Mapping the Domain of Troponin T Responsible for the Activation of Actomyosin ATPase Activity

Identification of Residues Involved in Binding to Actin*

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Abbreviations used are: ABZ, ortho-aminobenzoic acid; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxoethylenitrilo)tetraacetic acid]; PAGE, polyacrilamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tm, tropomyosin; Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; Tricine, N [2-hydroxy-,1,-bis(hydroxymethyl)ethyl]glycine; TRIS, tris(hydroxymethyl)aminomethane hydrochloride; wt, wild type; CB2, CB3, T1 and T2, fragments of TnT corresponding to, respectively, residues 77-157, 1-76, 1-157 and 158-263 of chicken skeletal muscle TnT isoform 3; TnT1-191, TnT157-263, TnT1-157, TnT1-76, TnT77-157, TnT 77-191 and TnT158-191, fragments of TnT corresponding to, respectively, amino acid residues 1-191, 157-263, 1-157,1-76, 77-157, 77-191 and 158-191 of chicken skeletal muscle TnT isoform 3; 5OH263WTm, recombinant tropomyosin with Gln263 substituted by 5-hydroxytryptophan.
ABSTRACT

The in vitro Ca\textsuperscript{2+}-regulation of the actomyosin Mg\textsuperscript{2+}-ATPase at physiological ratios of actin, tropomyosin and troponin occurs only in the presence of troponin T. We have previously demonstrated that a polypeptide corresponding to the first 191 amino acids of troponin T (TnT1-191) activates the actomyosin Mg\textsuperscript{2+}-ATPase in the presence of tropomyosin. In order to further characterize this activation domain, we constructed troponin T fragments corresponding to residues 1-157 (TnT1-157), 1-76 (TnT1-76), 77-157 (TnT77-157), 77-191 (TnT77-191) and 158-191 (TnT158-191). Assays using these fragments demonstrated that: a) residues 1-76 do not bind to tropomyosin or actin; b) residues 158-191 bind to actin cooperatively, but not to tropomyosin; c) the sequence 77-157 is necessary for troponin interaction with residue 263 of tropomyosin; d) TnT77-191 on its own activates de actomyosin ATPase activity as previously described for TnT1-191. TnT1-157, TnT1-76, TnT77-157, TnT158-191 and combinations of TnT158-191 with TnT1-157 or TnT77-157 showed no effect on the ATPase activity. We conclude that the activation of actomyosin ATPase activity is mediated by a direct interaction between amino acids 77-191 of troponin T, tropomyosin and actin.
INTRODUCTION

The contractile interaction between myosin and actin in skeletal muscle is regulated by changes in intracellular \( \text{Ca}^{2+} \) concentration and by the effects of such changes on the interactions between thin filament proteins. In skeletal muscle, tropomyosin (Tm) and the troponin complex (Tn) regulate this process. The troponin complex is formed by three subunits: troponin C (TnC), troponin I (TnI) and troponin T (TnT) (1). In the absence of \( \text{Ca}^{2+} \), the TnI subunit inhibits the actomyosin ATPase activity. The binding of \( \text{Ca}^{2+} \) to the regulatory sites of TnC removes TnI inhibition and leads to activation of actomyosin ATPase activity (for review see ref. 2 - 4). In addition to the central role played by TnI and TnC in this \( \text{Ca}^{2+} \)-dependent regulation, an increasing amount of evidence points to the important role of TnT in the regulation of muscle contraction. Besides its role in attaching the troponin complex to the thin filament (for review see ref. 5), TnT is required for the full inhibition of actomyosin ATPase activity in the absence of \( \text{Ca}^{2+} \) in vitro at physiological Tn:Tm:actin molar ratios (6-8). Furthermore, only in the presence of TnT does the binding of \( \text{Ca}^{2+} \) to TnC lead to an increase of the level of the actomyosin ATPase activity above the level of activity observed in the presence of tropomyosin and actomyosin (1,6-8).

The elongated shape of TnT (9) and its \( \text{Ca}^{2+} \)-dependent and \( \text{Ca}^{2+} \)-independent interactions with the thin filament (10) indicate its importance in the control of the position occupied by tropomyosin in the thin filament, a central theme in the proposed molecular
mechanisms of muscle contraction regulation (11-12). Experiments performed with chymotryptic fragments T1 and T2 of TnT (13) demonstrated that the NH2-terminal fragment T1 binds strongly to the COOH-terminal region of tropomyosin (14, 15, 16). The affinity of T1 for tropomyosin is mediated by residues present in cyanogen bromide fragment CB2 (17). The role of the NH2-region of TnT adjacent to CB2 in the regulation of muscle contraction is not clearly defined, although it has been suggested that this region overlaps the head-to-tail junction region of two adjacent tropomyosins (18). The COOH-terminal region of TnT (chymotryptic fragment T2) binds to the TnI/TnC dimer and to tropomyosin (15, 19-21). The interaction of T2 with tropomyosin in the presence of TnI and TnC is sensitive to Ca2+ concentration. In the absence of Ca2+, T2 binds to a region near to amino acid 190 of tropomyosin (14). In the presence of Ca2+, it is believed that modifications in the interaction between the T2 region and TnI/TnC lead to detachment of this region of TnT from tropomyosin (10, 22).

