Interaction of Deletion Mutants of Troponins I and T: COOH-Terminal Truncation of Troponin T Abolishes Troponin I Binding and Reduces Ca^{2+} Sensitivity of the Reconstituted Regulatory System†

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Received September 5, 1996; Revised Manuscript Received October 23, 1996⊗

ABSTRACT: The interaction between troponin I (TnI) and troponin T (TnT) remains the least understood binary interaction among the regulatory proteins of vertebrate striated muscle. To identify the specific binding domains of TnI and TnT and to evaluate the interactions of TnT with troponin C and tropomyosin (Tm), we generated an NH_2-terminal fragment of human fast skeletal β TnT (TnT_{1-201}; residues 1−201) using site-directed mutagenesis. The mutant protein failed to bind to rabbit skeletal muscle TnI as judged by HPLC, showed reduced TnC binding and reduced ternary troponin (Tn) complex formation, and exhibited a much reduced Ca^{2+} sensitivity in the reconstituted regulatory system. It is shown that the amount of Tn complex formed by TnT_{1-201} rather than the activity of the mutant Tn complex affected this Ca^{2+} sensitivity. Binding of the mutant to Tm was similar to that of intact TnT. These results support the view that the COOH-terminal segment of TnT is necessary for binding to TnI and TnC and Ca^{2+} sensitivity in the thin filament, whereas its NH_2-terminus strongly binds to Tm. To identify the regions of TnI which bind to muscle TnT, we used four recombinant fragments of fast skeletal muscle TnI containing amino acid residues 1−94 (TnI_{1-94}), 1−120 (TnI_{1-120}), 96−181 (TnI_{96-181}), and 122−181 ($TnI_{122-181}$) and a synthetic peptide, TnI_{98-114}, containing residues 98−114 corresponding to the inhibitory region. Only TnI_{1-120} showed weak binding to TnT but not to TnT_{1-201}. These results suggest that (i) a region within the NH_2-terminal 120 residues of TnI interacts with TnT and (ii) the COOH-terminal residues 202−258 of TnT contain the interaction site of TnI. Overall, our results also imply that residues 159−201 constitute the smallest region of TnT which contributes to the Ca^{2+} sensitivity of actoS1 ATPase in a reconstituted regulatory system.

The vertebrate thin filament protein troponin (Tn),[1] which is involved in the Ca^{2+}-regulated contraction of vertebrate striated muscles, is a complex of three structurally and functionally distinct subunits: troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC binds Ca^{2+}; TnI binds actin−tropomyosin (Tm) and prevents muscle contraction by inhibiting actin−myosin interaction, whereas TnT attaches the Tn complex to Tm. The binding of Ca^{2+} to TnC triggers muscle contraction which involves the propagation of the signal by protein−protein interactions and conformational changes in all thin filament proteins [for reviews, see Leavis and Gergely (1984), Zot and Potter (1987), Farah and Reinach (1995), and Tobacman (1996)]. The key event in this regulatory process is the Ca^{2+}-induced change in the interaction between TnC and TnI, but an effective Ca^{2+}-dependent regulation of actomyosin ATPase in a reconstituted system requires TnT (Greaser & Gergely, 1971). Recent studies indicate that TnT not only is a structural link between Tn and Tm but also increases the cooperativity of actin−Tm and provides Ca^{2+} sensitivity to the thin filament (Reiser et al., 1992; Schaertl et al., 1995; Wu et al., 1995). Mutations in cardiac TnT are known to cause a large proportion of familial hypertrophic cardiomyopathy (Watkins et al., 1995). Fast skeletal TnT has emerged as an excellent model system for understanding the tissue-specific and developmentally-regulated alternative splicing (Wu et al., 1994), and the changes in cardiac TnT isoform expression have been correlated with heart disease (Anderson et al., 1991).

TnT is a highly asymmetric protein which is poorly soluble at physiological ionic strength. Its chymotryptic digestion results in two soluble subfragments, T1 and T2, both of which have provided information on the interaction of TnT

† This work was supported by grants from the American Heart Association (90-892) and BRSG funds from the Tufts University School of Veterinary Medicine awarded to S.S. This publication is dedicated to Professor Kivie Moldave.

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⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: TnC, troponin C; TnI, troponin I; TnI_{1-94}, TnI_{1-120}, TnI_{96-181}, and $TnI_{122-181}$, TnI fragments consisting of amino acid residues 1−94, 1−120, 96−181, and 122−181, respectively; TnI_{98-114}, TnI inhibitory peptide consisting of amino acid residues 98−114; TnI_f, fast skeletal TnI; TnI_{ir}, inhibitory region of TnI; TnT, troponin T; Tm, tropomyosin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; HPLC, high-performance liquid chromatography; IOD, integrated optical density; IPTG, isopropyl β-D-thiogalactopyranoside; βME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; UTR, untranslated region.

S0006-2960(96)02243-X CCC: $12.00

with TnI, TnC, and Tm [for reviews, see Leavis and Gergely (1984), Zot and Potter (1987), and Farah and Reinach (1995)]. The fragment T1 (residues 1−158) spans the end-to-end overlap of Tm in the thin filament and constitutes the main Ca^{2+}-independent binding region for Tm (Ohtsuki, 1979; Pearlstone & Smillie, 1981; Tanokura et al., 1983). The fragment T2 (residues 159−259) mainly interacts with TnI and TnC. It is also the second binding region of Tm (Pearlstone & Smillie, 1981). Studies of Tanokura et al. (1983) and Pearlstone and Smillie (1981) suggest that the Tm binding region of T2 is located in its COOH-terminal 17 and 31 residue stretch, respectively. T2 interacts at a region around Cys-190 of Tm which is about one-third distance from the COOH-terminus of Tm (Morris & Lehrer, 1984), but this binding is disrupted by Ca^{2+} binding to TnC (Pearlstone & Smillie, 1983). Recently, Schaertl et al. (1995) demonstrated that in native Tn the T1 portion is responsible for the Ca^{2+}-independent increase in the size of the cooperative unit of the actin−Tm filament whereas the T2 portion contributes to the Ca^{2+} sensitivity of the thin filament.

