Identification and mutagenesis of a highly conserved domain in troponin T responsible for troponin I binding: Potential role for coiled coil interaction

RAYMUND STEFANCSIK*, PRakash K. JHA†, AND SATYAPRIYA SARKAR*‡

*Program in Cell, Molecular and Developmental Biology, and †Department of Anatomy and Cellular Biology, Tufts University Health Science Schools, 136 Harrison Avenue, Boston, MA 02111

ABSTRACT Troponin T (TnT), a thin filament myofibrillar protein, is essential for the Ca\(^{2+}\) regulation of striated muscle contraction in vertebrates, both in vivo and in vitro. To understand the role of TnT in this process, its interaction with two other troponin components, troponin I (TnI) and troponin C (TnC) was examined by using the yeast two hybrid system, which is a genetic approach to detect protein-protein interactions. Computer assisted analysis of phylogenetically distant TnT amino acid sequences unveiled a highly conserved protein domain that is characterized by a heptad repeat (HR) motif with a potential for α-helical coiled coil formation. A similar, potentially coiled coil forming domain is also conserved in all known TnI sequences. These protein motifs appeared to be the regions where TnI-TnT interaction may take place. Deletions and point mutations in TnT, which disrupted its HR motif, severely reduced or abolished TnI binding, but binding to TnC was not affected, indicating that the TnT-TnI and TnT-TnC binary interactions can be uncoupled. Remarkably, the truncated fragments of TnT and TnI in which the HR motifs were retained showed binary interaction in the yeast two hybrid system. It was also observed that the formation of the TnT-TnI heterodimers is favored over the homodimers TnT-TnT and TnI-TnI. These results indicate that the evolutionarily conserved HR motifs may play a role in TnT-TnI dimerization, presumably through the formation of α-helical coiled coils.

Muscle contraction is regulated primarily by intracellular Ca\(^{2+}\) levels. In vertebrate striated muscle the sensor of intracellular Ca\(^{2+}\) is the troponin (Tn) complex that is located in the thin filaments and is comprised of three subunits (for reviews see refs. 1–3). Troponin I (TnI) binds actin-tropomyosin and prevents muscle contraction by inhibiting the actomyosin ATPase activity. Troponin C (TnC), a member of the EF-hand family of proteins, binds Ca\(^{2+}\), and relieves TnI inhibition of actin-myosin interaction (see ref. 4 and references therein). Troponin T (TnT) is a structural link between the Tn complex and tropomyosin, and it also increases the cooperativity of actin-tropomyosin binding to myosin (5). TnT is essential for physiological Ca\(^{2+}\) regulation of muscle contraction. However, the structural basis for its role in this process is not clear. Recent mutagenesis studies have identified the region of fast skeletal TnT that contributes to the Ca\(^{2+}\) sensitivity of the thin filament-based regulatory system (6).

The three subunits of the Tn complex, each of which is encoded by members of separate multigene families in vertebrates, can form binary complexes in vitro. Because all these interactions are likely to be involved in the muscle contraction process, they have been studied extensively (1–3). Among these, the binary interaction between TnT and TnI is the most poorly understood as both proteins are sparingly soluble at physiological ionic strength. The available studies in the literature using either their proteolytic fragments that are soluble, or intact proteins at high salt concentrations, have implicated various regions of TnT, spanning residues 71–258, which interacted with TnI (7–10). Recent studies on the interaction of in vitro expressed deletion mutants of TnI and TnT by HPLC suggested that residues 1–120 of TnI bind to residues 202–258 of TnT (6). Clearly, the precise interaction sites of TnI and TnT remain to be examined for a better understanding of the vertebrate striated muscle contraction process.

In the present study, we have selected the yeast two hybrid system as a sensitive assay to examine interactions between TnT and TnI. This system is ideally suited to map the specific interacting domains of proteins known to form a complex in which the strength of the interaction can be correlated with the level of expression of an appropriate reporter gene (11–13).