Using recombinant fragments of TnT, our group has previously shown that in the absence of the TnI/TnC dimer, an isolated polypeptide corresponding to the first 191 amino acids of skeletal chicken muscle TnT (TnT1-191) is able to activate the Mg2+-ATPase activity of actomyosin, whereas isolated wild type TnT is not (22). The level of activation observed (~30%) was similar to those levels observed in experiments performed under similar conditions with the whole Tn complex in the presence of Ca2+, indicating that the amino acid sequence between residues 1 and 191 of TnT encompasses an activation domain. This domain must be responsible for the activation of actomyosin ATPase at high
Ca$^{2+}$ concentrations. To explain the observation that TnT1-191 is able to activate the ATPase activity while full-length TnT is not, we proposed a refinement of the original two-site binding model of Pearlstone and Smillie (10) used to describe troponin-binding to the thin filament. In the refined model (22), TnT was divided into three domains: a) an activation domain localized in the NH$_2$-terminal region of TnT; b) a central domain necessary for the full inhibition by TnI in the absence of Ca$^{2+}$; and c) a COOH-terminal domain that anchors the TnI/TnC dimer to the filament. We proposed that binding of Ca$^{2+}$ to TnC would promote the dissociation of the globular domain of troponin (formed by TnC, TnI and the COOH-terminal region of TnT) from the thin filament, thereby abolishing the inhibitory interactions. This would release the activation effects of the NH$_2$-terminal region of TnT.

In the present study we further map the activation domain of TnT in order to better understand its interactions with the thin filament. We constructed TnT fragments encompassing the region between the amino acids 1-191. Functional mapping was performed by examining the effect of TnT fragments on the actomyosin Mg$^{2+}$-ATPase activity. Direct interactions of these fragments with tropomyosin and actin were also studied.
MATERIALS AND METHODS

Construction, Expression and Purification of Recombinant TnT Fragments

DNAs encoding TnT fragments 1-76, 1-157, and 77-157 were constructed by oligonucleotide-mediated site-directed mutagenesis (23) using as template M13mp18-TnT-3 (6) which contains the chicken skeletal muscle TnT-3 cDNA (24; GenBank accession number M22156). Oligonucleotides 5'-AAAGACCTGATTTAACTGCAGGC-3' and 5'-TGTCCTCCATGTGAGCCTCATACAG-3' were used to change codons Glu77 and Gly158, respectively, into stop codons to produce M13mp18-TnT-3-1-76 and M13mp18-TnT-3-1-157 respectively. Using oligonucleotide 5'-GAACAAAGACCATATGGAACTGCAGG-3' and M13mp18-TnT-3-1-157 as template, a NdeI site at codons 75 and 76 was created, producing M13mp18-TnT-3-77-157. TnT-encoding DNA fragments were then cloned into the pET-3a expression vector (25) as previously described (22). A fragment encoding amino acids 77-191 was produced in a PCR reaction using the above oligonucleotide, oligonucleotide 5'-CATTAACCTATATAAAAATAGGCG-3' and the vector for expression of the fragment TnT-191 (vector pET-TnT1-191) (22) as template. The product was digested with Ndel and EcoRI and cloned into pET-3a previously digested with Ndel and EcoRI. Mutant sequences were confirmed by DNA sequencing (26). The recombinant proteins were expressed in E. coli strain BL21(DE3) pLysS (25) and purified as described (22). The identity of recombinant fragments TnT1-157, TnT1-76 and TnT77-157 was confirmed by Edman degradation. Protein concentrations were determined according to Hartree (27).
Expression and purification of recombinant TnT (TnTwt) and fragments TnT1-191 and TnT157-163 were carried out as previously described by Malnic et al. (22).

TnT158-191 Synthesis

The peptide TnT158-191 and the ABZ-containing fragment were synthesized using the solid phase method (28). Optimized coupling reaction conditions were applied according to peptidyl-resin-solvation theory (29). The peptides were purified by semi-preparative reverse phase-HPLC column and characterized by amino acid analysis, analytical HPLC and mass spectrometry.

Construction, Expression and Purification of Recombinant 5OH263WTm

A tryptophan codon was introduced at position 263 of the chicken-skeletal α-tropomyosin cDNA previously modified to code for a dipeptide Ala-Ser NH2-terminal fusion (30) using a PCR-based mutagenesis protocol (31) and using oligonucleotides 5’-AAGAGATCCAGCTTAAAGAAG-3’, 5’-TTCAGTTTCCAAGCATAAAGCTC-3’, 5’-CATTAACCTATAAAAATAGGCG-3’ and 5’-AGCTTTATGCTTGGAAACTGAAG-3. The amplified product was subcloned into pET-3a previously digested with BstEII and EcoRI. The nucleotide sequence was confirmed (26). Expression and purification of the mutant tropomyosin containing 5-hydroxytryptophan at position 263 (5OH263W) was performed as described by Farah and Reinach (32). 5OH263WTm concentration was determined according to Hartree (27).
Other Muscle Proteins

α-Tropomyosin was purified from chicken heart as described by Smillie (33). Actin (34) and myosin (35) were prepared from chicken pectoralis major and minor muscles. Recombinant TnI and TnC were expressed and purified as described (36). Reconstitution of the troponin complex was performed as described previously (6).

CD Spectra

TnT fragments dialyzed against 10 mM sodium phosphate (pH 7.0), 100 mM KCl and 1 mM DTT were used in circular dichroism studies performed on a Jasco 720 spectropolarimeter at 20°C. The apparent secondary structure content was calculated as described (37, 38).

Actomyosin ATPase Assays

The ATPase activity measurements were performed by mixing actin (4 µM), tropomyosin (0.57 µM), TnT or TnT fragments (concentrations are indicated in the figure legends) and myosin (0.2 µM) in 20 mM imidazole-HCl (pH 7.0), 3.5 mM MgCl$_2$, 0.5 mM EGTA, 60 mM KCl, 1 mM EDTA and 1 mM DTT as described (7, 22). Some assays were performed in the absence of tropomyosin and actin (see legend Figure 3 and Table 2). The samples were incubated for 15 minutes at 25°C before initiating the reaction by the addition of 2 mM Na$_2$ATP (pH 7.0). Reactions were stopped after 15 minutes and ATPase activity was determined by measuring the amount of inorganic phosphate released (39).
Actin-binding and Tropomyosin-actin-binding Co-sedimentation Assays

