A Recombinant Monocysteine Mutant (Ser to Cys-155) of Fast Skeletal Troponin T: Identification by Cross-Linking of a Domain Involved in a Physiologically Relevant Interaction with Troponins C and I†

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ABSTRACT: Troponin T (TnT), a subunit of the heterotrimeric troponin (Tn) complex, is essential for the Ca^{2+} regulation of vertebrate striated muscle contraction both in vivo and in vitro. With the exception of bovine cardiac TnT, all known vertebrate TnT isoforms lack a thiol group, a property which makes the wild-type proteins unsuitable as cross-linking substrate. We generated a mutant human fast skeletal TnT in which Ser^{155} was changed to Cys (TnT-Cys^{155}). Mutation of this residue in TnT as well as in vitro expression in *Escherichia coli* and purification of the recombinant mutant protein did not affect its biological properties in terms of in vitro binding to troponin I (TnI), troponin C (TnC), actin−tropomyosin (actin−Tm), and actomyosin ATPase activity. TnT-Cys^{155} was labeled with 4-maleimidobenzophenone (BP-TnT^{155}) and photo-cross-linked to TnI, TnC, Tm, and all of the thin filament proteins. BP-TnT^{155} did not cross-link to Tm and showed weak Ca^{2+}/Mg^{2+}-independent cross-linking with TnI in the binary complex and in the presence of all thin filament protein components. BP-TnT^{155} showed Ca^{2+}/Mg^{2+}-dependent cross-linking with TnC in the binary and ternary complexes and Ca^{2+}-favored cross-linking with TnI in the ternary complex. Thus, residue 155 of TnT is within 10 Å (the length of cross-linker) of TnC in the presence or absence of Ca^{2+} and comes within 10 Å of both TnI and TnC in the presence of Ca^{2+}. TnT residue 155 is in close proximity to or may even partly encompass the Tm binding site. These results suggest that TnT, in association with TnI, may participate in the "information transfer" mediated by the Ca^{2+} binding signal from TnC to Tm and the region around TnT residue 155 probably acts as a linker between troponin and actin−Tm in this signal transmission process. Our results also suggest that TnT contains at least one Ca^{2+}/Mg^{2+}-dependent TnC binding region located between its Tm and TnI binding regions. A recombinant truncated fragment of TnI, TnI_{96-181}, containing amino acid residues 96−181 and labeled with BP at Cys-133, failed to cross-link with TnT, indicating that the region around Cys-133 of TnI is not involved in binary interaction with TnT.

The contraction of vertebrate striated muscles is regulated by binding of Ca^{2+} to the troponin (Tn)[1] complex which is located in the thin filament and consists of three subunits that differ in structure and function: troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC binds Ca^{2+}; TnI binds actin−tropomyosin (actin−Tm) and inhibits muscle contraction in the absence of Ca^{2+}, whereas TnT attaches the Tn complex to Tm. TnT is essential for an effective Ca^{2+}-dependent regulation and also increases the cooperativity of actin−Tm and provides Ca^{2+} sensitivity to the thin filament (*1*−*4*). The binding of Ca^{2+} to TnC triggers muscle contraction by multiple interactions of thin filament proteins. However, the nature of these interactions and the precise mechanism and pathway by which the Ca^{2+} binding signal is transmitted are not completely understood (for reviews, see *5*−*8*). Among the interactions of the Tn subunits, information on those involving TnT is rather limited partly due to the relative insolubility of this protein and perhaps also because it lacks a suitable amino acid residue to be used for experimental approaches such as photo-cross-linking and fluorescence enhancement studies. The complex pattern of alternative splicing of fast skeletal TnT gene (*9*), the correlation of cardiac myopathies with the mutation in and expression of the cardiac TnT gene (*10*, *11*), and the reported specificity of cardiac TnT as a sensitive marker for

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[1] Abbreviations: BPMal, 4-maleimidobenzophenone; BP-TnT^{155}, BPMal-labeled troponin T at Cys-155; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; βME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tn, troponin; Tm, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; TnT-Cys^{155}, troponin T with a Ser to Cys mutation at amino acid residue 155; UTR, untranslated region; TnI_{96-181}, recombinant fragment of TnI consisting of amino acid residues 96−181; BP-TnI_{96-181}, recombinant TnI_{96-181} fragment labeled with BPMal at amino acid residue Cys-133; $TnT_{E.\ coli}$, βTnT purified from *E. coli* expression system.

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the detection of myocardial cell injury (for a review, see *12*) have all generated an interest in understanding the biology of TnT.

Several different biochemical and molecular techniques have been used to map the interacting domains of Tn subunits. These studies have provided the basic understanding of how the three subunits interact (for reviews, see *5*−*8*). However, the specific domains involved in the binary and ternary interactions as well as the nature and importance of these interactions in activating muscle contraction are not fully understood. This is mainly because only the crystal structure of TnC is known. Until the structures of TnI and TnT are solved, information on the Tn subunit interaction in a three-dimensional orientation may be obtained by other useful approaches such as cross-linking. Heterobifunctional cross-linking agents such as 4-maleimidobenzophenone (BP-Mal) have been used successfully in labeling TnC and TnI and studying their interaction with each other and with TnT (*4*, *13*−*22*). This approach could not be undertaken with TnT because no known vertebrate TnT, with the sole exception of bovine cardiac TnT (*23*), contains a thiol group. The two isoforms of bovine cardiac TnT have one thiol group each (Cys-39, Cys-34) but in a region which is not known to be involved in a specific interaction with any thin filament protein. A single Cys residue is present in the recently reported TnT molecules from the invertebrates (*24*, *25*).