Studies on the binary interaction of TnI−TnT are limited because both are highly charged basic proteins and are only sparingly soluble at physiological ionic strength. Nevertheless, their interaction has been confirmed by several techniques such as cross-linking, circular dichroism, gel filtration, affinity chromatography, and FPLC [for reviews, see Leavis and Gergely (1984) and Zot and Potter (1987)]. The specific domains involved in the binary TnI−TnT interaction and its nature and functional importance are poorly understood. Photo-cross-linking experiments have revealed that TnT remains in contact with TnI in the thin filament independently of Ca^{2+} concentration (Sutoh & Matsuzaki, 1980) and TnI residues 40−98 may be involved in this binding (Hitchcock-DeGregori, 1982; Chong & Hodges, 1982a). Results using the cyanogen bromide peptides of TnT have shown that the T2 fragment binds TnI as effectively as intact TnT but smaller CNBr fragments bind TnI only weakly with the affinity decreasing in the order: 176−230 > 239−259 or 152−175 (Pearlstone & Smillie, 1985). Their work and that of other groups (Hitchcock et al., 1981; Chong & Hodges, 1982a) suggest that TnT residues 159−259 and possibly also residues 71−151, a highly helical part of TnT, bind to TnI.

With regard to mutation of TnT, only NH_2-terminal truncation of the protein has been studied which showed that the interaction of TnT with Tm is drastically reduced by the deletion of residues 70−150 but there is little effect on deletion of residues 1−69 or 151−158 (Fisher et al., 1995). The NH_2-terminal truncation of TnT also did not alter the Ca^{2+} sensitivity of thin filament assembly (Fisher et al., 1995). This is consistent with the results of Schaertl et al. (1995) and also with earlier findings that the deletion of the first 45 and even up to 69 N-terminal residues of TnT has little effect on most of its functional properties (Hill et al., 1992; Pan et al., 1991). In this report, we show that the deletion of COOH-terminal residues 202−258 of human fast skeletal TnT (TnT_{1-201}) did not affect its binding to Tm but binding to TnI was completely abolished, binding to TnC and Tn complex formation were considerably reduced, and the Ca^{2+} sensitivity of the regulatory system reconstituted with the mutant protein was strongly reduced. These results indicate that the COOH-terminal region of TnT provides Ca^{2+} sensitivity to the thin filament and is likely to be involved in TnI and TnC binding. Taken together with the widely accepted view that residues 159−259 of TnT impart Ca^{2+} sensitivity to the thin filament (Schaertl et al., 1995), a function also retained by the mutant TnT_{1-201}, our results have identified the shortest region of TnT (residues 159−201) which is involved in this regulation. Analysis of the binding of TnT with four recombinant deletion fragments and the inhibitory peptide of TnI suggests that the COOH-terminal region of TnT and in particular residues 202−258 bind to TnI comprising a portion of its inhibitory region and the adjacent region in its N-terminus.

MATERIALS AND METHODS

Construction of TnT_{1-201} Mutant. The deletion mutant TnT_{1-201} was generated by polymerase chain reaction (PCR)-based site-directed mutagenesis. Human fast skeletal β TnT cDNA (Wu et al., 1994), which was subcloned in T7 polymerase promoter-based vector pET17b for high-level expression (Wu et al., 1995), was used as DNA template. PCR was performed as described previously (Jha et al., 1994) using primer 1 (5′AAAGCGCTGTCCTCCATGGGCGC-CAACTAC) and primer 2 (5′ TGGATCCGGGTCTACCA-GAGCTACTTGGCCTTG). Primer 1 was designed to bind at the unique internal *Nco*I site of TnT cDNA in pET17b whereas primer 2 introduced the stop codon and generated a *Bam*HI site for subcloning. By this approach, only a small region of cDNA, about 0.2 kb, was amplified by PCR, and this reduced the chance of polymerase errors. The PCR-amplified DNA was digested with *Nco*I and *Bam*HI and ligated to the original construct pET17b-TnT into the same sites. The mutation was confirmed by restriction mapping and DNA sequencing.

Construction and Purification of TnI Mutants. Generation of rabbit TnI_f deletion mutants and their purification from *E. coli* as well as the chemical synthesis of TnI_{98-114} peptide were reported previously (Jha et al., 1996).

Synthesis of Recombinant TnT_{1-201} in E. coli and Its Purification. TnT_{1-201} was synthesized in *E. coli* strain BL21(DE3). Cells were grown in NZCYM medium (Sambrook et al., 1989) at 37 °C until A_{600} was 0.6−0.8, and then allowed to grow for an additional 6 h without induction. The synthesis of TnT_{1-201} was constitutive and optimal under these conditions. The level of synthesis of the recombinant protein did not change in the presence of 0.1 mM IPTG. Higher concentrations of IPTG inhibited the synthesis of TnT_{1-201} as has been observed for the synthesis of intact TnT (Wu et al., 1995). Several recent reports (e.g., Hill et al., 1992) have also found that the induction of TnT synthesis by IPTG leads to a reduced level of this recombinant protein, perhaps because it is toxic to *E. coli*. Cells were lysed by lysozyme and deoxycholic acid and treated with DNase I as described by Sambrook et al. (1989). The cell lysate was centrifuged at 27000*g* for 30 min, and the recombinant protein was found almost exclusively in the supernatant. The supernatant was made to 50 mM Tris·HCl, pH 7.5, and subjected to ammonium sulfate fractionation. Most of the TnT_{1-201} was present in the 40−60% ammonium sulfate fraction whereas the rest of it partitioned into 20−40 and 60−80% fractions. The pellets from all these fractions, obtained by centrifugation as above, were pooled and suspended in buffer A [6 M urea, 20 mM Tris.Cl, pH 8.0, 2 mM EDTA, and 0.5 mM dithiothreitol (DTT)]. After extensive dialysis against buffer A, the samples were loaded