Actin (20 μM), á-tropomyosin and TnT fragments (see figure legends for concentrations) were combined in 20 mM imidazole-HCl (pH 7.0), 3.5 mM MgCl₂, 0.5 mM EGTA, 60 mM KCl and 1 mM DTT as described (22). The mixtures were homogenized and centrifuged at 315,000 × g for 15 minutes at 4°C. Pellets were gently rinsed three times and resuspended in an equivalent volume of the same buffer. In qualitative assays, binding was detected by analyzing equivalent volumes of mixtures before centrifugation and samples corresponding to the supernatant and pellet after centrifugation in 15% SDS-PAGE (40) or 12.5% Tricine/SDS-PAGE (41). Quantitative experiments using labeled ABZTnT158-191 were carried out as described above except that 25 mM MOPS (pH 7.0) was used as a buffer instead of 20 mM imidazole-HCl (pH 7.0). Binding was detected by measuring the fluorescence intensity of the mixture, supernatant and pellet samples diluted to 1.5 ml final volume and adjusted to 1 M KCl using an F-4500 Hitachi spectrofluorometer at 25°C. The excitation wavelength was 319 nm and the emission wavelength was 418 nm with slit widths of 5 nm.

Effects of Actin and Tropomyosin on the Fluorescence Spectra of ABZTnT158-191

The effect of the presence of actin (1 μM) and tropomyosin (1 μM) on the emission spectra of ABZTnT158-191 (1 μM) was determined in a solution containing 20 mM MOPS (pH 7.0), 3.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 60 mM KCl or 1M KCl (specified
in figure legends). Samples were excited at 319 nm. The emission spectra were monitored at 25°C with slit widths of 5 nm. All spectra were corrected for dilution.

Gel Filtration Assays

Gel filtration of mixtures of tropomyosin and ABZTnT158-191 were carried out using a Superose 6HR 10/30 column (Pharmacia Biotech) coupled to an AKTA-Pharmacia FPLC system. The column was equilibrated with 0.1 mM KCl, 20 mM MOPS (pH 7.0) and 1 mM DTT at room temperature. Samples (1 ml) containing 3 μM tropomyosin or 3 μM ABZTnT158-191 or a mixture of 3 μM tropomyosin and 3 μM ABZTnT158-191 were applied to the column and eluted with the same buffer. Fractions containing tropomyosin were identified using 15% SDS-PAGE (40). Fractions containing ABZTnT158-191 were identified by fluorescence spectroscopy as described above.

Effects of Troponin Complex and TnT Fragments on the Fluorescence Spectra of 5OH263WTm

The effect of the addition of isolated TnT fragments or troponin complexes reconstituted with wild type TnT or TnT157-263 (see figure 7 and Table 3 for concentrations) on the emission spectra of 5OH263WTm was determined in 25 mM MOPS (pH 7.0), 60 mM NaCl, 5 mM MgCl₂ and 1 mM DTT in the presence and in the absence of 7 μM actin using a F-4500 Hitachi spectrofluorometer at 25°C. Samples were excited at 312 nm and the fluorescence detected between 320 nm and 420 nm using slit bandwidths of
5 nm. In parallel, binding was confirmed by analyzing TnT-5OH263WTm-actin or Tn-5OH263WTm-actin mixtures in co-sedimentation assays as described above except that 25 mM MOPS (pH 7.0) was used as a buffer instead of 20 mM imidazole-HCl (pH 7.0).

RESULTS

Insert Figure 1.

TnT Fragments

We have previously shown that residues 1-191 of TnT are able to activate the actomyosin ATPase in the presence of tropomyosin and in the absence of TnC and TnI (22). In order to identify the sequences responsible for this effect we constructed five fragments of chicken skeletal TnT-3 (Figure 1). We produced three recombinant fragments based on the known chymotryptic fragment T1 (fragment TnT1-157) and on the cyanogen bromide fragments CB2 and CB3 (fragments TnT77-157 and TnT1-76 respectively) (42). In addition, recombinant fragment TnT77-191 (amino acids 77-191) and a synthetic fragment TnT158-191 were produced. In contrast to wild type TnT, all five fragments
constructed in this work were soluble at low ionic strength (60 mM KCl) (data not shown).

The far UV CD spectra of the fragments studied in this work are shown in Figure 2 and the apparent secondary structure contents are summarized in Table 1. TnT1-157, TnT77-157 and TnT1-76 and present estimated \( \alpha \)-helix contents of approximately 44%, 68% and 9% respectively. These \( \alpha \)-helix contents are in general agreement with those previously reported for non-recombinant fragments T1, CB2 and CB3 of rabbit skeletal muscle TnT (20, 43). TnT1-191, TnT77-191 and TnT158-191 presented estimated \( \alpha \)-helix contents of 30%, 50% and 2% respectively. Since TnT1-76 and TnT158-191 possess little residual molar ellipticity, the \( \alpha \)-helix contents estimated for fragments TnT1-191, TnT1-157 and TnT77-191 seem to be due to the highly helical CB2 region (residues 77-157).

**Activation Properties of TnT Fragments**

The effect of the isolated TnT fragments on the actomyosin Mg\(^{2+}\)-ATPase activity was studied in the presence of tropomyosin and in the absence of TnC and TnI. Our titration of actomyosin ATPase activity with these fragments confirmed that TnT1-191 activates the actomyosin ATPase activity to higher levels than the basal level observed in the presence of tropomyosin (Figure 3). The same level of activation, an increase of approximately 40% over basal activity, was observed in the titration with TnT77-191 (Figure 3). Maximum activation was observed at a ratio of TnT1-191 or TnT77-191 to tropomyosin of approximately 1.5. No activation was observed in titrations with fragments.
TnT1-76, TnT1-157, TnT77-157 or TnT158-191. Also, no activation was observed with a combination of fragments TnT1-157 + TnT158-191 or TnT77-157 + TnT158-191. These results demonstrated that the activation domain is located between amino acids 77-191 of TnT.