Studies using the CnBr fragments of TnT for interaction with other Tn subunits have shown a complex pattern because these fragments contain overlapping binding domains of Tm, TnI, and TnC. The N-terminal rodlike fragment of TnT containing amino acid residues 1−158, together with a C-terminal region consisting of residues 243−259, is involved in binding to Tm, whereas, the C-terminal globular T2 fragment consisting of residues 159−259 interacts with TnI and TnC (*6*, *16*, *26*−*30*). The N-terminal portion containing residues 159−221 of the T2 fragment has been proposed as the major, Ca^{2+}-sensitive, TnC binding region whereas the C-terminal portion of the fragment, residues 206−258, is considered the second, Ca^{2+}-insensitive, binding region for TnC (*15*, *27*). Previous studies by us, using a recombinant truncated fragment of TnT which contained residues 1−201, suggested that TnT residues 202−258 constitute the site for TnI binding, and a portion of TnT residues 1−201 is involved in TnC interaction (*31*). To gain further insight into the binding domains of TnT which interact with TnI and TnC, we have generated a monocysteine mutant of human fast skeletal β TnT (TnT-Cys^{155}) using recombinant DNA techniques. This developmentally regulated isoform of TnT is the major isoform in the mammalian fast skeletal fetal muscle and is constitutively expressed both in fetal and in adult muscles (*32*). The mutant protein was purified from the *E. coli* expression system without any loss in its biological properties. TnT-Cys^{155} was labeled with BPMal (BP-TnT^{155}) and photo-cross-linked to TnI, TnC, and Tm. BP-TnT^{155} did not cross-link to Tm. It showed a weak Ca^{2+}/Mg^{2+}-independent cross-linking with TnI in binary complexes and formed a Ca^{2+}/Mg^{2+}-dependent cross-linked product with TnC both in the binary and in the ternary complexes. It also showed Ca^{2+}-favored cross-linking with TnI in the ternary complex. Our results, using a thiol mutant of TnT for the first time, suggest that residue 155 of TnT is within 10 Å of TnC in the presence or absence of Ca^{2+} and comes within 10 Å of both TnI and TnC in the presence of Ca^{2+}. These results also indicate that TnT contains at least one Ca^{2+}/Mg^{2+}-dependent TnC binding region located between its Tm and TnI binding regions. Furthermore, they provide some insights into the transmission of "information transfer" mediated by a Ca^{2+} binding signal from TnC to other thin filament proteins.

MATERIALS AND METHODS

Construction of TnT-Cys^{155} Mutant. The point mutation which converted the 155th codon (excluding the initiating Met codon) encoding Ser (AGC) to Cys (TGC) was generated by polymerase chain reaction (PCR)-based site-directed mutagenesis. Human fast skeletal β TnT cDNA (*32*), subcloned in T7 polymerase promoter-based vector pET17b for high-level expression (*33*), was used as DNA template. PCR was performed as described previously (*34*) using primer 1 (5′GCTGTCCTCCATGGGCGCCAACTA-CAGC*T*GCTAC) and primer 2 (5′GCTAGTTATTGCT-CAGCGG). Primer 1 was designed to bind at the unique internal *Nco*I site (underlined) of TnT cDNA in pET17b and contained the underlined A→T point mutation whereas primer 2 was complementary to vector sequence. 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 (a new *Pvu*II site was created due to the point mutation) and DNA sequencing.

Synthesis of TnT-Cys^{155} in E. coli and Its Purification. TnT-Cys^{155} was synthesized in *E. coli* strain BL21(DE3) which was grown in NZCYM medium (*35*) at 37 °C. After A_{600} of the culture reached ∼0.6, it was allowed to grow for 6 h without induction. TnT- Cys^{155} synthesis was constitutive and optimal under these conditions as reported previously by us for wild type TnT (*31*). The cells from a 1 L culture were harvested by centrifugation at 5000*g* for 10 min. Cell lysis was carried out by incubating with lysozyme and deoxycholic acid followed by DNase I treatment as described by Sambrook et al. (*35*). All centrifugations were carried out at 27000*g* for 30 min unless mentioned otherwise. The pellet was subsequently suspended in 50 mL of solution A [20 mM Tris·HCl, pH 8.0, 0.1 M NaCl, 2 mM EDTA, 1.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)], centrifuged, and homogenized manually in a glass homogenizer for 1 h in 50 mL of solution B (6 M urea, 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, and 1.5 mM DTT) and centrifuged. TnT-Cys^{155} partitioned at 60:40 ratio in insoluble (pellet) and soluble (supernatant) fractions. Immunoblot analysis using a monoclonal antibody against TnT revealed that the recombinant protein from the soluble fraction was more immunoreactive than that present in the insoluble fraction (Figure 1A, lanes 1−3). Therefore, only the soluble fraction of the cell homogenate was used for TnT-Cys^{155} purification. The soluble fraction was made to 50 mM Tris·HCl, pH 7.5, and subjected to ammonium sulfate fractionation. Most of the TnT-Cys^{155} was retained in the 30−90% ammonium sulfate fraction. The pooled fractions were suspended and extensively dialyzed against solution B and loaded onto a DE-52 column (Whatman, bed volume 15 mL L^{-1} *E. coli* culture) equilibrated with the same solution. The column was run at 0.5 mL/min and washed with 15 mL of buffer B containing 0.15 M NaCl. TnT-

Cys^{155} was eluted stepwise with 15 mL each of buffer B containing 0.38 and 0.45 M NaCl, respectively. The purified protein was concentrated by ultrafiltration, 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. Rabbit skeletal muscle TnI and TnC were used for the formation of binary and ternary complexes. The isolation of proteins and the formation of T−C and T−I−C complexes and their analysis by native PAGE were carried out as previously described (*4*, *31*). The TnI−TnT complex formation was analyzed by high-performance liquid chromatography (HPLC) as described previously (*31*).