Computer assisted analysis of phylogenetically distant TnT amino acid sequences unveiled a highly conserved protein domain that is characterized by a heptad repeat (HR) motif with a potential for α-helical coiled coil formation. A similar, potentially coiled coil forming domain is also conserved in all known TnI amino acid sequences. These protein motifs appear to be the regions where TnI-TnT interaction may take place. Deletions and point mutations in TnT, which disrupted its HR motif, severely reduced or abolished TnI binding, but binding to TnC was not affected, indicating that the TnT-TnI and TnT-TnC binary interactions can be uncoupled. Remarkably, the truncated fragments of TnT and TnI in which the HR motifs were retained showed binary interaction in the yeast two hybrid system. It was also observed that the formation of the TnT-TnI heterodimers is favored over the homodimers TnT-TnT and TnI-TnI. These results indicate that the evolutionarily conserved HR motifs may play a role in TnT-TnI dimerization, presumably through the formation of α-helical coiled coils.

MATERIALS AND METHODS

Plasmid Constructions. All plasmids were grown in Escherichia coli strain XL1Blue, or SURE (Strategene). The fol-
Materials and Methods

following constructs are summarized in Fig. 1. The cDNA of the wild-type human fast skeletal TnTβ (14) was subcloned into plasmids pEG202 and pGJ4–5 (15) (a kind gift of Roger Brent, Massachusetts General Hospital, Boston) fused in frame to the DNA binding domain of the bacterial repressor LexA, which generated pEG-TnT-wt (Fig. 1) and pJG-TnT-wt, respectively. Mutagenesis was carried out by inverse PCR by using the High-Fidelity PCR kit of Boehringer Mannheim. To create the plasmid pEG-TnT-G203S204 (Fig. 1), we amplified a fragment containing amino acid residues 150–258, as described by Golemis et al. (15). Each heterologous fusion protein used in the two hybrid assays was checked for its inability to activate transcription and was also examined for proper transport into the nucleus by using the transcriptional repression assay (21, 15). To measure the expression level of the LEU2 reporter gene, yeast cells were plated onto selective minimal medium whereas LEU2 Leu represents the average plating efficiency of a particular yeast strain without Leu in the growth medium. All the two hybrid assays were carried out as described by Golemis et al. (15). Yeast cells were grown in complete (yeast extract/peptone/dextrose) or selective minimal medium (synthetic minimal dextrose, SD) (19). We used the transformation protocol of Schiestl and Gietz (20). The yeast two hybrid assays were carried out as described by Golemis et al. (15). Extracts of human TnT were used (http://theory.lcs.mit.edu/~bba/paircoil.html).

RESULTS

A rabbit fast skeletal TnI cDNA (17) was subcloned into pJG4–5 and pEG202 (15) creating pJG-TnI-wt and pEG-TnI-wt, respectively. A fragment, corresponding to amino acid residues 58–108, was PCR amplified with primers 5′-CCGATATCCGAGGTTGCA-3′ and 5′-CGAGCTCTCGAAGTTGAGATTCC-3′. The PCR product was digested with EcoRI and XhoI sites and cloned into pJG4–5, producing pJG-TnI58–108.

To create pJG-TnI-G203S204, we used pJG-TnI-wt as a template and the following primer pair complementary to the TnI mutant in which amino acid residues 203–214

\[ \text{5′-GTCAATCTCCAGCTGGTGCCGGGTCTCCC-3′} \]

was used (http://theory.lcs.mit.edu/~bba/paircoil.html). The resulting fragment was double digested with EcoRI and XhoI sites and cloned into pJG-TnI-wt, producing pJG-TnI58–108.

A rabbit skeletal TnC cDNA (18) was subcloned into pJG4–5 generating pJG-TnC. The fragment carrying the TnC cDNA was excised with NdeI (followed by filling in with T4 DNA polymerase) and EcoRI, then subcloned into EcoRV-EcoRI digested pLITMUS29 (New England Biolabs). The resulting plasmid was digested with NcoI, followed by a T4 DNA polymerase treatment, and digested again, now with XhoI. The resulting DNA fragment was subcloned into the EcoRI (made blunt end with T4 DNA polymerase)-XhoI site of pJG4–5.