**Insert Figure 3 and Table 2**

We also studied the effects of these fragments on actomyosin ATPase activity in the absence of tropomyosin (Table 2). Our results showed no activation caused by the presence of TnT1-191 and TnT77-191. In fact, we observed an inhibition of ATPase activity in these assays. In the absence of tropomyosin, the fragments inhibit to the same extent that full-length TnT does in the presence of tropomyosin. To exclude the possibility that activation activity of TnT77-191 and TnT1-191 is due to a non-specific effect, we analyzed the direct influence of TnT77-191 and TnT1-191 on the Mg^{2+}-ATPase activity of myosin in the absence of actin. These results (summarized in Table 2) demonstrate that activation depends on the presence of both tropomyosin and actin.

**Interaction of TnT Fragments with Actin and Tropomyosin**

All TnT fragments constructed in this present work are soluble in 60 mM KCl, permitting the study of their interactions with thin filaments at physiological salt concentrations. Performing qualitative co-sedimentation binding assays with TnT1-76, TnT77-157 and TnT1-157, we confirmed that the region of TnT that encompasses residues 1-76 does not bind to actin or tropomyosin-actin, whereas the regions delimited by amino acid residues 1-157 and 77-157 bind to tropomyosin-actin under the conditions
tested (data not shown). We also observed that fragments TnT77-157 and TnT1-157 do not bind to actin in the absence of tropomyosin. Most interestingly, fragments TnT77-191 and TnT158-191 do bind to isolated actin (Figure 4 and data not shown). These results indicate that there is an actin-binding site between residues 158-191 of TnT.

**Insert Figure 4.**

To analyze the interaction of TnT158-191 with tropomyosin and actin, we labeled TnT158-191 at its NH2-terminal extremity with ABZ (ortho-aminobenzoic acid). ABZTnT158-191 showed the same behavior as TnT158-191 in co-sedimentation assays (data not shown), indicating that the addition of the ABZ probe did not significantly affect the solubility and the interaction of this fragment with the thin filament. In agreement with the actin co-sedimentation results, the fluorescence emission spectrum of ABZTnT158-191 is affected by the presence of actin at 60 mM KCl (Figure 5A). The significant reduction in fluorescence intensity is consistent with a direct interaction between TnT158-191 and actin. In control experiments in which actin was added to ABZTnT158-191 in the presence of 1 M KCl, no changes in fluorescence emission spectrum were observed (data not shown).

**Insert Figure 5.**

We could not demonstrate a direct interaction between ABZTnT158-191 and tropomyosin in the absence of actin. The addition of tropomyosin has no effect on ABZTnT158-191 fluorescence at 60 mM KCl (Figure 5A). When a mixture of ABZTnT158-191 and tropomyosin were applied to a gel filtration column, tropomyosin and ABZTnT158-191 appeared as two distinct peaks (Figure 5B). The peaks
corresponding to tropomyosin and to ABZTnT158-191 eluted at the same positions as that of tropomyosin alone and ABZTnT158-191 alone, indicating a lack of interaction between tropomyosin and the region correspondent to residues 158-191 of TnT under the conditions tested. This result is in agreement with previous studies that showed that a fragment of TnT corresponding to residues 159-227 of rabbit skeletal TnT did not bind to immobilized tropomyosin (17).

**Insert Figure 6.**

To explore the binding of TnT158-191 to actin and to tropomyosin-actin, we performed quantitative co-sedimentation assays with increasing concentrations of ABZTnT158-191. In control experiments performed with ABZTnT158-191 alone, no significant amount of TnT fragment was detected in the pellet. Figure 6 shows the binding curve of ABZTnT158-191 to actin and to tropomyosin-actin. Assuming that the binding of TnT158-191 to actin occurs at a molar ratio 1:1, the calculated apparent dissociation constant is 8.1 X 10^{-6} M^{-1}. Interestingly, the S shape of the binding curve shown in Figure 6 suggests that this binding is cooperative. The Hill coefficient was calculated to be 2.1. The binding curve of ABZTnT158-191 to tropomyosin-actin (molar ratio 1:7) was very similar to the binding curve of ABZTnT158-191 to actin (Figure 6).

*Effects of Troponin Complex and TnT fragments on the Fluorescence Spectra of 5OH263WTm*

To study the region of tropomyosin that interacts with the amino terminal half of TnT, we produced a recombinant tropomyosin with an intrinsic fluorescent 5-
hydroxytryptophan probe replacing the original glutamine at position 263 (5OH263WTm). While 5OH263WTm binds to F-actin in co-sedimentation assays (data not shown), its emission spectrum is unaltered by this interaction (Figure 7). On the other hand, the addition of troponin to 5OH263WTm or actin-5OH263WTm causes a significant decrease in 5OH263WTm fluorescence intensity (Figure 7). Therefore, 5OH263WTm fluorescence can be used as a specific probe for the TnT-Tm interaction.

Insert Figure 7 and Table 3.

Figure 7 shows spectra obtained in fluorescence assays using actin-5OH263WTm with wild-type troponin complex, troponin complex reconstituted with recombinant TnT157-263 fragment and isolated TnT1-157. These results, as well as those involving the other TnT fragments, are summarized in Table 3. These assays demonstrate that: i) while the binding of actin to 5OH263WTm had no effect on its emission spectra, significant decreases in fluorescence intensity were caused by the presence of wild-type troponin complex, TnT1-191 and TnT77-191, both in the presence and in the absence of actin. These decreases were more significant in the presence of actin (~20%) than in its absence (~12%) (Table 3), most likely reflecting more extensive binding in the presence of actin under the conditions of the assay. Interestingly, TnT1-157 promoted a decrease of approximately 20% in the fluorescence signal, independent of the presence of actin. ii) The fluorescence emission spectra of 5OH263WTm was not affected by the presence of troponin complex reconstituted with fragment TnT157-263 of TnT under conditions in which this complex is bound to actin-5OH263WTm. This indicates that the variation in the fluorescence observed is specific for the interaction of 5OH263WTm with the NH2-
terminal portion of TnT (Figure 7). iii) As expected, fragment TnT1-76 did not affect the fluorescence of 5OH263WTm since it does not bind to actin-Tm (Table 3). iv) All fragments of TnT that contain the region corresponding to fragment CB2 (TnT1-191, TnT1-157, TnT77-157 and TnT77-191) were able to bind to 5OH263WTm–actin in cosedimentation binding assays (data not shown). Surprisingly, all these TnT fragments except TnT77-157 (CB2) promoted a decrease in fluorescence intensity of 5OH263WTm. These data suggest that the binding of TnT residues 77-157 (CB2) to tropomyosin-actin is modulated by neighboring amino acid sequences along the primary structure of TnT. Only in presence of these sequences is a change in the fluorescence intensity of 5OH263WTm observed. It is noteworthy that the two fragments that are able to activate the ATPase activity of actomyosin (TnT1-191 and TnT77-191) encompass both the tropomyosin binding (residues 77-157) and the actin-binding (residues 158-191) sites.
DISCUSSION