onto a DE-52 (Whatman) column (bed volume 15 mL L^{-1} *E. coli* culture) equilibrated with buffer A. The column was run at 0.5 mL/min. After washing with 15 mL each of buffer A containing 0.1 and 0.3 M NaCl, respectively, the bound TnT_{1-201} was eluted stepwise with 15 mL each of buffer A containing 0.37 and 0.45 M NaCl, respectively. The eluted protein was concentrated, and its purity was assessed by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE).

Formation and Analysis of TnI−TnT, TnT−TnC, and Tn Complexes. TnI, TnT, and TnC were prepared from rabbit skeletal muscle as described by Potter (1982). For the formation of Tn complexes and TnI−TnT binary complexes, equimolar amounts of each subunit were mixed. Since binary complex formation of TnT−TnC appears weak on gels, the two subunits were mixed in a molar ratio of 2:1 (TnT:TnC). The complexes were dialyzed extensively against buffer B (3 M urea, 20 mM Hepes, pH 7.2, 0.15 M NaCl, 0.1 mM $CaCl_2$, and 2.5 mM DTT) and buffer C (20 mM Hepes, pH 7.0, 0.1 M NaCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM DTT). For TnI−TnT binary complex formation, buffer C contained 0.18 or 0.3 M NaCl. TnT−TnC and Tn complexes were analyzed by native PAGE as previously described (Jha et al., 1996). TnI−TnT complex formation was analyzed by high-performance liquid chromatography (HPLC) using a Shodex KW 803 gel filtration column (Shoko Co. Ltd., Japan). HPLC was carried out in a Rainin Instrument. The flow rate was 0.5 mL/min, and A_{280} was monitored. The eluant buffer (buffer C) contained either 0.18 M or 0.3 M NaCl.

Immunoblotting. Samples of TnI−TnT and Tn complexes were subjected to PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody against rabbit fast skeletal TnT (Sigma, St. Louis, MO). Membranes were incubated with anti-mouse IgG−HRP (Sigma), and the enzymatic activity was detected by color development with freshly prepared 3,3′-diaminobenzidine (Sambrook et al., 1989).

Acto−Myosin Subfragment 1 (S1) ATPase Assay. Filamentous (F)-actin, S1, and Tm were prepared from skeletal muscles following previously published methods [see Jha et al. (1994) and references cited therein]. TnT_{1-201} and TnT were reconstituted into the Tn complex using rabbit muscle TnC and TnI in equimolar ratio. The ATPase activity was determined in 10 mM imidazole, pH 7.0, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, and 4 mM ATP using F-actin:S1:Tm:Tn at a molar ratio of 7:2:1:1 (Jha et al., 1994, 1996). The ATPase activity was also determined in the presence of 2 mM EGTA.

Densitometer Scanning. TnT−TnC and Tn complexes in the gels were densitometrically scanned using Millipore Bioimage Wholeband Software (Millipore Inc., Bedford, MA). The bands were quantitated by obtaining the integrated optical density (IOD) which takes into account the area and the optical density. The IOD value of the complex formed by the muscle Tn components was taken as 100% in calculating the percent complex formation by the mutant TnT.

Cosedimentation of TnT with Tm. TnT and TnT_{1-201} were combined with F-actin and Tm in a molar ratio of 7:1:1 in F-actin buffer (2 mM Hepes, pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP, 50 mM KCl, and 2 mM $MgCl_2$) and centrifuged at 340000*g* for 20 min at 4 °C. Identical volumes of unspun samples and centrifuged samples (partitioned into pellet and supernatant) were analyzed by SDS−12% PAGE and immunoblotting.

