x 10^6 M^-1 and L = 4.1 ± 0.1. For thin filaments with tropomyosin and TnT-(1–153), Kp = 2.41 ± 0.07 x 10^6 M^-1 and L = 3.3 ± 0.2. Data points indicate the averages from three experiments for each condition, with standard errors of the measurements approximately the size of the symbols, or less.

 tropomyosin is on the actin outer domain (the B-state, characteristic of actin-troponin-tropomyosin filaments in the absence of Ca2+ (1, 2)), but also sterically interferes, albeit to a lesser extent, when the tropomyosin is on the outer edge of the actin inner domain (the C-state, which is the predominant state in the presence of troponin plus Ca2+) (4, 44). Only when tropomyosin shifts further onto the actin inner domain (the M-state) is there no steric interference between tropomyosin and myosin (44). Therefore, the ATPase, in vitro motility, and myosin binding data in the current study could result from the troponin tail strengthening association of tropomyosin with actin in either the B-state or the C-state position. As precedents for the latter possibility, specific mutations in either tropomyosin (33) or actin (55) result in C-state filaments that are profoundly inhibitory, even more so than the current results with TnT-(1–153). Furthermore, if the B-state depends upon TnI-actin interactions, then actin-tropomyosin-TnT-(1–153) filaments should not be in the B-state.

To assess these issues, thin filaments were examined by electron microscopy with helical reconstruction (Fig. 4). Fila-
ments containing tropomyosin plus the troponin tail contained additional density, corresponding to the tropomyosin strand, which is not observed in reconstructions of bare actin filaments (3, 57). Regardless of whether the tropomyosin was from skeletal muscle or from cardiac muscle, this additional density contacted with the actin outer domain in indistinguishable, B-state positions. This can also be seen in helical projections and Z-sections of the reconstructions (Fig. 5). For comparison, it is significant that in the absence of any portion of troponin, skeletal muscle tropomyosin binds in the C-state position (57) (at the outer edge of the inner domain), and cardiac tropomyosin localizes to the same B-state position (57) now observed when TnT-(1–153) was present. Therefore, the cardiac troponin tail does not shift the predominant position of the cardiac tropomyosin, but rather strengthens its binding to actin. In contrast, the cardiac troponin tail shifts the position of skeletal muscle tropomyosin on actin, from the C-state to the B-state position.

**DISCUSSION**

A principal observation in this report is that, in the presence of tropomyosin, the TnT NH$_2$ terminus inhibits the interactions between myosin and the thin filament, suppressing strong myosin-actin binding, the maximal actomyosin ATPase rate, and the rate of filament sliding. Previous studies have clearly indicated that the inhibitory properties of troponin involve the globular, TnI-containing region of the troponin complex (for review, see Refs. 49 and 58). However, the current results indicate that striking inhibition is produced independently by another region of the extended troponin molecule: the troponin tail fragment TnT-(1–153) examined in this report. This unexpected result has implications both for the function of the troponin tail region, and for the overall mechanism of thin filament-mediated regulation.

The first functional characterizations of the troponin tail region employed rabbit fast skeletal muscle TnT chymotryptic fragment TnT-(1) (59–61), now known to consist of residues 1–165 of the predominant fast skeletal isofrom (Protein Information Resource code TPRBTS). TnT-(1) does not interact with TnC or TnI, and extends away from the globular region of troponin to form an elongated structure that binds to a long segment of tropomyosin (62). TnT-(1) increases the cooperativity with which myosin S1 converts actin-tropomyosin filaments to the M- or active state (63). In a new report by Maytum and colleagues, (84) TnT-(1) was found to be inhibitory to myosin S1, similar to the actions of the cardiac construct TnT-(1–153) examined in the present article. Similarly, Ohtsuki and coworkers (65, 66) found that TnT-(1) inhibited both superprecipitation and actomyosin ATPase rates in the presence of tropomyosin, TnI, and TnC. This agreement supports the generality of our findings that the troponin tail, in the absence of the troponin I-containing globular domain, has substantial inhibitory effects.