The aim of this study was to investigate the phenomena of activation of actomyosin ATPase activity. We studied the properties of fragments of TnT that encompass the region corresponding to the first 191 amino acid residues of TnT. The fact that all of the TnT fragments constructed here were soluble allowed us to study and map the interactions of the first 191 amino acid residues of TnT with tropomyosin and actin. In this study, we: i) confirmed that TnT is responsible for the activation of actomyosin ATPase activity; ii) demonstrated that the activation domain is located between the residues 77 and 191 of TnT; iii) demonstrated that there is an actin-binding site between residues 158 and 191 of TnT; iv) demonstrated that amino acid residue 263 of tropomyosin interacts with residues 77-157 of TnT.

In agreement with previous studies (15, 17), our results demonstrated that the region between residues 77-157 (CB2) is a tropomyosin-binding site. However, our binding assays performed with TnT fragments and 5OH263WTm (Figure 7) suggested that neighboring amino acid sequences along the primary structure of TnT modulate this interaction. The hypothesis that the CB2 region (residues 77-157) interacts non-specifically with tropomyosin and that CB3 (residues 1-76) confers specificity to binding of T1 (1-157) to tropomyosin has been suggested previously (44). These authors observed that CB2 interacts weakly and non-specifically with tropomyosin while the T1-tropomyosin interaction is stronger and more specific. Our results suggest that residues 158-191 of TnT have a similar effect on the modulation of the interaction of amino acid
residues 77-157 with tropomyosin.

Studies of the binding of TnT to actin have been impaired due to the insolubility of full-length TnT. However, a set of studies has demonstrated that the presence of TnT increases the affinity of tropomyosin for actin (45-47). Hill et al. (46) showed that a deletion of the first 69 residues of cardiac TnT did not affect this property, while the affinity was significantly reduced when the first 158 amino acid residues of cardiac TnT were removed (corresponding to the first 135 residues of chicken skeletal TnT-3). Previous studies performed at high ionic strength (400 mM KCl) had detected a weak interaction of TnT with actin (48, 49). Employing actin co-sedimentation assays with T1, T2 and wild type TnT at 150 mM KCl, Heeley et al. (45) showed that the T2 fragment interacts with actin. In the present work, we localized this actin-binding site between amino acids 158-191 of TnT. Binding of this fragment to actin is a cooperative process. While this cooperativity is only observed at non-physiological ratios of TnT:actin, it suggests that this direct TnT-actin interaction may modify the structure of the thin filament.

The first evidence of the role of TnT in the activation process arose with the experimental observation that the activation of actomyosin activity at high Ca^{2+} concentration occurs only when TnT is present in the troponin complex (1, 6-8). Recently, our group (22) demonstrated that an isolated fragment of TnT corresponding to amino acids 1-191 has the ability to activate the actomyosin ATPase in the presence of tropomyosin and in the absence of TnI and TnC. The level of activation observed was similar to those levels observed in the presence of the whole troponin complex and at high Ca^{2+} concentration (pCa 4) (22). In this work, ATPase assays were carried out with a set of TnT
fragments derived from the first 191 amino acids of the molecule. We found that only fragments TnT1-191 and TnT77-191 have the activation property. These observations suggest that the first 76 amino acids of TnT are not involved in this process. Walsh et al. (50) demonstrated that the removal of the first 11 amino acids of tropomyosin, which results in a non-polimerizable tropomyosin that does not bind to actin in the absence of troponin, has no effect on the cooperativity of Ca^{2+}-activation of the actomyosin ATPase. This observation was interpreted as evidence that TnT would play a central role in the cooperativity of the activation process perhaps through its interactions with two adjacent tropomyosins. Studies using troponin complexes reconstituted respectively with cardiac TnT lacking the first 94 amino acids (51) and rabbit skeletal TnT lacking the first 45 amino acids (52), showed that the removal of the NH2-terminal extremity of TnT has no significant effect on the cooperativity of activation of the actomyosin ATPase. Since the fragments studied in the present study lack TnC and TnI binding sites (amino acids 216-263 of TnT) (22), assays of Ca^{2+} regulation by the troponin complex were not performed. However, our results are consistent with the notion that the CB3 region of TnT does not play an essential role in the activation process.

Interestingly, we observed that only fragments of TnT that preserve both the tropomyosin binding site (amino acids 77-157) (15, 17) and the actin binding-site (amino acids 158-191) were able to activate the actomyosin ATPase activity. In the context of the steric-blocking model (11), these fragments may lead to an alteration of the position of tropomyosin on the actin filament. Korman and Tobacman (53) recently demonstrated the importance of the interaction of troponin with actin in the activation of the actomyosin
ATPase. They observed that a point mutation in sub-domain 3 of actin affected only the
Ca²⁺-activation process without affecting the affinity of actin for troponin-tropomyosin. Our
results also suggest that the cooperative interaction of TnT with actin may be associated
with the cooperativity of Ca²⁺-binding to regulated thin filaments and Ca²⁺-activation of
the actomyosin ATPase.