Yeast Strains and Methods. Saccharomyces cerevisiae strain EGY48 (a kind gift of Roger Brent, Massachusetts General Hospital, Boston) was used in our studies (15). Yeast cells were grown in complete (yeast extract/peptone/dextrose) or selective minimal medium (synthetic minimal dextrose, SD) (19). We used the transformation protocol of Schiestl and Gietz (20). The yeast two hybrid assays were carried out as described by Golemis et al. (15). Each heterologous fusion protein used in the two hybrid assays was checked for its inability to activate transcription and was also examined for proper transport into the nucleus by using the transcriptional repression assay (21, 15). To measure the expression level of the LEU2 reporter gene, yeast cells were plated onto selective minimal medium containing galactose with or without Leu. The plating efficiency of each yeast strain carrying different plasmid constructs was determined by counting the number of colonies on plates after plating serial dilutions. The relative plating efficiency (RPE) was calculated as follows: RPE (%) = (PE-1Leu + PE-1Leu+)/100, whereas PE-1Leu is the average plating efficiency of a particular yeast strain without Leu in the growth medium whereas PE-1Leu+ represents the average plating efficiency with Leu in the growth medium. All the two hybrid assays were repeated at least five times.
to represent the functionally important protein domains. A conserved domain spanning over 60–70 amino acid residues was identified in TnT sequences from mammals, birds, Caenorhabditis elegans, Drosophila, and the protochordate ascidian Halocynthia roretzi (Fig. 2a). The most important feature of this domain is the presence of a HR with periodic occurrence of Leu and other hydrophobic residues in positions “a” and “d” in a seven amino acid repeat unit “abcdefg” (underlined in Fig. 2a, and spanning over 40 amino acid residues). This type of structural motif can, in principle, form an α-helical coiled coil with another protein containing HR. As TnT interacts with two other subunits in the Tn complex, we also searched for similar potential candidate domains in TnI and TnC. We observed some similarity between parts of TnT and TnI but not between TnT and TnC or TnI and TnC. This similarity appeared to be statistically significant according to the Poisson scores derived by the BESTFIT program of the GCG package (results not shown).

However, the different TnT and TnI HR domains show considerable variation in the estimated probability for forming α-helical coiled coils. For example, the human cardiac TnT HR motif shows very high coiled coil forming probability reaching the theoretical limit of 1. In contrast, the human fast skeletal TnT HR shows only a moderate value of 0.4. The TnI sequences also show a broad range of predicted coiled coil forming capacity. For example, the HR region of the rabbit fast skeletal TnI gives the maximal PAIRCOIL value of 1, whereas the HR of the Drosophila TnI has a value of 0.2.

The potential role of the HR domains in TnT-TnI binary interaction was tested by using the yeast two hybrid system.

**Yeast Two Hybrid Analysis of the Interactions between TnT and TnI Mutants.** We designed mutations in TnT to test whether the HR motif is necessary for TnI binding. The mutations were located in the center of the HR of TnT to maximize the possible effect on any interaction mediated by them. Several additional aspects in designing the mutations were also considered: (i) Deletion of 12 amino acid residues 203–214 involving the three most conserved Leu-s of the TnT HR domain (TnT203–214, Figs. 1 and 2a) will cause a loss of one complete heptad unit plus five amino acids. Among the three Leu-s deleted, two are at “d,” while one is at an “a” position. Mutations replacing Leu-s at “d” positions are known to have considerable variation in the estimated probability for forming coiled coil.

The potential role of the HR domains in TnT-TnI binary interaction was tested by using the yeast two hybrid system.