Photo-Cross-Linking. The method of Tao et al. (*18*) was used with minor modifications (*4*) for BPMal labeling and photo-cross-linking. Rabbit muscle TnI, TnC, and also TnT-Cys^{155} were unfolded in a medium as described by Tao et al. (*18*) with the modifications that 0.5 M NaCl and 10 mM DTT were also used. Labeling of TnT-Cys^{155} with BPMal was carried out in their 1:3 molar ratio. Photo-cross-linking of BP-TnT^{155} (8 μM) with Tm (8 μM), TnI (8 μM), and/or TnC (10 μM) with or without F-actin was carried out in a photochemical reactor (Rayonet RPR-100, Southern New England Ultraviolet, Hamden, CT). Five hundred microliters of reconstituted sample was prepared and dialyzed against the irradiation buffer [20 mM Hepes, pH 7.5, 0.1 M NaCl, and 2 mM DTT at 4 °C (*4*, *18*)] for 40 min. Three replicate aliquots, each 100 μL, were irradiated in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA or 10 mM $MgCl_2$, and a nonirradiated aliquot of 100 μL was maintained. A recombinant C-terminal fragment of TnI, TnI_{96-181}, containing amino acid residues 96−181 and a single Cys residue at 133, previously reported by us (*4*, *31*), was labeled with BPMal, and was also used for cross-linking studies. EDTA (25 mM) was added to all the reaction mixtures before subjecting them to SDS−PAGE using 12% or 4−20% gradient polyacrylamide gels.

Other Biochemical Assays. The acto−S1 ATPase activity of the reconstituted Tn complex in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA, cosedimentation of TnT, TnI, or TnT+TnI with F-actin+Tm, immunoblotting using a monoclonal antibody against rabbit fast skeletal TnT (Sigma, St. Louis, MO), and densitometric scannings were performed essentially as described previously (*4*, *31*, *34*).

RESULTS

Generation, E. coli Expression, and Purification of TnT-Cys^{155}. The mutation was identified by restriction mapping and confirmed by bidirectional sequencing of cloned PCR-amplified DNA. The expression was optimized using the *E. coli* strain DE3 which we have previously used successfully for wild-type TnT expression (*33*). Several investigators (*36*) have previously shown that the N-terminal methionine is cleaved off by *E. coli* in in vitro expressed troponin subunits. We have, therefore, considered the mutant to be authentic with N-terminal methionine deleted. TnT-Cys^{155} was purified as indicated under Materials and Methods and was found to be ~95% homogeneous by SDS−PAGE (Figure 1B). The mutant cross-reacted with a monoclonal antibody against rabbit fast skeletal TnT (Figure 1A) which also confirmed its identity and immunoreactivity.

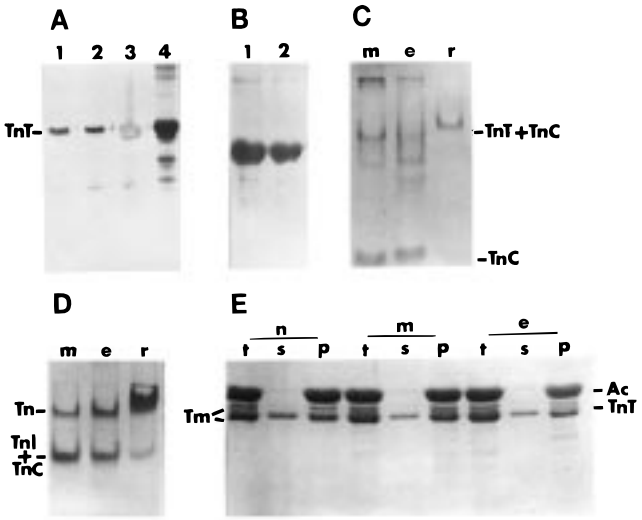

FIGURE 1: Purification and biological properties of TnT-Cys^{155}. For details, also see Materials and Methods. Panel A: Immunoblot of the proteins from *E. coli* cells expressing TnT-Cys^{155} after cell lysis and urea extraction. A monoclonal antibody against rabbit fast skeletal TnT was used as the primary antibody. Lane 1, total extract; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, semipurified rabbit fast skeletal TnT used as the positive control. Two microliter samples containing 50 ng of proteins (lanes 1−3) and 300 ng of muscle TnT (lane 4) were loaded. Panel B: SDS−PAGE of purified TnT-Cys^{155}. Lane 1, purified sample concentrated by ultrafiltration (15 μg); lane 2, TnT-Cys^{155} eluted from DE-52 column (8 μg). Panels C and D: TnT−TnC complex (panel C) and TnI−TnT−TnC complex (panel D) formation by various TnTs in native polyacrylamide gel. TnT-Cys^{155} (m), *E. coli* expressed wild-type human fast skeletal muscle TnT (e), and rabbit fast skeletal muscle TnT (r) were examined for complex formation. The binary and ternary complexes and uncomplexed TnC are shown. Uncomplexed TnT and TnI did not enter the gel. Fifty microliter samples containing TnT (8 μM), TnI (8 μM), and/or TnC (10 μM) was loaded in each gel well. Panel E: Cosedimentation of TnT-Cys^{155} (m) and $TnT_{E.\ coli}$ (e) with actin−Tm. The negative control (n) contained no TnT. Reaction mixtures contained actin (28 μM), Tm (4 μM), and TnT (4 μM) in a total volume of 50 μL. Equal volumes of unspun (t) and spun samples partitioned into the supernatant (s) and pellet (p) were loaded in adjacent lanes and subjected to SDS−PAGE analysis. Ac, actin.

Biological Properties of TnT-Cys^{155}. TnT isolated from rabbit fast skeletal muscle (muscle TnT; an adult isoform of TnT predominantly of α type) and human fast skeletal TnT expressed in *E. coli* ($TnT_{E.\ coli}$; a fetal isoform of TnT of β type) were used as positive controls in assaying the biological properties of the mutant protein. TnT-Cys^{155} has been derived from $TnT_{E.\ coli}$. Previously, we have shown that $TnT_{E.\ coli}$ is only about half as active as muscle TnT in almost all biological properties examined (*33*). Similar results were also obtained by Pan and Potter (*37*) using a truncated TnT β fragment containing the C-terminal 108 residues. Muscle TnT, $TnT_{E.\ coli}$, and TnT-Cys^{155} formed a TnT−TnC complex of intermediate mobility in native polyacrylamide gels containing 0.5 mM $CaCl_2$ (Figure 1C) but not in gels with 5 mM EDTA (results not shown). As expected, the TnC−TnT complex formed by $TnT_{E.\ coli}$ and TnT-Cys^{155} was relatively weak as compared to muscle TnT based on the intensity of the band in gel runs and also by the presence of a high amount of uncomplexed TnC in the sample (Figure 1C). Densitometric scanning and normalization of data with regard to the muscle TnT lane revealed that complex formed by $TnT_{E.\ coli}$ and TnT-Cys^{155} represented about 23 and 24%,