The COOH-terminal region of tropomyosin interacts with the T1 region of TnT
(amino acids 1-157) (16). Previous studies have demonstrated that the region of
tropomyosin involved in this interaction is delimited by amino acids 190-284 (18) and
Hammell and Hitchcock-DeGregori (54) demonstrated that residues 258-284 of skeletal
muscle tropomyosin are essential for the binding of troponin-tropomyosin to actin. Using a
recombinant tropomyosin, we showed that residue 263 of tropomyosin interacts with
residues 77-157 of TnT both in the presence and in the absence of actin.

The results presented in this work strongly suggest that the Ca²⁺-activation of
striated muscle actomyosin ATPase activity is caused by interactions of the activation
domain of TnT (residues 77-191) with actin-tropomyosin. After the removal of the
inhibitory interactions of the troponin complex, the activation domain is likely to maintain
tropomyosin in a specific position on the actin filament (55). This activation probably
involves specific TnT interactions with the region near amino acid 263 of tropomyosin and
with actin.
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REFERENCES

1. Greaser, M. L. and Gergely, J. (1971) J. Biol. Chem. 246, 4226-4233.
2. Zot, A. S. and Potter, J. D. (1987) Ann. Rev. Biophys. Biophys. Chem. 16, 535-559.
3. Farah, S. C. and Reinach, F. C. (1995) FASEB J. 9, 755-767.
4. Tobacman, L. S. (1996) Ann. Rev. Physiol. 58, 447-481.
5. Perry, S. V. (1998) J. Musc. Res. Cell Motil. 19, 575-602.
6. Farah, C. S., Miyamoto, C. A., Ramos, C. H. I., Da Silva, A. C. R., Quaggio, R. B., Fujimori, K., Smillie, L. B., and Reinach, F. C. (1994) J. Biol. Chem. 269, 5230-5240.
7. Malnic, B. and Reinach, F. C. (1994) Eur. J. Biochem. 222, 49-54.
8. Potter, J. D., Sheng, Z., Pan, B. and Zhao, J. (1995) J. Biol. Chem. 270, 2557-2562.
9. White, S. P., Cohen, C., and Phillips Jr., G. N. (1987) Nature 325, 826-828.
10. Pearlstone, J. R. and Smillie, L. B. (1983) J. Biol. Chem. 258, 2534-2542.
11. Huxley, H. E. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 361-376.
12. Haselgrove, J. C. (1972) Cold Spring Harbor Symp. Quant. Biol. 37 341-352.
13. Ohtsuki, I. (1979) J. Biochem. 86, 491-497.
14. Mak, A. S. and Smillie L. B. (1981) J. Mol. Biol. 149, 541-550.
15. Pearlstone, J. R. and Smillie, L. B. (1982) J. Biol. Chem. 257, 10587-10592.
16. Flicker, P. F., Phillips Jr, G. N. and Cohen, C. (1982) J. Mol. Biol. 162, 495-501.
17. Jackson, P., Amphlett, G. W. and Perry, V. (1975) Biochem. J. 151, 85-97.
18. Pato, M. D., Mak, A. S. and Smillie, L. B. (1981) J. Biol. Chem. 256, 602-607.
19. Pearlstone, J. R. and Smillie, L. B. (1978) *Can. J. Biochem.* **56**, 521-527.

20. Pearlstone, J. R. and Smillie, L. B. (1985) *Can. J. Biochem.* **63**, 212-218.

21. Tonokura, M. and Ohtuski, I. (1983) *J. Biochem.* **95**, 1417-121.

22. Malnic, B., Farah C. S. and Reinach, F. C (1998) *J. Biol. Chem.* **273**, 10594-10601.

23. Kunkel, T. A. (1987) *Methods Enzymol.* **154**, 367-382.

24. Smillie, L. B., Golosinska, K. and Reinach, F. C. (1988) *J. Biol. Chem.* **263**, 18816-18820.

25. Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60-89.

26. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.

27. Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422-427.

28. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149-2154.

29. Cilli, E. M., Oliveira, E., Marchetto, R., Nakaie, C. R. (1996) *Org. Chem.* **61**, 8992-9000.

30. Monteiro, P. B., Lataro, R. C., Ferro, J. A., and Reinach, F. C. (1994) *J. Biol. Chem.* **269**, 10461-10466.

31. Higuchi, R. (1990) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, London, United Kingdom.

32. Farah, S. C. and Reinach, F. C. (1999) *Biochemistry* **38**, 10543-0551.

33. Smillie, L. B. (1982) *Methods Enzymol.* **85**, 234-241.

34. Pardee, J. D. and Spudich, J. A. (1982) *Methods Enzymol.* **85**, 164-182.
35. Reinach, F. C., Masaki, T., Shafiq, S., Obinata, T., and Fishman, D. A. (1982) J. Cell Biol. 95, 78-84.

36. Quaggio, R. B., Ferro, J. A., Monteiro, P. B., and Reinach F. C. (1993) Protein. Sci. 2, 1053-1056.

37. Provencher, S. W. (1982) Comput. Phys. Commun. 27, 213-227.

38. Provencher, S. W. (1982) Comput. Phys. Commun. 27, 229-242.

39. Heinomem, J. K. and Lahti, R. J. (1981) Anal. Biochem. 113, 313-317.

40. Laemmli, U. K. (1970) Nature 368, 65-67.

41. Schaegger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368-379.

42. Pearlstone, J. R., Carpenter, M. R., Johnson, P. and Smillie, L. B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1902-1906.