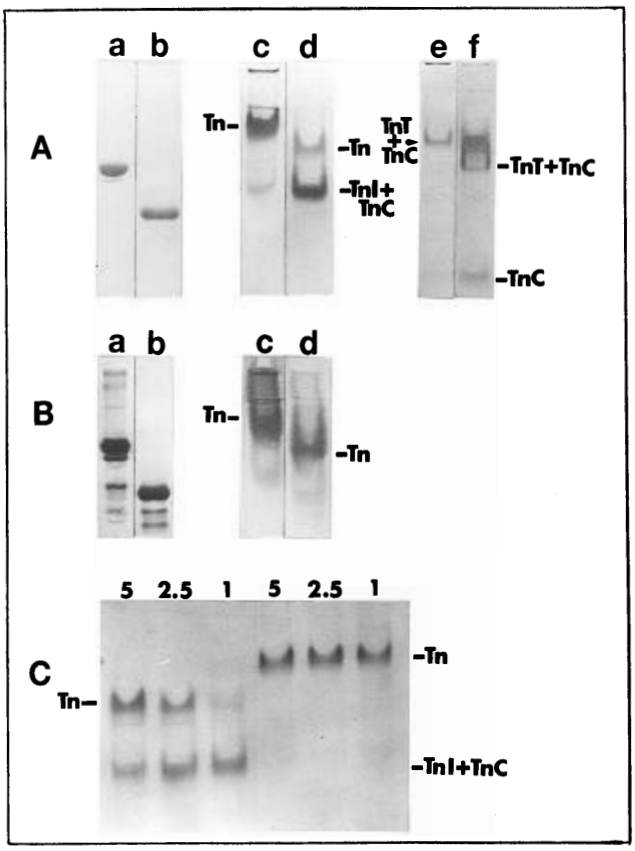

FIGURE 1: Purification, immunoblotting, and TnT+TnC binary complex and TnI+TnT+TnC ternary complex formation by TnT and TnT_{1-201}. For details, see also Materials and Methods. The ternary complex (Tn), binary complexes (TnT+TnC and TnI+TnC), and free TnC are indicated. (Panel A) Lanes a, b, SDS−PAGE of purified TnT and TnT_{1-201}, respectively; lanes c, d, Tn complex formation by TnT and TnT_{1-201}, respectively; lanes e, f, TnT+TnC complex formation by TnT and TnT_{1-201}, respectively. (Panel B) Lanes a−d, immunoblot of lanes a−d in panel A using TnT_f antibody. (Panel C) Tn complex formation in native gel by TnT_{1-201} (first three lanes from left) and TnT (last three lanes) as a function of increasing molar concentration of TnT (1, 2.5, and 5 as indicated on top of lanes), keeping the concentrations of TnI and TnC the same.

RESULTS

Construction and Purification of TnT_{1-201}. The deletion mutation was confirmed by restriction mapping and bidirectional sequencing of the cloned PCR-amplified TnT_f cDNA. Since it is known that N-terminal methionine is cleaved off by *E. coli* in *in vitro* expressed troponin subunits and their fragments (Farah et al., 1994), we have considered the mutant protein to be authentic with N-terminal methionine deleted. The protein was purified from the soluble fraction of the *E. coli* lysate by a combination of ammonium sulfate fractionation and DE-52 chromatography (for details, see Materials and Methods). The purity of the protein was assessed to be about 99% by SDS−PAGE (Figure 1A, lane b). The TnT_{1-201} cross-reacted with TnT_f antibody (Figure 1B, lane b), which confirmed its identity and purity.

Formation and Analysis of TnT−TnC and Tn Complexes. Both intact TnT and TnT_{1-201} formed TnT−TnC complexes of intermediate mobility in native polyacrylamide gels containing 0.5 mM $CaCl_2$ (Figure 1A, lanes e, f) but not in

Table 1: Regulation of the Acto−S1 ATPase Activity [mol of P_i (mol of S-1)$^{-1}$ min^{-1}] by Troponin Reconstituted with TnT and TnT_{1-201}[a]

troponin reconstituted with	ATPase activity +Ca^{2+}	ATPase activity +EGTA	Ca^{2+} sensitivity (%)
no troponin	51.1	51.1	00.0
TnT	60.4	24.4	59.6
TnT_{1-201} (in 1:1:1 ratio with TnI and TnC)	62.2	54.9	11.7
TnT_{1-201} (in 5:1:1 ratio with TnI and TnC)	58.9	41.8	29.0

[a] The calcium sensitivity was calculated as follows: (1 − EGTA rate/Ca^{2+} rate) × 100.

gels with 5 mM EDTA (results not shown). The complex formed by TnT_{1-201} was weak as compared to intact TnT based on the intensity of the complex in gel and also by the presence of a high amount of uncomplexed TnC in the sample (Figure 1A, lane f). Densitometric scanning of the TnT−TnC complex and uncomplexed TnC revealed that the complex formed by TnT_{1-201} represented about 22% of that formed by intact TnT. TnT_{1-201} also formed a high amount of another complex that migrated slower than the binary complex (Figure 1A, lane f), and presumably represents free TnT_{1-201} that dissociated from the complex during electrophoresis.

A similar analysis indicated that the ternary Tn complex formed by TnT_{1-201} was also rather weak as compared to intact TnT (Figure 1A, lanes c, d). This was confirmed by immunoblotting (Figure 1B; lanes c,d). Densitometric scanning revealed that this complex represented 10% of that formed by TnT. When Tn complexes were assayed for the ATPase activity, the mutant Tn complex showed about 12% Ca^{2+} sensitivity as compared to the complex formed by intact TnT which showed about 60% Ca^{2+} sensitivity (Table 1). Their absolute ATPase activities in the presence of Ca^{2+} were similar under our assay conditions (Table 1). In order to distinguish whether the low Ca^{2+} sensitivity of the mutant Tn complex was due to the formation of less ternary complex or due to its lower activity *per se*, we reconstituted the Tn complex by using increasing molar concentrations of TnT or TnT_{1-201}, but keeping TnI and TnC constant. Densitometric scanning revealed that TnT complexed with TnI and TnC in a molar ratio of 1:1:1, 2.5:1:1, or 5:1:1 had similar amounts of complex formed (Figure 1C), indicating that excess TnT did not increase the amount of the complex formed. On the other hand, TnT_{1-201} with TnI and TnC in 1:1:1, 2.5:1:1, and 5:1:1 molar ratios formed ternary complexes representing 11, 48, and 59%, respectively, of that formed by TnT at these molar ratios (Figure 1C). However, even at 5-fold excess, there was a significant amount of the binary TnI−TnC complex (Figure 1C), indicating a much lower affinity of TnT_{1-201} for TnI−TnC as compared to intact TnT. Almost identical patterns were obtained whether TnT_{1-201}, TnI, and TnC were reconstituted together or when TnT_{1-201} was added to a preformed TnI−TnC binary complex (results not shown). At a molar ratio of 5:1:1, the mutant Tn complex was about half as Ca^{2+} sensitive as that formed by the TnT (Table 1).