Table 1: Regulation of Acto−S1 ATPase Activity [mol of P_i (mol of S-1)$^{-1}$ min^{-1}] by Troponin Reconstituted with Muscle TnT, $TnT_{E.\ coli}$, and TnT-Cys^{155} [a]

troponin reconstituted with	ATPase activity +Ca^{2+}	ATPase activity +EGTA	Ca^{2+} sensitivity (%)
no troponin	51.1	51.1	0.0
muscle TnT	60.4	24.4	59.6
$TnT_{E.\ coli}$	50.0	33.3	33.4
TnT-Cys^{155}	54.4	37.8	30.5

[a] The calcium sensitivity was calculated as follows: (1 − EGTA rate/Ca^{2+} rate) × 100. The assays were carried out simultaneously with those using a deletion mutant of TnT, TnT_{1-201} containing residues 1−201, previously reported by us (*31*). Therefore, the ATPase activities of muscle TnT, TnT-Cys^{155}, and $TnT_{E.\ coli}$, reported here, and of TnT_{1-201} (*31*) are directly comparable.

respectively, as compared to the complex formed by muscle TnT. Bands of slower and faster mobility than TnT−TnC complex were also observed with both $TnT_{E.\ coli}$ and TnT-Cys^{155}. Presumably, they represent the dissociated TnT or TnC from the weak TnT−TnC complex during electrophoresis. Both $TnT_{E.\ coli}$ and TnT-Cys^{155} also showed a comparable and weak interaction with muscle TnI as detected by HPLC. Whereas muscle TnI and TnT formed a distinct peak of TnI−TnT binary complex as previously reported by us (*31*), those formed by $TnT_{E.\ coli}$ and TnT-Cys^{155} appeared only as a "shoulder peak" (results not shown).

Similarly, the Tn complex formed by $TnT_{E.\ coli}$ and TnT-Cys^{155} was relatively weak as compared to muscle TnT (Figure 1D). Densitometric scanning and normalization of data with reference to the muscle TnT lane revealed that the Tn complex formed by $TnT_{E.\ coli}$ and TnT-Cys^{155} represented 39 and 34%, respectively, as compared to that formed by muscle TnT. When Tn complexes were assayed for acto−S1 ATPase activity, both $TnT_{E.\ coli}$ and TnT-Cys^{155} showed about half Ca^{2+} sensitivity as compared to the complex formed by muscle TnT under our assay conditions (Table 1). It should be noted that the acto−S1 ATPase assay using the reconstituted components shows considerable variation in activity (*38*). The addition of Tm is known to alter the relatively high Ca^{2+}-insensitive ATPase activity of myosin and actin (*7*, *39*, *40*). Upon addition of the Tn complex to this system, the ATPase is activated at micromolar Ca^{2+} concentrations but is inhibited at submicromolar Ca^{2+} concentrations (*1*). The Ca^{2+} sensitivity of the acto−S1 ATPase activity perhaps reflects the most important physiologically relevant parameter of the reconstituted system. The biological activity of βTnT, a fetal isoform, as well as its thiol mutant, is presented in terms of both ATPase value and Ca^{2+} sensitivity (Table 1). The results are consistent with the current view (*33*, *37*) that in vertebrates the fetal TnT isoforms have lower biological activity than the adult isoforms, e.g., muscle TnT used in this study.

Cosedimentation experiments were performed using both $TnT_{E.\ coli}$ and TnT-Cys^{155} to examine their ability to bind to actin−Tm. Under the experimental conditions selected, TnT would sediment only if it was bound to actin−Tm. Unbound TnT would not sediment upon centrifugation. At a actin:Tm:TnT molar ratio of 7:1:1, $TnT_{E.\ coli}$ and TnT-Cys^{155} cosedimented with actin−Tm (Figure 1E). This pattern of cosedimentation is essentially similar to that reported previously with muscle TnT (*31*).

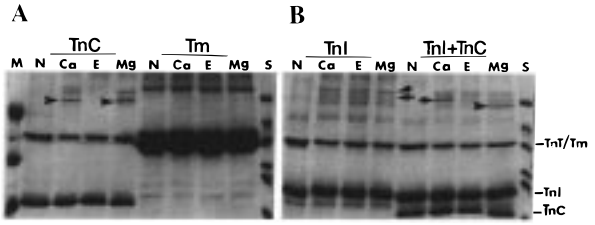

FIGURE 2: Photo-cross-linking of BPMal-labeled TnT-Cys^{155} with TnC, Tm (panel A), or TnI and TnI−TnC (panel B). Four experimental conditions in which photo-cross-linked patterns were compared are as follows: N, no irradiation; Ca, with 0.1 mM $CaCl_2$; E, with 2 mM EGTA; and Mg, with 10 mM $MgCl_2$. TnT−TnC cross-linked product is shown by arrowheads, whereas TnT−TnI−TnC product is indicated with an arrow. M, molecular size markers of 46, 30, and 21.5 kDa (Amersham, Arlington Heights, IL); S, molecular size markers of 66, 45, 36, 29, 24, and 20.1 kDa (Sigma, St. Louis, MO). For details, also see Materials and Methods. An aliquot of the cross-linked reaction mixtures (65 μL) after addition of EDTA and loading buffer was concentrated to 20 μL, using ultrafiltration through a 10 kDa molecular mass cutoff filter (Millipore Inc., Bedford, MA), and loaded in each gel well.