43. Pearlstone, J. R. and Smillie, L. B. (1977) Can. J. Biochem. 55, 1032-1038.

44. Brisson, J. R., Golosinska, K., Smillie, L. B. and Sykes, B. D. (1986) Biochemistry 25, 4548-4555.

45. Heeley, D. H., Golosinska, K. and Smillie, L. B. (1987) J. Biol. Chem. 262, 9971-9978.

46. Hill, L. E., Mehegan, J. P., Butters, C. A. and Tobacman, L. S. (1992) J. Biol. Chem. 267, 16106-16113.

47. Fisher, D., Wang, G., and Tobacman, L. S. (1995) J. Biol. Chem. 270, 25455-25456.

48. Potter, J. D. and Gergely, J. (1974) Biochemistry 13, 2697-2703.

49. Hitchocok, S. E. (1975) Eur. J. Biochem. 52, 255-263.

50. Walsh, T. P., Trueblood, C. E., Evans, R. and Weber, A. (1985) J. Mol. Biol. 82, 265-269.
51. Hinkle, A., Goranson, A., Butters, C. A. and Tobacman, L. S. (1999) *J. Biol. Chem.*

**274**, 7157-7164.

52. Pan, B. S., Gordon, A. M. and Potter, J. D. (1991) *J. Biol. Chem.* **266**, 12432-2438.

53. Korman, V. L. and Tobacman, L. S. (1999) *J. Biol. Chem.* **274**, 2291-22196.

54. Hammell, R. L. and Hitchcock-DeGregori, S. E. (1996) *J. Biol. Chem.* **271**, 4236-4242.

55. Squire, J. M. and Morris, E. P. (1998) *FASEB J.* **12**, 761-771.
FIGURE LEGENDS

Figure 1: **Schematic representation of the fragments of chicken fast skeletal muscle TnT-3 used in this work.** Recombinant wild type TnT (TnTwt) and recombinant fragments TnT1-191 and TnT157-263 were described previously (22). The recombinant fragments TnT1–157, TnT1-76, TnT77-157, TnT77-191, the synthetic fragment TnT158-191 and TnT158-191 with an extrinsic probe ABZ (ortho-aminobenzoic acid) attached to its NH2-terminal extremity (ABZTnT158-191) were constructed in this study. Fragments TnT1-157 and TnT157-263 correspond to chymotryptic fragments T1 and T2 of rabbit skeletal TnT (TnT157-263 is one amino acid residue longer at its NH2-terminal than T2) (42). TnT77-157 and TnT1-76 correspond to cyanogen bromide fragments CB2 and CB3 respectively of rabbit skeletal TnT (42).

Figure 2: **Far ultraviolet CD spectra of fragments of TnT.** Circular dichroism of TnT fragments in 10 mM sodium phosphate (pH 7.0), 100 mM KCl and 1 mM DTT. CD experiments were performed as described in Materials and Methods.

Figure 3: **Effect of isolated TnT fragments on actomyosin Mg2+-ATPase activity.** Actomyosin ATPase activity was determined at increasing ratios of TnT fragments to
tropomyosin as indicated in the abscissa. The results were expressed as a percentage of the tropomyosin-actomyosin Mg\textsuperscript{2+}-ATPase activity observed in the absence of troponin (100% ATPase activity). The data are the average ± SD of at least 5 independent determinations at each molar ratio of TnT/ tropomyosin and TnT fragment/ tropomyosin, except for the data for TnT77-157+ TnT158-191 (average of 3 determinations). Assays were carried out as described in Material and Methods using the following conditions: actin (4 μM), α-tropomyosin (0.57 μM), and myosin (0.2 μM) in 20 mM imidazole-HCl (pH 7.0), 3.5 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 60 mM KCl, 1 mM EDTA and 1 mM DTT.

Figure 4: **Binding of TnT158-191 to actin and tropomyosin-actin.** The protein mixture before ultracentrifugation (M), and the supernatant (S) and pellet (P) after ultracentrifugation were analyzed using 12.5% Tricine/SDS-PAGE. Assay conditions: actin (20 μM), tropomyosin (2.86 μM) and TnT158-191 (8.58 μM) in 20 mM imidazole-HCl (pH 7.0), 60 mM KCl, 3.5 mM MgCl\textsubscript{2}, 2 mM â-mercaptoethanol and 1 mM EDTA as described in Material and Methods. The assays were performed with a TnT158-191:actin molar ratio of 3:7.

Figure 5: **A. Effect of actin and tropomyosin on the fluorescence spectrum of ABZTnT158-191.** Typical fluorescent emission spectra of TnT158-191 labeled at its NH\textsubscript{2}-terminal with ortho-aminobenzoic acid (ABZ) alone (___), with actin (____) and with tropomyosin (___ _ _) are shown. The excitation wavelength was 319 nm. Assay conditions: 1 μM ABZTnT158-191, 1 μM α-tropomyosin (when present) and 1 μM actin (when
present) in 20 mM MOPS (pH 7.0), 60 mM KCl, 3.5 mM MgCl₂, 1 mM EDTA and 1 mM DTT.

B. Gel filtration of tropomyosin and fragment ABZTnT158-191. Gel-filtration assays were carried using a Superose 6 HR column (Pharmacia Biotech) (total volume 23.56 ml), as described in Materials and Methods. The column was equilibrated with 25 mM MOPS (pH 7.0), 60 mM KCl and 1 mM DTT at room temperature. A sample (1 ml) of a mixture of 3 mM tropomyosin and 3 mM ABZTnT158-191 was applied to the column and eluted with the same buffer. The presence of ABZTnT158-191 and tropomyosin in the fractions was monitored by fluorescence intensity measurements and by SDS-PAGE (insert). The continuous line indicates the absorbance at 280 nm (total protein). The discontinuous line indicates the fluorescence emission intensity of ABZTnT158-191. Peaks a and b indicate the fractions where tropomyosin (Tm) and ABZTnT158-191, respectively, were eluted. In assays carried out with tropomyosin alone and ABZTnT158-191 alone, elution occurred at the same respective positions.