Interaction of TnT and TnT_{1-201} with TnI and Its Recombinant Fragments. The binary interaction of TnT_{1-201} and TnT with TnI and its recombinant fragments was analyzed by HPLC using a Shodex KW 803 gel filtration column (see also Materials and Methods). Under the assay conditions,

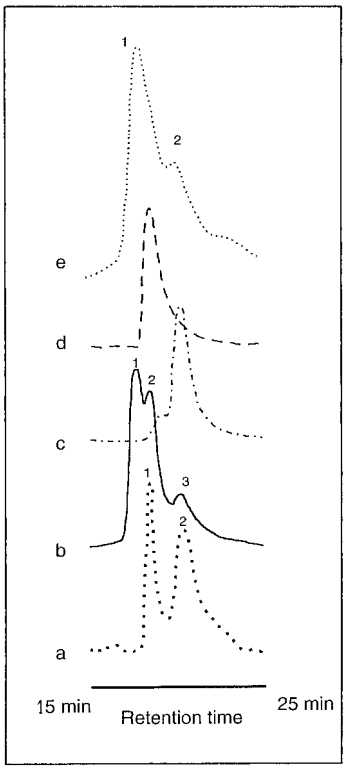

FIGURE 2: Binary interaction of TnT and TnT_{1-201} with TnI as detected by HPLC using a Shodex KW 803 gel filtration column. Elution profiles a−d, binary interaction studied in the presence of 0.3 M NaCl: (a) TnT_{1-201} (peak 1) and TnI (peak 2); (b) TnT (peak 2) and TnI (peak 3) interact to form a binary complex (peak 1); (c) TnI alone; and (d) TnT alone. Elution profile e, binary interaction of TnI and TnT in the presence of 0.18 M NaCl. Peak 2 represents TnI whereas the broad peak 1 represents unresolved free TnT and TnT−TnI binary complex.

free proteins eluted in various HPLC runs at almost reproducible time points which were 18.7, 18.2, 20.6, 22.9, 21.8, 23.8, 24.2, and 28.3 min, respectively, for TnT, TnT_{1-201}, TnI, TnI_{1-120}, TnI_{1-94}, TnI_{96-181}, and $TnI_{122-181}$, respectively (Figures 2 and 3; also results not shown). However, there were minor variations in the elution times of these proteins in each HPLC run. Therefore, free proteins were run as controls in every run. In most cases, the elution profile of the uncomplexed protein from a mixture was directly superimposable on its individual elution profile. The elution time of TnI and its recombinant fragments could be correlated with their molecular mass. TnT_{1-201} eluted with a retention time that was not consistent with its molecular mass perhaps due to the asymmetric nature of the protein. For example, TnT_{1-201} (23.7 kDa) eluted slightly earlier (18.2 min) than TnT (18.7 min) which has a molecular mass of 30.5 kDa. We found that binary complex formation between TnI and TnT was efficient when 30 μM of each protein was mixed. At lower protein concentration (15 and 22 μM), binding occurred at slightly reduced levels. The interaction of TnI and TnT was similar whether their incubation buffer contained Ca^{2+} (2 mM) or EDTA (2 mM).

At a NaCl concentration of 0.3 M, the binary complex of muscle TnT and TnI was observed as a new peak which appeared at 17.5 min, preceding the peaks of free TnT and TnI (Figures 2b and 3b). However, TnT_{1-201} and TnI showed no interaction (Figure 2a; Table 2). No binding was detected even when TnT_{1-201} was combined with TnI in a 4:1 molar ratio (results not shown). Similarly, TnT did not interact

Table 2: Interaction of TnT and TnT_{1-201} with TnI and Its Recombinant Fragments Based on HPLC Analysis[a]

TnT and its recombinant fragment	TnI and its fragments					
	TnI	TnI_{1-94}	TnI_{1-120}	TnI_{96-181}	$TnI_{122-181}$	TnI_{98-114}
TnT (at equimolar ratio)	+++	−	+	−	−	−
TnT (at 5-fold molar excess)	ND	ND	+	ND	ND	ND
TnT_{1-201} (at equimolar ratio)	−	ND	−	ND	ND	ND
TnT_{1-201} (at 4- or 5-fold molar excess)	−	ND	−	ND	ND	ND

[a] The symbles −; +; ++; and +++ represent no, weak, moderate, and strong interaction, respectively. ND, not done. See also text.

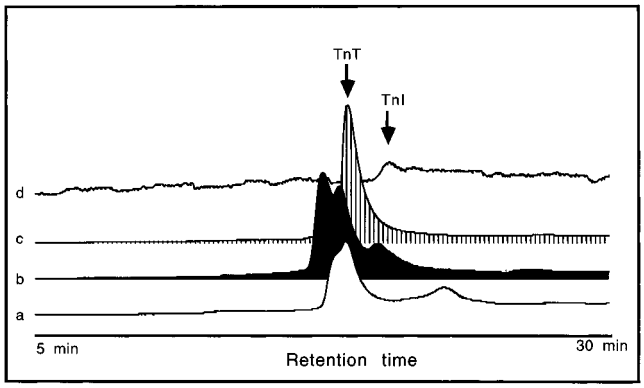

FIGURE 3: Interaction of TnT with recombinant NH_2-terminal fragment of TnI in the presence of 0.3 M NaCl. HPLC was carried out as described in the legend to Figure 2. TnT and TnI peaks are marked with arrows: (a) TnT and TnI_{1-120} interact as apparent from a "shoulder" peak which eluted preceding the free TnT peak; (b) TnT and TnI interact to form a binary complex which elutes preceding the free TnT peak; (c) free TnT; and (d) free TnI.

with TnI_{1-94}, TnI_{96-181}, $TnI_{122-181}$, and TnI_{98-114} (results not shown). However, there appeared to be a weak interaction between muscle TnT and TnI_{1-120} based on the widening of the TnT peak and the appearance of a "shoulder" (Figure 3a; Table 2). We could not detect increased complex formation even at a 5:1 molar ratio of TnI_{1-120}:TnT. Since TnI_{1-120} showed a weak interaction with TnT, we investigated the interaction of this TnI fragment with deletion mutant TnT_{1-201}. As expected, there was no interaction between these two protein fragments at 1:1 or 5:1 molar ratios (Table 2).