Photo-Cross-Linking of TnT-Cys^{155} with Thin Filament Proteins. TnT-Cys^{155} was labeled with BPMal and irradiated in the presence of either Tm, TnI, or TnI−TnC. Each incubation sample was subdivided into four aliquots. Three of them were irradiated in the presence of Ca^{2+}, Mg^{2+}, or EGTA, and a nonirradiated control was maintained (Materials and Methods). The cross-linked product was identified by comparing with the nonirradiated control and also by the molecular mass of the apparent cross-linked complex. Irradiation of BP-TnT^{155} with Tm resulted only in background cross-linking (Figure 2A), whereas irradiation of BP-TnT^{155} with TnC resulted in a major cross-linked product in the presence of either Ca^{2+} or Mg^{2+} (Figure 2A, arrowheads). This band was not observed in nonirradiated control or in the irradiated sample containing EGTA. Thus, Cys^{155} of TnT is close to TnC, and this interaction is Ca^{2+}/Mg^{2+}-dependent. The apparent molecular mass of this cross-linked product was ∼60 kDa which could be due to the well-documented anomalous migration of Tn components in SDS−PAGE, especially when a bulky cross-linker was attached to the polypeptide (*18*, *31*). A comparison with the molecular size markers indeed showed (Figure 2A,B) that free TnT, TnI, and TnC migrated at apparent molecular masses of ∼37, 23, and 21 kDa, respectively, although they have calculated molecular masses of 30.5, 21, and 17.9 kDa, respectively. Irradiation of BP-TnT^{155} with TnI resulted in the formation of heterogeneous and relatively weak cross-linked bands (Figure 2B, arrows). These products were independent of Ca^{2+}/Mg^{2+}, as they were detected in all irradiated samples, including that in the presence of EGTA (Figure 2B).

Cross-linking of BP-TnT^{155} with TnI and TnC in a ternary complex resulted in at least two major cross-linked products. The first one was present in the sample containing Mg^{2+} (Figure 2B, arrowhead) and corresponded in size and appearance to the cross-linked product observed when BP-TnT^{155} was irradiated with TnC alone (Figure 2A, arrowheads). This apparent TnT−TnC cross-linked product was also present, albeit in much lower amounts, in the sample containing Ca^{2+} (Figure 2B). The second major cross-linked product of ∼66 kDa (Figure 2B, arrow) was observed in the presence of Ca^{2+}. This product was not observed in the control sample and appeared to be present only at very low

levels in the presence of Mg^{2+} or EGTA (Figure 2B). On the basis of electrophoretic mobility, this product appeared to be a binary complex of TnI and TnT. The above results indicated that the TnT−TnI cross-linked species was formed at a higher level in the presence of TnC and this binary complex formation was observed predominantly when both TnC and Ca^{2+} were present. In contrast, the TnT−TnC cross-linking could take place in the presence of Ca^{2+} or Mg^{2+} both in binary and in ternary complexes. Some cross-linked products showing lower electrophoretic mobility than that expected from the molecular mass of the binary or ternary complexes were also observed, especially in samples containing TnC. Such cross-linked products have been reported previously in the literature (*18*). They probably arise due to the cross-linking of TnT-Cys^{155} at different sites on TnC giving rise to products that differ in shape and, therefore, are expected to migrate at different mobilities in gels.

The cross-linking pattern of TnT-Cys^{155} with other Tn subunits was also examined in the presence of all thin filament proteins, i.e., F-actin, Tm, TnC, TnI, and TnT-Cys^{155}. In general, very low levels of cross-linked species were formed in the presence of F-actin and Tm. Two Ca^{2+}/Mg^{2}-independent cross-linked products of different mobilities were observed. One of these appeared to represent a TnI−TnT cross-linked product (Figure 3A, arrowhead). On the basis of the molecular mass, the slower migrating cross-linked species of ∼80 kDa (Figure 3A, arrow) appeared to be a cross-linked product of either actin−TnT or Tm−TnT, the latter being formed only when actin was also present, because Tm and TnT did not cross-link in the binary complex (Figure 2A). In a previous report, Tao et al. (*18*) examined the interaction of Tn subunits with Tm by using BPMal-labeled Tm. They observed that the level of cross-linking is decreased drastically when F-actin was also incorporated in the sample containing Tn subunits and BPMal-labeled Tm (see Figure 1 in ref *18*). The apparent dissimilarity in the cross-linking pattern of Tm subunits in our studies, with or without F-actin, may be partly due to the low yields of the cross-linked product, particularly in the presence of F-actin. Alternatively, it is quite likely that the cross-linking pattern in the binary/ternary complex is different from that in the reconstituted thin filament system.

To further support our cross-linking results obtained with TnT-Cys^{155}, we labeled a recombinant fragment of TnI, TnI_{96-181}, containing the C-terminal residues 96−181. As this fragment contains the C-terminal and inhibitory regions of TnI, it is also termed TnI_{C+1}. This fragment represents the most important region of TnI in terms of biological activity (*4*, *7*, *36*), and contains a single Cys residue (Cys^{133}) as compared to the full-length TnI which has three Cys residues (Cys^{48}, Cys^{64}, and Cys^{133}). Studies by several groups, including us (*7*, *31*, *39*), have shown that this region of TnI interacts with TnC and actin but not with TnT. As TnT-Cys^{155} showed a lower interaction with Tn subunits, muscle TnT was used for cross-linking with BP-TnI_{96-181}. Indeed, it was observed that BP-TnI_{96-181} did not cross-link with muscle TnT under our assay conditions (Figure 3A). These results indicate that (i) Cys-133 of TnI is not close to TnT, and the region around this residue of TnI does not interact with TnT; and (ii) cross-linking of Tn subunits takes place only due to specific interactions.