Figure 6: Binding of ABZTnT158-191 to actin and actin-tropomyosin. Data for the assays for ABZTnT158-191 binding to actin (10 μM) (circles) and to tropomyosin-actin (1.4 μM tropomyosin and 10 μM actin) (squares) were obtained in co-sedimentation assays with increasing concentrations of ABZTnT158-191. Pellets were ressuspended in buffer containing 1 M KCl and the amount of ABZTnT158-191 bound was determined by fluorescence as described in Material and Methods. Assay conditions: 20 mM imidazole-HCl (pH 7.0), 3.5 mM MgCl₂, 0.5 mM EGTA, 60 mM KCl e 2 mM â-mercaptoethanol.
The data are averages of at least three independent determinations and their respective standard deviations. The abscissa indicates the total ABZTnT158-191 concentration and the ordinate indicates the concentration of ABZTnT158-191 detected in the pellet after ultracentrifugation. The line represents the best fit obtained from the data without tropomyosin using the Hill equation: \( Y = \frac{C^n}{(C^n + K_D^n)} \), where \( Y \) is the fractional binding of ABZTnT158-191, \( C \) is the concentration of ABZTnT158-191, and \( K_D \) is the dissociation constant. Control experiments were performed in the absence of actin (triangles).

Figure 7: Effect of thin filament proteins and TnT fragments on the emission spectra of tropomyosin 5OH263WTm. Typical emission spectra of tropomyosin 5OH263WTm (+/- actin) in the presence of TnT1-157, TnT77-191, whole troponin complex and troponin complex reconstituted with fragment TnT157-263 are shown. The spectra were obtained as described in Materials and Methods under the following conditions: 25 mM MOPS (pH 7.0), 60 mM NaCl, 5 mM MgCl2, 1 mM DTT and 1 mM CaCl2 or 1 mM EGTA at 25ºC. Proteins concentrations: 1 µM 5OH263WTm, 3 µM complex troponin or TnT fragments and 7 µM actin. ______ 5OH263WTm; ___-.__-__ 5OH263WTm and troponin or TnT fragments, ______ 5OH263WTm and Tn or TnT fragments in the presence of Ca2+, ___ ____ 5OH263WTm and actin; .......... 5OH263WTm, actin and troponin or TnT fragments, - - - - - 5OH263WTm, actin, troponin or TnT fragments in the presence of Ca2+. Fluorescence intensity is expressed in arbitrary units.
Table 1: **Estimated Secondary Structure Contents of TnT Fragments.**

|         | α-helix | β-sheet | Other* |
|---------|---------|---------|--------|
| TnT1-191| 30      | 26      | 44     |
| TnT1-157 (T1) | 44 (35.2) | 17 | 39     |
| TnT77-191| 50      | 11      | 39     |
| TnT77-157 (CB2) | 68 (77.9) | 0 (19.4) | 32 (2.7) |
| TnT1-76 (CB3) | 9 (10.5) | 47 (20.6) | 44 (68.9) |
| TnT158-191| 2       | 53      | 45     |
| ABZTnT158-191| 9       | 47      | 44     |

Secondary structure composition was estimated (37, 38) from the CD spectra shown in Figure 2. Values are expressed as percentages. *“Other” is the sum of the predictions for β-turn and “remainder” structures as defined in (37, 38). In the parenthesis are previously reported values for cyanogen bromide fragments and chymotryptic fragments of rabbit skeletal TnT (20, 43).
Table 2: Effect of isolated wild type TnT, TnT1-191 and TnT77-191 on myosin, actomyosin and tropomyosin-actomyosin ATPase activity.

|                     | Absence of TnT | TnTwt | TnT1-191     | TnT77-191     |
|---------------------|----------------|-------|--------------|--------------|
| Tropomyosin-actomyosin ATPase | 100% ± 8.9 %    | 95.6% ± 10.1% | 140.5% ± 7.4% | 143.6% ± 4.6% |
| Actomyosin ATPase   | 106.4% ± 14.8% | 72.7% ± 9.9%  | 61.4% ± 6.1%  | 68.5% ± 10.5% |
| Myosin ATPase      | 11.4% ± 2.8%   | 11.5% ± 0.9%  | 11.4% ± 1.3%  | 10.8% ± 1.2%  |

The data shown are presented as percentage of ATPase activity observed for tropomyosin-actomyosin (100% activity). Assay conditions: 1.14 ìM of TnT or TnT1-191 or TnT77-191, 0.57 ìM α-tropomyosin (when present), 4 ìM actin (when present) and 0.2 ìM myosin in 20 mM imidazole-HCl (pH 7.0), 3.5 mM MgCl₂, 60 mM KCl, 1 mM EDTA and 1mM DTT.
Table 3: Effects of troponin complex and TnT fragments on the fluorescence intensity of the emission spectra of 5OH263WTm.

| Fragment                  | + Actin | - Actin |
|---------------------------|---------|---------|
|                           | - Tn    | + Tn    | - Tn    | + Tn    |
| TnTwt + TnI + TnC         | 100%    | 81%     | 100%    | 88%     |
| TnT157-263 + TnI + TnC    | 100%    | 108%    | 100%    | 99%     |
| TnT1-191                  | 100%    | 80%     | 100%    | 89%     |
| TnT1-157                  | 100%    | 76%     | 100%    | 82%     |
| TnT1-76                   | 100%    | 101%    | 100%    | 98%     |
| TnT77-157                 | 100%    | 97%(96%)* | 100%    | 96% (97%)* |
| TnT77-191                 | 100%    | 79%     | 100%    | 92%     |
| TnT158-191                | 100%    | 102%    | 100%    | 98%     |

The data shown were obtained from the spectra presented in Figure 7 (Tn:5OH263WTm:actin = 3:1:7) and represent typical results obtained from at least 2 independent assays. ( )*: Results obtained in assays performed at TnT77-157:5OH263WTm:actin = 10:1:7.
Mapping the domain of troponin T responsible for the activation of actomyosin aTPase activity. Identification of residues involved in binding to actin
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