The binary interaction of TnI−TnT was also checked by HPLC at 0.18 M NaCl. We did not use any lower salt concentration as both TnI and TnT precipitate below 0.15 M NaCl (Horwitz et al., 1979). At 0.18 M NaCl, the individual peaks of all protein samples became smoother and wider. The interaction of TnT and TnI could be visualized only by the fact that their peaks merged into a broad, shouldered peak (Figure 2e). Since no new peak pattern was apparent, we considered the binary interaction at 0.18 M NaCl to be less pronounced than that observed at 0.3 M NaCl.

Binding of TnT_{1-201} to Tm. The ability of TnT_{1-201} to bind Tm was examined by cosedimentation. The conditions were such that only TnT bound with actin−Tm would sediment (as pellet) upon centrifugation. In the absence of interaction, only actin−Tm will be pelleted and TnT will be in the supernatant. At a molar ratio of 7:1:1 of actin:Tm:TnT, TnT_{1-201} cosedimented with F-actin−Tm in a manner similar to TnT as both TnT_{1-201} and TnT were present in the pellet only and not in the supernatants (Figure 4B,C). The supernatant fraction of both TnT and TnT_{1-201} samples showed the presence of a band (Figure 4B,C) which appears to represent free Tm, since a band of the same mobility was present in the supernatant fraction of a sample containing actin−Tm only (Figure 4A). Immunoblotting using antibody to TnT_f also suggested that the cosedimentation patterns of TnT_{1-201} and TnT were similar as both proteins were present in the pellet only and not in the supernatant (results not shown).

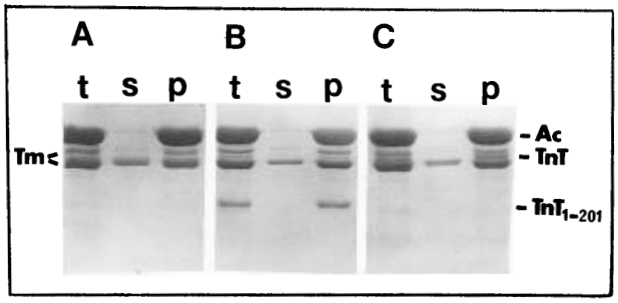

FIGURE 4: Cosedimentation of TnT_{1-201} with F-actin−Tm and its analysis by SDS−PAGE. For details, see also Materials and Methods. Equal volumes of unspun (t) and spun samples containing the supernatant (s) and pellet (p) fractions separately of each reaction mixture were loaded in adjacent lanes. F-Actin (Ac), Tm, TnT, and TnT_{1-201} are indicated. (Panel A) Actin−Tm only. (Panel B) Actin−Tm and TnT_{1-201}. (Panel C) Actin−Tm and TnT.

DISCUSSION

Proteolytic fragments have greatly contributed to studies of the interactions of TnT with other thin filament proteins. However, one must exercise caution in extrapolating these results to the intact protein due to the differences in their size, charges, and possible resulting differences in their folding and binding properties. Larger fragments of a protein often better preserve the native structure and mimic the structure−function properties of the parent protein as has been shown for TnT, TnI, and TnC (Farah et al., 1994; Pearlstone & Smillie, 1995; Fisher et al., 1995; Jha et al., 1996). Several investigators have reported the critical role of the COOH-terminal region of TnT in TnI and TnC binding based on studies employing proteolytic fragments, but there is no report on the mutagenesis of this region in the literature. Considering the above, we generated a COOH-terminal truncation of TnT. We designed this mutation in such a way that (i) the slightly variable inter- and intraspecies COOH-terminal regions of vertebrate TnT isoforms, previously identified by us (Wu et al., 1994), are deleted; (ii) the heptad hydrophobic repeat region of TnT [Parry, 1981; Pearlstone & Smillie, 1981; for a review, see Farah and Reinach (1995)] is deleted; and (iii) the protein ends at a Lys residue since this residue is remarkably conserved as the last residue among all TnT isoforms (Wu et al., 1994). The mutant protein TnT_{1-201} was purified to >95% homogeneity as detected by SDS−PAGE and immunoblotting.

TnT_{1-201} formed a Ca^{2+}-dependent binary complex with TnC with a considerable lower affinity. Only about 20% of

the TnT_{1-201} formed a complex with TnC under conditions that yield 100% complex formation with intact TnT. This weaker interaction appears relevant because a CNBr fragment containing TnT residues 176−230 is known to bind TnC (Zot & Potter, 1987). By COOH-terminal truncation, about half of these residues were deleted in TnT_{1-201}. When equimolar ratios or increased molar ratios of TnT relative to TnI and TnC were mixed, almost identical amounts of the ternary complex were formed (Figure 1C). However, the amount of ternary complex formed by TnT_{1-201} increased with its increasing molar ratio (Figure 1C) and was maximum at a molar ratio of 5:1:1, the highest relative molar ratio examined by us. The Ca^{2+} sensitivity of actoS1 ATPase activity of Tn complex reconstituted with TnT_{1-201} was about one-fifth of that observed with a complex containing intact TnT. Considering the above results which showed that TnT_{1-201} formed higher amounts of Tn complex at its higher molar concentration, we carried out actoS1 ATPase activity by forming the Tn complex with TnT_{1-201} at a higher molar ratio relative to TnI and TnC. At a molar ratio of 5:1:1, the mutant Tn complex was nearly half as Ca^{2+} sensitive as that formed by intact TnT (Table 1). Thus, the actoS1 ATPase activity correlates with the amount of complex formed by TnT_{1-201} (Figure 1C, Table 1). On the other hand, the cosedimentation of TnT_{1-201} with F-actin−Tm was similar to that of TnT (Figure 4). This is consistent with the view that the N-terminal region of TnT predominantly interacts with Tm. Taken together, the above findings strongly support the view that whereas the COOH-terminal region of TnT plays an important role in providing Ca^{2+}-sensitive regulation of muscle contraction and interaction with TnI and TnC, the N-terminal region strongly interacts with Tm (Morris & Lehrer, 1984; Fisher et al., 1995; Schaertl et al., 1995).