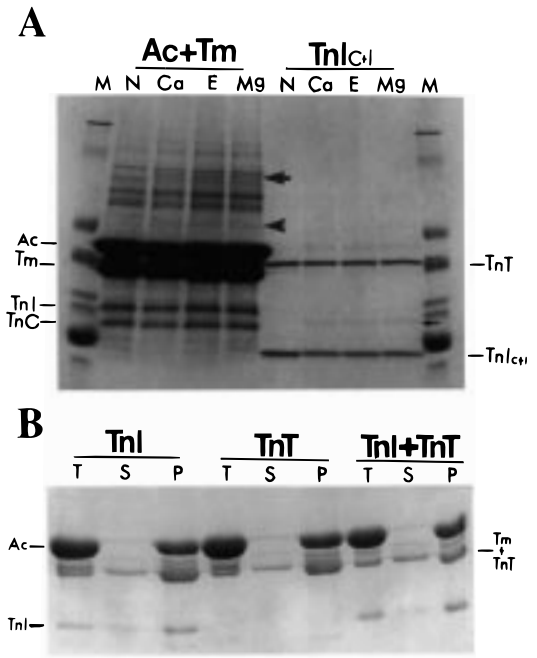

FIGURE 3: Interaction of TnT-Cys^{155} with TnI and TnC in the presence of F-actin and Tm. (A) Photo-cross-linking of BP-TnT^{155} with TnC and TnI in the presence of Tm and F-actin (indicated as A_c+Tm), and photo-cross-linking of BP-TnI_{96-181} with muscle TnT (indicated as TnI_{C+1}). For details, also see Materials and Methods. The four experimental conditions (N, Ca, E, and Mg) used in cross-linking were the same as described in the legend to Figure 2. TnT−TnI complex is shown by an arrowhead. A slow migrating product of ∼80 kDa, which may represent either TnT−actin or TnT−Tm product, is indicated by the arrow. The small arrow in panel TnI_{C+1} shows a band present in irradiated samples only, and this may be a dimer of TnI_{96-181}. Samples were processed as per the legend to Figure 2 and run in 4−20% gradient gels. M, molecular size markers of 250, 148, 60, 42, 30, 22, 17, and 6 kDa (Novex, San Diego, CA). (B) Cosedimentation of TnT-Cys^{155} with TnI in the presence of F-actin and Tm. For details, also see Materials and Methods and legend to Figure 1E. Cosedimentation with F-actin and Tm was carried out with TnT-Cys^{155} alone, TnI alone, and TnT-Cys^{155} + TnI. T, unspun; S, supernatant fraction; P, pellet fraction of the samples. The positions of actin (Ac), TnI, TnT, and Tm in the gel runs are indicated.

In view of the detectable but weak cross-linking of TnT-Cys^{155} with TnI in the binary complex (Figure 2B), cosedimentation in the presence of F-actin and Tm, using the combinations TnT-Cys^{155} alone, muscle TnI alone, and TnT-Cys^{155} + muscle TnI, was also carried out as a supplementary approach to analyze the TnI−TnT binary interaction. Both TnI and TnT-Cys^{155}, used either individually or in combination, showed identical and almost complete cosedimentation with F-actin−Tm (Figure 3B). Thus, this method, although demonstrating strong binding of TnT and TnI individually to F-actin−Tm, was limited for studying TnT−TnI binary interaction. These results also indicate that cross-linking may currently be the method of choice for studying weak TnI−TnT interaction.

DISCUSSION

Among the Tn subunits, the crystal structure of only TnC is known. Electron microscopic study of the Tm−Tn complex has revealed that the Tn complex has both a globular and a rodlike part (*28*). Furthermore, the globular region of the Tn complex consists of TnC, TnI, and a

carboxy-terminal portion of TnT (*41*). Therefore, to investigate the interaction patterns of the three subunits in a three-dimensional orientation, it is imperative to use alternative techniques. The use of a heterobifunctional cross-linking agent such as BPMal appears to be one of the most convenient and reliable approaches for such investigations. Previously, cross-linking approaches have been utilized by attaching the heterobifunctional probe such as BPMal to TnC and TnI by us and other groups (*4*, *13*−*22*). As TnC has one thiol group (Cys-98) and TnI has three (Cys-48, Cys-64, and Cys-133), these two proteins have been extensively used for labeling with the cross-linker. On the other hand, no known vertebrate TnT except bovine cardiac TnT has a thiol group. Our studies with TnT-Cys^{155} are the first in which a heterobifunctional cross-linker has been attached to a recombinant thiol mutant of TnT for investigating its interaction with other thin filament proteins.

Previous studies in the literature using the CnBr fragments of TnT and several different approaches including cross-linking have shown that TnT contains overlapping binding domains that interact with Tm, TnI, and TnC in a complex manner. TnT residues 1−158 (T1 fragment) and 243−259 bind to Tm (*26*−*28*) whereas residues 159−259 (T2 fragment) interact with TnI and TnC (for a review, see *6*). Studies with smaller CnBr fragments of TnT have shown that they bind to TnI in the following descending order: 159−259, 176−230, 239−259, or 152−175 (*16*, *42*). TnT fragments 152−209, 135−185, and 71−151 also bind to TnI (*16*, *30*). Interestingly, many of these fragments such as 152−209 and 176−230 bind to TnC also (*16*, *29*). TnT residues 159−259 contain an extensive binding region for TnC. It has been proposed that the N-terminal portion of this fragment, residues 159−221, is the major, Ca^{2+}-sensitive TnC binding region whereas the C-terminal portion of the fragment, residues 206−258, is the second, Ca^{2+}-insensitive binding region for TnC (*15*, *27*). Our own studies, using a recombinant truncated fragment of TnT containing amino acid residues 1−201, have shown that whereas TnT residues 202−258 may be a binding site for TnI, at least a part of TnT residues 1−201 is involved in TnC interaction in addition to residues 202−258 (*31*).

To further dissect the TnI and TnC binding domains of TnT, we have generated a Ser→Cys mutation at TnT residue 155. TnT residue Ser-155 was converted to Cys-155 because (i) Ser→Cys mutation is benign due to the chemical, structural, and functional similarity of these two amino acids, (ii) a comparative sequence analysis revealed that Ser-155 is conserved only among fast skeletal TnT and not in slow skeletal, cardiac, or invertebrate TnT (*24*, *32*), (iii) it is a potential phosphorylation site but phosphorylation has no known physiological significance in TnT; besides, the Ser-155 site is perhaps the least important among three phosphorylatable sites in fast skeletal TnT, and (iv) it is located in a region where Tm, TnI, and possibly TnC may also interact (*26*, *29*, *30*).