![Fig. 2. (a) Amino acid sequence alignment of the HR domain in TnT polypeptides. Conserved Leu-s or hydrophobic residues in positions “a” and “d” of the HR units (“abcdefg”) are shaded. Amino acid residues Leu(L)\(^{203}\), Trp(W)\(^{204}\), Leu(L)\(^{207}\), Arg(R)\(^{214}\) of the human fast skeletal TnT and between amino acids 20 and 110 of the human fast skeletal TnI. Within this part of TnI there is a subregion, which is characterized by HR (Fig. 2b). Also, this sequence is highly conserved in TnI sequences from both vertebrate and invertebrate species.](Image 119x105 to 465x368)
dramatic effect on protein-protein interaction in coiled coils (25). Deletion of a HR and five additional amino acid residues in an otherwise continuous repeat should alter the heptad phasing in such a way that it is likely to disrupt coiled coil interactions due to the resulting azimuthal shift of the stripe of apolar residues in the “a” and “d” positions (26). (ii) A mutant with only a two amino acid substitution involving the replacement of a Leu and a Trp at positions 203 and 204 with Gly and Ser (TnT-G203S204, Fig. 2a), respectively, is likely to produce subtle changes in the HR motif. The Leu substituted in this mutant resides in the most conserved region of the TnT HR region. As Gly possesses the smallest possible side chain, and it is not usually found in the “d” positions of HR motifs, this mutation is likely to affect coiled coil formation. Substitution of the neighboring Trp with Ser in this mutant should further decrease the overall hydrophilicity of this two amino acid region. (iii) In a point mutation, one of the Leu-s at an “a” position of the HR domain, Leu at 207 is changed to Arg (TnT-R207, Fig. 1). Arg has a basic side chain with high surface probability and is hydrophilic. Therefore, this mutation is also likely to affect coiled coil interaction.

The ability of these mutants to bind TnI was compared with that of the wild-type TnT in the yeast two hybrid system. Deletion of amino acid residues 203–214 of the fast skeletal TnTβ sequence (TnTΔ203–214, Figs. 1 and 2a) caused at least 90% reduction in the level of expression of the reporter gene (Fig. 3a), suggesting a comparable decrease in TnT-TnI interaction. The ability of the TnT-G203S204 mutant to bind TnI is essentially abolished (Fig. 3a). Furthermore, in the point mutation where Leu207 in TnT was substituted by Arg (TnT−R207), TnI binding was reduced by 41% (Fig. 3a).

Next, the question whether truncated fragments of TnT and TnI containing the HR domains, can form a binary complex in the yeast two hybrid system was addressed. Coexpression of a recombinant fragment of TnT (amino acid residues 150–258, TnT150–258) with a recombinant TnI fragment (amino acid residues 58–108, TnI58–108) resulted in ~25-fold increase in reporter gene activation as compared with the negative control (Fig. 3b) indicating a strong interaction involving the truncated fragments. The negative control in this case is pG-TnI58–108 coexpressed with the LexA DNA-binding domain (plasmid pEG202 with no insert). Also, the truncated fragments gave similar level of reporter gene activation as obtained with the intact polypeptides. Moreover, the combination of TnT150–258 and TnI58–108 interacted as efficiently as the combination of full-length TnT and TnI58–108 (Fig. 3b). In contrast, the mutants TnTΔ203–214 and TnT-G203S204 failed to interact with the HR fragment TnI58–108 (Fig. 3b). Similarly, TnT-R207 shows reduced binding to TnI58–108 (Fig. 3b). These results strongly suggest that (i) the HR domains of TnT and TnI per se have the intrinsic capacity for binary interactions; and (ii) mutations involving the disruption of the HR domain of TnT inhibit binary interaction with TnI and its truncated HR fragment.

**Mutations in the Conserved HR Region of TnT Do Not Affect TnC Binding.** We also tested whether the aforementioned mutations in the HR of TnT have any effect on TnC binding. None of the TnT mutants including TnTΔ203–214 and TnT-G203S204 show any reduction in TnC binding, as compared with the wild-type TnT (Fig. 3c).

The fact that binary interaction of TnC with the TnT mutants was the same as observed with wild-type TnT, strongly suggests that the stability of the TnT polypeptides was not affected by the mutations in the HR domain. However, this possibility was also tested by carrying out the transcriptional repression assay (15), which determines whether the wild-type and mutant TnT fusion proteins are produced at sufficiently high levels, and whether they are transported efficiently into the nucleus of the yeast cells. The wild-type and all the mutant TnT fusion proteins, except TnT150–258, are expressed and transported into the yeast nucleus at comparable levels. For