As judged by HPLC, there seemed to be a total loss of interaction between TnT_{1-201} and TnI, indicating that the truncated COOH-terminal region of the mutant (residues 202−258) plays a critical role in the interaction with TnI. If previous reports in the literature on CNBr fragments of TnT are considered together, the following TnT residues appear to be involved in the binding to TnI: 159−259, 176−230, 239−259, 152−175, and 71−151 (Chong & Hodges, 1982b; Hitchcock et al., 1981; Pearlstone & Smillie, 1985; Zot & Potter, 1987). Among these, residues 176−230 also bind to TnC (Zot & Potter, 1987) which is supported by our results. Residues 71−151 and 152−175 do not appear to be important for TnI binding because these regions were present in TnT_{1-201} and yet the mutant did not bind to TnI. By exclusion, TnT residues 239−259 and at least a portion of the region consisting of residues 176−230, which have been deleted from TnT_{1-201}, may be the binding region to TnI. Although HPLC provides an efficient and rapid approach for investigating the *in vitro* TnI−TnT interaction, it is quite possible that TnT_{1-201} may interact weakly with TnI at a level below the detection limit of HPLC. This point should be considered because TnT_{1-201} formed a small amount of Tn complex at 1:1:1 ratio with TnI and TnC. A much higher amount of the Tn complex was formed at a 5:1:1 ratio of TnT_{1-201}:TnI:TnC. The observed Tn complex formation by TnT_{1-201} may be due to a weak interaction between TnT_{1-201} and TnI coupled with a strong interaction of TnI with TnC. It is also possible that TnI and TnC interaction causes a conformational change in one or both of them which leads to their interaction with TnT_{1-201}.

Recent work of Schaertl et al. (1995) has shown that the T2 fragment of TnT (residues 159−259) introduces Ca^{2+} sensitivity to the thin filament and that the T1 fragment (residues 1−158) is not required for this function. Several other reports strongly support this view (Fisher et al., 1995; Hill et al., 1992; Morris & Lehrer, 1984). The mutant TnT_{1-201} has only residues 159−201 corresponding to the T2 fragment, and yet it contributes to the Ca^{2+} sensitivity of the reconstituted regulatory system. TnT_{1-201} does not appear to interact with TnI, yet it is incorporated in the Tn complex which exhibits Ca^{2+} sensitivity. Also, with increasing relative molarity of TnT_{1-201}, correspondingly more Tn complex was formed which showed higher Ca^{2+} sensitivity in actoS1 ATPase assay. However, even at a 5:1:1 molar ratio of TnT_{1-201}:TnI:TnC, a significant amount of the binary TnI−TnC complex was present (Figure 1C), indicating that TnT_{1-201} has a low affinity for TnI and TnC. It appears likely that higher amounts of the mutant Tn complex with Ca^{2+} sensitivity approaching that of the native Tn complex could have been obtained if TnT_{1-201} had more affinity for TnI−TnC (or that TnT_{1-201} was present in >5-fold excess). To sum up, TnT_{1-201} in general and residues 159−201 in particular seem to possess the functional property of TnT of introducing Ca^{2+} sensitivity to the reconstituted regulatory system. However, due to the deletion of residues 202−258 in TnT_{1-201}, the likely binding region of TnI and TnC, the affinity of TnT_{1-201} for TnI and TnC is diminished. Taken together, we propose residues 159−201 of TnT as the region which contributes to the Ca^{2+}-dependent regulation of actoS1 ATPase in a reconstituted system.

Since binding of TnT_{1-201} to intact TnI was abolished, as judged by HPLC, we could not use this mutant protein for its interaction with various recombinant fragments of TnI. Instead, intact muscle TnT was used. The weak binding of TnI_{1-120} but not TnI_{1-94} to TnT suggested that the region of TnI which binds to TnT consists of its N-terminal portion together with all or a portion of the inhibitory region of TnI (TnI_{ir}). A lack of binding of TnT with TnI_{98-114}, TnI_{96-181}, and $TnI_{122-181}$ indicated that TnT does not bind to the inhibitory region alone or the COOH-terminal region of TnI. These findings are consistent with the suggestion that TnI residues 57−106 (part of the N-terminal region and part of TnI_{ir}) and TnT residues 197−250 have a heptad hydrophobic repeat capable of forming a coiled-coil structure with each other (Parry, 1981; Pearlstone & Smillie, 1981). Upon sequence analyses of TnI and TnT, we observed that these heptad hydrophobic repeats not only are conserved among various species but also are conserved among the three types of striated muscles: fast skeletal, slow skeletal, and cardiac. Our results show that the TnI fragment TnI_{1-120}, which contains an intact conserved heptad hydrophobic repeat (residues 58−107), is the only TnI fragment which interacts with TnT. Interestingly, a similar conserved heptad hydrophobic repeat is present in residues 196−241 of TnT. Therefore, it is suggested that the above-mentioned heptad hydrophobic repeats are involved in the interaction of TnI and TnT. This view is further supported by our finding that TnT_{1-201}, which lacks the intact conserved repeat, did not bind to either TnI or TnI_{1-201}. Our observations on TnT_{1-201} and binding of TnT with recombinant TnI fragments, together with the relevant published work, are summarized schematically in Figure 5. Only important binding sites are shown