The mutant TnT-Cys^{155} showed essentially similar biological characteristics as the wild-type TnT purified from *E. coli* ($TnT_{E.\ coli}$) with respect to binary TnI−TnT and TnT−TnC complex formation, ternary Tn complex formation, cosedimentation with actin−Tm, and acto−S1 ATPase activity (Figure 1; Table 1). These results indicated that neither the Ser→Cys mutation nor the in vitro expression in *E. coli* and subsequent purification has affected the biological activity of the protein. In rabbit skeletal muscle TnT, Ser-1, Ser-149/150, and Ser-155/156 can be phosphorylated (*43*, *44*). In human fast skeletal muscle TnT, the corresponding residues are Ser-1, Ser-148/149, and Ser-154/155, among which Ser-155 was mutated to Cys in our work. The similarity in biological activity of mutant and wild-type TnT is consistent with the observations that the phosphorylation of TnT is perhaps not of physiological importance (*45*).

We selected BPMal as the cross-linker because it does not affect the biological properties of the labeled protein and apparently represents the true interaction between the cross-linked proteins (*18*, *23*). Irradiation of BP-TnT^{155} with Tm resulted only in background cross-linking (Figure 2A,B). Although the T1 fragment (residues 1−158) of TnT interacts with Tm, the interaction primarily involves residues 71−151 of TnT which may form a coiled-coil with Tm. Cys^{155} is outside this coiled-coil region and, therefore, forms only a background level of cross-links.

With respect to cross-linking of BP-TnT^{155} with Tn subunits, the following features are noted: BP-TnT^{155} formed heterogeneous and weak cross-links with TnI. This cross-linking was independent of Ca^{2+}, as the cross-linked bands were present at similar levels in irradiated samples containing Ca^{2+}, Mg^{2+}, or EGTA (Figure 2B, arrows). The observed weak cross-linking pattern may be significant, because TnT-Cys^{155}, which is a derivative of the TnT β isoform, shows relatively weak interaction with TnI and TnC in the binary and ternary complexes and a reduced ATPase activity as compared to muscle TnT, the adult isoform (Figure 1, Table 1). The wild-type and recombinant βTnT's also have significantly reduced biological activity than the adult isoform, as previously reported by us (*33*). Previous results on CnBr fragments and those obtained by cross-linking with the probe attached to TnI suggested that residues 71−175 of TnT bind TnI (*16*, *30*). Cross-linking of TnI with BP-TnT^{155} is consistent with these reports. Furthermore, these results are also in agreement with the view that the TnI−TnT interaction is primarily independent of Ca^{2+} (*14*; for a review, see *6*). Irradiation of BP-TnT^{155} with TnC resulted in a clearly detectable cross-linked product which was Ca^{2+}/Mg^{2+}-dependent (Figure 2A, arrowheads). Thus, Cys^{155} of TnT is within 10 Å (the length of BPMal) of TnC, and the two proteins interact in a Ca^{2+}/Mg^{2+}-dependent manner. These results suggest that at least one TnC binding site on TnT (residues ∼152−195) is located between the binding sites for Tm (TnT residues 71−151) and TnI (TnT residues 196−241). This view is consistent with the observation that TnT residues 159−259, 176−230, and, in particular, 152−209 bind TnC (*6*, *29*). Our results are also consistent with those reported by Leszyk et al. (*46*), who found that residues 84−135 of TnC cross-linked to TnT residues 152−230. Their results also suggested that TnT residues 175−178 were very close to Cys-98 of TnC, to which they had attached the BPMal. Taken together with our results, it appears that residue 155 in TnT^{155} is quite close to Cys-98 of TnC. The cross-linking pattern of BP-TnT^{155} with TnI and TnC in a ternary complex was also consistent with the aforementioned results. For example, a major cross-linked product was observed in the presence of Mg^{2+}(Figure 2B, arrowhead) which corresponded in size and appearance to the cross-linked TnT−TnC product (Figure 2A, arrowheads). This

product was also detectable in the presence of Ca^{2+}, but the major cross-linked product was that of TnT−TnI in the presence of Ca^{2+} (Figure 2B, arrow). Thus, the formation of a TnT−TnI cross-linked product was favored in the presence of Ca^{2+} and especially when TnC was also present. These results are consistent with those obtained by Hitchcock-De Gregori (*47*), who showed that Ca^{2+} binding to TnC affects the TnI−TnT interaction. Our results also indicate that TnC remains within 10 Å (the length of BPMal) of TnT^{155} in the presence and absence of Ca^{2+}. Furthermore, in the presence of Ca^{2+}, the structure of TnC or the binding sites of TnC for TnI/TnT are altered in such a way that TnI−TnT interaction is promoted in the vicinity of TnT^{155}, a region very close to or even partly encompassing the TnT−Tm binding region. The specificity of TnT−TnI photo-cross-linking in our studies is supported by the fact that BP-TnI_{96-181}, a truncated recombinant fragment of TnI containing amino acid residues 96−181, and labeled at the single Cys-133 residue, did not cross-link when used as a negative control with muscle TnT (Figure 3A). Finally, regarding the reduced level of cross-linking of BP-TnT^{155} with Tn subunits in the presence of F-actin and Tm (Figure 3A), our results are consistent with the observation reported in the literature (*18*) that the cross-linking of BP-Tm with Tn subunits was reduced drastically in the presence of F-actin. Our cross-linking results, reported in this study, are also relevant to the problem of activation of muscle contraction and the role of TnT in this process. For example, the recent analysis of the ATPase activity of deletion mutants of TnT by Malnic et al. (*48*) revealed that residues 1−191 of TnT activate, whereas, residues 196−263 inhibit, ATPase and that the activation was observed only when intact TnT, TnC, TnI, and Ca^{2+} were present.