**Fig. 3.** Interaction between various recombinant TnT, TnI, and TnC polypeptides as measured by percent RPE (RPE%) in the yeast two hybrid system. RPE% was calculated as described in the Materials and Methods. Bars = SD. (a) Interaction of wild-type and mutant TnT proteins with TnI. Wild-type TnT (TnT-wt) was coexpressed with wild-type TnT (TnT-wt) or the mutant TnTs (TnT-G203S204, TnTΔ203–214 and TnT-R207) in yeast, and the activation of the LEU2 reporter gene was measured as RPE%. A wild-type TnT coexpressed with pG4–5, the transcriptional activator alone was included as a negative control (TnT-wt + pG4–5). (b) Interaction of the truncated TnI58–108 fragment containing the HR domain with various TnT constructs. TnI58–108 was coexpressed with either TnT150–258 or TnT-wt, or TnTΔ203–214, or TnT-G203S204, or TnT-R207. As a control, TnI58–108 was also coexpressed with the bacterial LexA repressor DNA binding domain alone (pEG202). (c) Interaction of TnC with wild-type and mutant TnT proteins. TnC was coexpressed with wild-type TnT (TnT-wt) or the mutant TnTs (TnT-G203S204 and TnTΔ203–214), and the activation of the LEU2 reporter gene was measured as RPE%. A wild-type TnT expressed without any interactor protein is included as a negative control (TnT-wt alone).
example, TnT-G^{203S,204} and TnTΔ203–214 reduced transcription from the LexA-operator-GAL1 promoter by 73% and 80%, respectively. This level is similar to the 82% repression observed with the wild-type TnT construct. The lower expression level of TnT^{250–258}, which gave 46% repression, does not interfere with the interpretation of the two hybrid assays by using this construct, because there is a significant interaction between this mutant and the TnI^{58–108} fragment resulting in high level of reporter gene activation (Fig. 3b). Thus, the strong interaction of this TnT fragment with the HR fragment of TnI appears to overcome its lower expression phenotype.

**Homo- vs. Heterodimer Formation by TnT and TnI.** As many proteins containing HR domains can form homodimers as well as heterodimers (27), we next tested whether the heterodimer TnT-TnI is preferred to the formation of the homodimers, TnT-TnT and TnI-TnI. It was observed that the heterodimer formation is strongly favored, the homodimers TnT-TnT and TnI-TnI showing ~5% and 38% reporter gene activity, respectively, compared with the activation level obtained for the heterodimer (Fig. 4). Interestingly, the higher homodimer forming ability of TnI, as compared with that of TnT, correlates with its higher PAIRCOIL score (0.4 vs. 1, respectively).

**DISCUSSION**

In this report, we have identified an evolutionarily conserved domain that is characterized by an HR motif in members of the TnT protein family. Previous examination of several proteins forming coiled coil interactions have shown that the residues at “a” and “d” in the seven amino acid repeat unit “abcdefg” of the HR motif are hydrophobic and constituted the solvent unexposed helix interface of the α-helical coiled coil (for reviews, see refs. 26–28).

The presence of an HR motif in a single TnT amino acid sequence was noticed earlier by Pearlstone and Smillie (10). Although these authors (10) speculated that the HR in the rabbit TnT amino acid sequence may be important for TnI binding, this possibility was not tested experimentally. Hence, the role of the HR motif in TnT-TnI binary interaction has not been established prior to this study. Recently, it was reported that fast skeletal TnT binds to dystrophin by coiled coil- or leucine zipper-mediated interaction (29). However, the *in vivo* relevance of this finding is not clear, and the authors also did not investigate what region of TnT was involved in the interaction with dystrophin.

Our results supporting the view that the HR domain in TnT is necessary for binary TnT-TnI interaction is based on the following considerations: (i) all of the HR domains in both TnT and TnI polypeptides (Fig. 2 a and b) are potentially capable of forming α-helical coiled coil as shown by analysis by using the PAIRCOIL prediction program (see also Results); (ii) deletion or point mutations that alter or disrupt the HR motif in TnT abolish or inhibit TnT-TnI interaction (Fig. 3a); (iii) truncated TnT and TnI fragments containing the HR domains show similar level of binary interaction as that obtained with intact polypeptides (Fig. 3b); (iv) truncated TnI fragment containing the HR domain shows decreased interaction with TnT HR mutants in contrast to the strong interaction observed with native TnT (Fig. 3c); and (v) the formation of the TnT-TnI heterodimer occurs in preference to the TnI-TnI and TnT-TnT homodimers.