On the other hand, the detailed studies of Maytum et al. (84) suggest that TnT-(1) stabilizes the C-state of the thin filament, rather than the B-state now observed with the cardiac troponin tail fragment. Thus, the two troponin tail fragments appear to act by different mechanisms. One possible explanation is that selected experimental methods differ in the two studies: TnT-(1) has not been examined by electron microscopy, nor have the effects of TnT-(1–153) on S1-thin filament binding kinetics been determined. These methods generally agree, but not always (57). However, the most likely explanation for the discrepancy is that structural differences between the peptides result in different behavior. TnT-(1–153) of the current study shares high homology to skeletal TnT-(1) only in one region: cardiac TnT residues 74–133 are 80% homologous to skeletal muscle TnT residues 54–133. The more NH$_2$-terminal parts of the peptides are not homologous, and TnT-(1) contains 31 conserved residues at its COOH terminus that are not included in TnT-(1–153). These dissimilarities may explain why the cardiac TnT fragment inhibits myosin S1 ATPase activity more than does skeletal muscle TnT-(1), and also why the cardiac fragment stabilizes the B-state rather than the C-state. (Note that these two functional differences are distinct; the degree of ATPase inhibition does not always correlate with the presence of the B-state (33, 55, 57, 67).) Unfortunately, a cardiac TnT fragment similar to skeletal muscle TnT-(1) has poor solubility (22), making the importance of the additional COOH-terminal residues in skeletal muscle TnT-(1) difficult to test.

Subtle differences in the amino acid sequence of either tropomyosin or actin can lead to a change in the equilibrium position of tropomyosin on actin (57). Therefore, it is not surprising that different fragments of TnT in either the presence or the absence of S1 may perturb M-state to C-state to B-state equilibria to different extents. Interestingly, very different
properties were reported for an NH₂-terminal fragment of skeletal muscle TnT that is still longer than TnT-(1) (27) conserved residues. Unlike the results with both cardiac TnT-(1–153) and skeletal TnT-(1), skeletal fragment TnT-(1–191) causes a modest increase in the myosin-actin-tropomyosin ATPase rate (68), similar in findings with holo-TnT (69, 70). Although the regulatory effects of TnT-(1–191) (and holo-TnT) were not examined in detail, the available data suggest that different regions of the troponin tail have distinct effects on the thin filament, and sometimes opposite effects on myosin-thin filament interactions. The same conclusions can be inferred from more indirect approaches to these issues: deletional (71, 72) or tail peptide properties are affected by non-native structure. These several considerations, and the complex nature of thin filament activation, require that interpretations be made with caution.

Nevertheless, when considered in sum the current and previous results suggest that the troponin tail produces specific interactions that stabilize tropomyosin binding to actin in the B-state position, and separate interactions that stabilize the C-state. When studied as an isolated peptide, this can result in an equilibrium favoring one or the other state, depending on the construct. This conclusion advances understanding of the regulatory mechanism when it is combined with previous results, which have shown that when Ca²⁺ binds to tropomyosin, selected TnI sites move further from actin (79–81) and there is decreased troponin density near actin (20). Ca²⁺-mediated detachment of part of Tn from actin has been generally viewed as critical for the switching of the thin filament out of the B-state. The current work does not refute this viewpoint, but rather shows that it is incomplete; it is now clear that Ca²⁺ must also reverse other interactions that strongly stabilize the B-state, interactions involving TnT-(1–153).

Because both TnT-(1–153) and the inhibitory peptide of TnI promote formation of the B-state, it is unclear how the thin filament switches to the more activated C-state in the presence of Ca²⁺. We have suggested previously (48, 51, 57, 82) that the B to C transition involves a passive release of TnI from actin. TnT may contribute to this process, as stated above, is that these effects are because of greater stabilization of the B-state when TnT-(1–153) is present. However, this need not be the only mechanism, and there may be several functional differences between tropomyosin alone and tropomyosin in complex with the tropo-

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