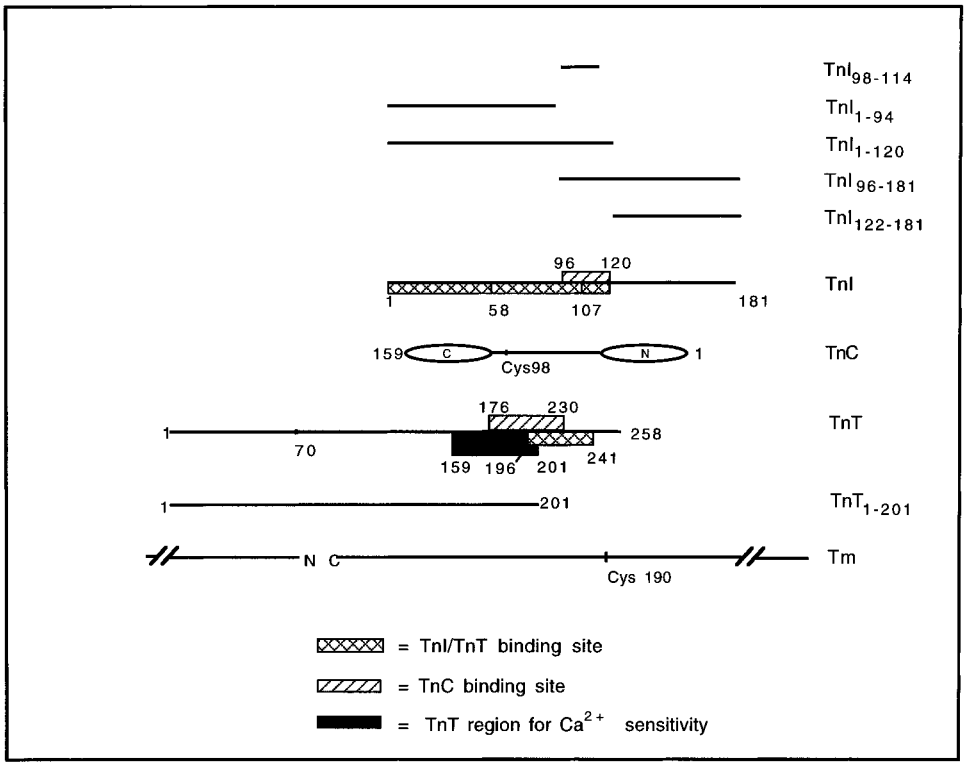

FIGURE 5: Schematic representation of intersubunit interactions of components of the Tn complex in light of this work and previously published literature. Only major sites of interactions are shown for brevity and clarity. The recombinant fragments are indicated by lines. TnC is shown as a dumbbell-shaped molecule. TnI and TnT are represented by lines with the location of important intersubunit binding sites shown as boxes. Cysteine (Cys) 98 in TnC and Cys190 in Tm are also shown.

for clarity. This scheme shows the binding site of TnT on TnI (within its NH_2-terminal 120 residues) as revealed by this work, and also indicates the location of the heptad hydrophobic repeat (residues 58−107) in TnI. Similarly, the putative binding region of TnI on TnT (residues 196−241, the conserved heptad hydrophobic repeat, as suggested by this work) is shown. The region of TnT which has been identified by us as contributing to Ca^{2+} sensitivity (residues 159−201) is also shown.

Interestingly, the COOH-terminal truncation of TnT and the lower level of Tn complex formation by TnT_{1-201}, as reported here, may have functional significance and a potential role in understanding the disease of striated muscles. For example, a splice site mutation in intron 15 of the human cardiac TnT gene produces a COOH-terminal truncation of the protein (Thierfelder et al., 1994). It has been suggested that reduced levels of cardiac TnT can cause familial hypertrophic cardiomyopathy whereas altered stoichiometry of sarcomeric proteins is possibly involved in secondary forms of cardiac hypertrophy (Thierfelder et al., 1994). Interestingly, in TnT_{1-201}, the region encoded by exon 15, which shares high similarity with the corresponding region in the cardiac TnT, is deleted.

In summary, by generating a large recombinant NH_2-terminal fragment of TnT and by studying its interaction with Tm, TnI, and TnC coupled with interaction studies involving TnT with the recombinant fragments of TnI and sequence analyses, we have identified a region where TnI and TnT predominantly interact. This region includes the COOH-terminal residues 202−258 of TnT and a region within the N-terminal 120 residues of TnI. These regions in both TnI and TnT have heptad repeats of hydrophobic residues which may be involved in their interaction most likely by forming a coiled-coil. We have shown that the N-terminal region of TnT is predominantly involved in Tm binding whereas the C-terminal region, particularly residues 202−258, contains binding sites of TnI and TnC. The C-terminal region also introduces Ca^{2+} sensitivity to the thin filament. We have also identified residues 159−201 as the region of TnT which is involved in contributing Ca^{2+} sensitivity to the reconstituted regulatory system. Finally, lower affinity ternary complex formation by the COOH-terminal deletion mutant of TnT suggests relevance to disease of striated muscles in which altered stoichiometry of proteins may be playing a role.

ACKNOWLEDGMENT

We thank Ms. E. Gowell for excellent help in HPLC analysis, Mr. R. Stefancsik for many helpful discussions, Dr. R. Willson for help with densitometer scanning, Dr. M. Gordon for comments on the manuscript, and Ms. Barbara Lewis for help in the preparation of the manuscript.

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BI9622433