We have recently identified evolutionarily highly conserved heptad repeat (HR) domains among the members of TnI and TnT (*49*). Using both deletion and point mutations which disrupt the HR motif in TnT and by utilizing the sensitive yeast two-hybrid system, we have shown that the TnT mutants selectively fail to interact with TnI but these mutations do not affect TnC binding. Furthermore, the HR-containing domains of TnT (inclusive of residues 196−241) and TnI (residues 58−108) also show binary interaction as efficiently as the intact proteins (*49*). Whereas these results strongly suggest a coiled-coil heterodimer formation between TnI and TnT, such an interaction remains to be established by X-ray crystallography or NMR studies.

On the basis of the aforementioned results and the cross-linking studies reported here and considered together with the published literature, we propose a model for the transmission of the Ca^{2+} binding signal from TnC to other thin filament proteins as follows (Figure 4). TnT residues 196−241 and TnI residues 58−108 remain in constant interaction (*31*) both in the presence and in the absence of Ca^{2+} (*14*; for a review, see *6*). This interaction, which is not affected by TnC, is presumably mediated by a highly conserved HR region present in both proteins (*49*). Upon Ca^{2+} activation/binding, the structure of TnC and/or its binding site on TnI/TnT is altered which in turn strengthens the TnI−TnT interaction close to TnT^{155}. This Ca^{2+}-favored enhanced TnT−TnI interaction, which we have identified by photo-cross-linking in the ternary complex, therefore extends at least up to TnT^{155} (i.e., extends from residues

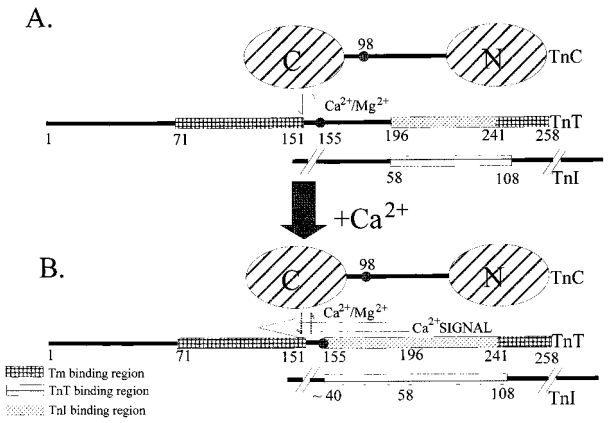

FIGURE 4: Schematic representation of the interaction of the troponin components in the absence (A) and presence of Ca^{2+} (B). This representation is based on the cross-linking results presented in this paper and the published literature. The interaction sites and the corresponding legends are provided. Various interaction sites have been demarcated by specific amino acid residues only for simplicity and should not be considered as completely defined. For brevity, Tm and actin are not shown. The recently mapped binding sites for actin−Tm and TnC in the C-terminal of TnI (39) are not shown here for simplicity. The pathway of Ca^{2+} signal transmission is indicated by a long horizontal arrow. The thin reciprocal arrows indicate a strong interaction between TnT and TnC in a binary complex, whereas the broken reciprocal arrows indicate a reduction in this interaction in a ternary complex. TnT residue 155 is shown as a circle outside the Tm binding region of TnT, although the TnT−Tm interaction may include this residue too. The binary interaction involving amino acid residues 196−241 of TnT and 58−108 of TnI (panels A and B) is mediated by conserved HR domains in these two proteins presumably by a coiled-coil interaction (49). This binary interaction may act as a structural element in the globular head of the Tn complex.

196−241 to 155−241), approaching the Tm binding region of TnT (Figure 4). TnI residues ~40−58 may participate in this extended interaction with TnT. It is quite likely that the Ca^{2+} signal is then transmitted to Tm and is relayed by actin to the entire thin filament. As our results showed that TnT−TnC cross-linking takes place with Ca^{2+} or Mg^{2+}, this interaction probably does not play a direct role in the signal transmission process. Consistent with our findings, the interaction of residues ~40−96 of TnI with TnT and its role in activating muscle contraction have been proposed recently (*39*). Although the transmission of the Ca^{2+} signal from TnC to Tm−actin via TnI, TnT, or both has been proposed previously in the literature (*4*, *3*, *39*, *50*; for a review, see *7*), our experimental approach of labeling a monocysteine TnT mutant for cross-linking has provided some insights into the process of signal transmission. Furthermore, our results have implicated the region around TnT residue 155 as a transmission bridge between TnC−TnI and Tm−actin. These results are especially interesting because we recently reported that residues 159−201 constitute the smallest TnT region which provides Ca^{2+} sensitivity to the thin filament (*31*). Although this model is consistent with many observations reported in the literature, future experiments which could be predicted and designed are needed to further test the validity of the model.

In summary, we have generated a biologically active thiol mutant of TnT, reported for the first time, and used it for understanding the interactions of TnT with TnI, TnC, and Tm in order to gain insights into the role of TnT in the

structure and function of the Ca^{2+} regulatory Tm−Tn complex. The Ser→Cys mutation in TnT-Cys^{155} as well as the *E. coli* expression and purification of the recombinant protein did not affect its biological properties. BP-TnT^{155} did not cross-link to Tm, but showed Ca^{2+}/Mg^{2+}-independent cross-linking with TnI, Ca^{2+}/Mg^{2+}-dependent cross-linking with TnC in the binary and ternary complexes, and Ca^{2+}-favored cross-linking with TnI in the ternary complex. Our results suggest that residue 155 of TnT is within 10 Å (the length of cross-linker) of TnC in the presence or absence of Ca^{2+} and comes within 10 Å of both TnI and TnC upon Ca^{2+} activation. A model showing the physiological relevance of these interactions in transmission of the Ca^{2+} binding signal from TnC to other thin filament proteins has been proposed. Taken together with previous reports in the literature, our cross-linking data suggest the presence of at least one TnC binding region in TnT which is sandwiched between the binding regions for Tm and TnI and which is probably involved in Ca^{2+} signal transmission as well in providing Ca^{2+} sensitivity to the thin filament. Finally, the lack of cross-linking between BP-TnI$_{96-181}$, a truncated fragment of TnI which was labeled at Cys 133 and muscle TnT, indicated that the region around TnI residue 133 is not involved in binary interaction with TnT.

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