The observation that a deletion of 12 amino acid residues in the center of the HR motif in TnT abolishes binary complex formation with TnI (Fig. 2r) is most likely due to the disruption of the HR motif in TnT. However, in view of the possibility that even a modest 12 amino acid sequence deletion may cause sufficient structural changes in protein conformation of TnT that prevented its interaction with TnI, we created single or double amino acid substitutions that are likely to alter primarily the hydrophobicity of the HR (see also Results). The complete or partial loss of reporter gene activation observed with these mutants (Fig. 3a) is consistent with the view that Leu at “a” and “d” positions are important for HR motif mediated coiled coil interaction (26–28). In the Leu→Arg substitution at position 207, the observed partial reduction in binding to TnI is probably due to fact that the aliphatic parts of the bulky Arg residue may compensate for increased hydrophilicity of the Arg. Also, as there are seven other Leu-s besides the one that is changed to Arg, these Leu-s may provide the necessary hydrophobic interactions for the observed residual TnT-TnI association. This view is also consistent with the HR interaction hypothesis that assumes a cooperative effect of Leu residues in coiled coil interaction (30). With regard to the double amino acid substitution in the mutant TnT-G^{203S,204}, both of these substitutions (Leu→Gly at position 203; Trp→Ser at position 204) are likely to decrease sharply the overall hydrophobicity of this two amino acid region. Because in the human slow skeletal TnT isoform a Trp→Ser substitution occurs naturally in the homologous position (31), the total loss of interaction with TnI for this mutant appears to be primarily due to alteration in the hydrophobicity of the side chain at position 203.

Electron microscopic study of tropomyosin and Tn indicate that the Tn complex has both a globular and a rod-like part (32). Furthermore, crystallographic studies have shown that the globular region of the Tn complex consists of TnI, TnC, and a carboxy-terminal portion of TnT (33). The identification of the TnT-TnI interaction site as the HR domain of TnT spanning amino acid residues 196–242 is consistent with the above-mentioned structural features of Tn. It is quite likely that the HR domains of TnT and TnI function as a structural building element within the globular “head” of Tn. Our observation that the TnT-TnI heterodimer is preferred among the possible interacting dimers in the two hybrid system (Fig. 4) is also consistent with this view. However, it should be noted that, until x-ray crystallographic or NMR studies are available, it is not possible to establish that the TnT-TnI interaction is coiled coil.

Various *in vitro* experiments using the proteolytic fragments of TnT and their interaction with TnI have also suggested that the carboxy-terminal half of TnT interacts with TnI (9) (7). Recently, Jha *et al.* (6) showed that the deletion of 57 carboxy-terminal residues of TnT eliminates TnI binding. Our results...
showing the involvement of HR domains of TnT and TnI in binary interaction are consistent with the above-mentioned reports.

The conserved HR domains of TnT and TnI show similarity at a statistically significant level, suggesting that the HR motifs in TnT and TnI may have a common ancestry.

Mutations in the HR of TnT do not affect TnC-TnT binary complex formation (Fig. 3c), presumably because TnC does not contain any HR motif. The normal interactions observed in TnT-TnI and TnC-TnT binary complex formation are consistent with the above-mentioned studies.

In summary, we have identified an evolutionarily conserved HR motif in TnT and TnI, and demonstrated that the interaction of TnT and TnI is mediated by this HR, presumably acting as a common dimerization motif. These studies also point to complex but distinct patterns of protein-protein interactions involving the three subunits of the Tn complex that play a role in the vertebrate muscle contraction process.

It is quite likely that the HR motif-mediated interaction of TnT and TnI also plays a role in the transmission of the Ca2+ binding signal from TnC to the thin filament and structural organization of the complex.

We thank Dr. Roger Brent for providing yeast strain EGY48 and the plasmids pEG202 and pJG4–5; Dr. David Damassa for help with statistical analysis; Dr. C. Cohen for valuable discussions; Drs. Z. Grabarek, P. Leavis, and M. Gordon for helpful comments on the manuscript; and Ms. V. Olson for help in the preparation of the manuscript. These studies were supported by grants from the National Institutes of Health (5P01 HD23081), the American Heart Association, and Basic Research Support Grant funds from the Tufts University School of Veterinary Medicine.