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Tissue-specific interactions of TNI isoforms with other TN subunits and tropomyosins in *C. elegans*: The role of the C- and N-terminal extensions

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Abstract

The aim of this study is to investigate the function of the C-terminal extension of three troponin I isoforms, that are unique to the body wall muscles of *Caenorhabditis elegans* and to understand the molecular interactions within the TN complex between troponin I with troponin C/T, and tropomyosin. We constructed several expression vectors to generate recombinant proteins of three body wall and one pharyngeal troponin I isoforms in *Escherichia coli*. Protein overlay assays and Western blot analyses were performed using antibodies. We demonstrated that pharyngeal TNI-4 interacted with only the pharyngeal isoforms of troponin C/T and tropomyosin. In contrast, the body wall TNI-2 bound both the body wall and pharyngeal isoforms of these components. Similar to other invertebrates, the N-terminus of troponin I contributes to interactions with troponin C. Full-length troponin I was essential for interactions with tropomyosin isoforms. Deletion of the C-terminal extension had no direct effect on the binding of the body wall troponin I to other muscle thin filament troponin C/T and tropomyosin isoforms.

Abbreviations used: TN, troponin; TNI, troponin I; TNC, troponin C; TNT, troponin T; TM, tropomyosin

1. Introduction

Muscle contraction is the result of a series of protein-protein interactions. In striated muscle, the thin filament complex of troponin (TN) and tropomyosin (TM) regulates contraction. The troponin complex components include the calcium-binding protein, troponin C (TNC), the tropomyosin (TM)-binding protein, troponin T (TNT) and troponin I (TNI) which is involved in inhibition of the actomyosin ATPase activity [1, 2]. Recent studies on crystals of the core TN complex suggest that in the absence of calcium, TNI-binding to actin holds the TN-TM complex in a "closed" state thereby preventing myosin from binding to actin [3, 4].

Calcium ions (Ca^{2+}) released into the muscle cells following neural stimulation bind to TNC, which undergoes a conformation change that alters its relationship with TNI, resulting in the release of TNI-binding to actin. Biochemical and physicochemical studies of muscle contraction have been carried out using proteins from different tissues of vertebrates. Multiple forms of troponin subunits have been identified in cardiac [5], slow skeletal [6] and fast skeletal muscles [7]. The detection of TNI isoforms by specific antibody staining provides a convenient and reliable method of muscle cell typing and the presence of TNI in serum can be used for the detection of disease in skeletal and cardiac muscles [8]. The immunological detection of serum cardiac TNI is widely used in cardiology as an index of myocardial damage [9]. Slow and fast isoforms are present in the same skeletal muscle cell as a consequence of cross innervations, hormone intervention, and in pathological conditions [10]. Thus, specific TNI isoforms probably play important roles in determining the distinctive functional properties of various muscle types from different animals.

Specific amino acid sequences in muscle proteins have been shown to interact with specific sites. The first report on TNI fragments that bind TNC was done by Syska et al. [11]. They report that TNI fragment of the rabbit skeletal containing residues 1-21 and 96-116 bind TNC, and the latter also bind to actin. It has also been summarized in the rabbit skeletal TNT that the N-terminal residues 160-260 bound to TNC, and the C-terminal residues 216-263 might be the TNI interaction site[12]. Thus, specific interaction sites in the components form part of a functional complex. Comparisons of these amino acid sequences reveal that a high sequence homology of the TNC-binding site is present towards the N-terminal region of TNI isoforms containing N-terminal extensions that are seen in invertebrates and vertebrate hearts. It has been reported that bisphosphorylation of the N-terminal extension of human cardiac TNI alters the Ca^{2+}-binding properties to TNC [13]. Similarly the high sequence homology of the actin/TNC-binding site exists within the central portions of TNI isoforms from many animals. These results of biochemical

studies were confirmed when atomic structures of the core TN complex recently became available [3, 4]. Probably, the regulatory complex of TN components shares common structural bases within the core complex but with some variation depending on muscle or animal type. Although many detailed analyses of interaction sites between the TN components still continues, few report on the overall interactions between regulatory components and include all isoforms derived from a single genome.

TNI isoforms are expressed in two patterns: tissue specific and developmentally regulated. Previously, we have shown that three genes, namely, *tni-1*, *tni-2/unc-27* and *tni-3* are expressed in the body wall muscles, and that *tni-4* exhibits pharyngeal-specific expression in *Caenorhabditis elegans* [14]. An approach to understand the role of TNI in the regulation of muscle contraction is to study the structural and functional differences between the various TNI isoforms. In our previous study, we also compared the amino acid sequences of four TNI isoforms of *C. elegans* with those of *Drosophila*, crayfish, and the cardiac, slow skeletal and fast skeletal muscles of rabbit [14]. Comparisons of these amino acid sequences reveal that a high sequence homology of the TNC-binding and the actin/TNC-binding sites exists in all *C. elegans* TNI isoforms. Interestingly, three body wall type TNI-1, TNI-2 and TNI-3 isoforms of *C. elegans* contain unique C-terminal extensions. C-terminal extensions containing approximately 20 glutamic acid residues are common in all body wall TNI isoforms but not in pharyngeal TNI isoform of *C. elegans*, the TNI isoforms of *Drosophila*, crayfish and rabbit [14]. The biological importance of the C-terminal extension remains unknown structurally and functionally.

In this study, we focused on the importance of the C-terminal extensions of three body wall TNI isoforms as well as the tissue-specific interaction of TNI isoforms with other thin filament components. To investigate the interactions of the TNI isoforms and those fragments with other thin filament proteins, we constructed recombinant proteins of the N-, C-, and C-terminal extension-deletion fragments of TNI isoforms and performed Western blot analysis using antibodies. The C-terminal extensions had no direct role in the TNC, TNT and TM interactions. Molecular binding assays of TNI with other thin filament components provide an insight into how thin filament regulatory proteins evolved to specialize in their interactions to regulate muscle contractions. This is the first report on how the components of the TN complex of *C. elegans* interact with other thin filament components.

2. Materials and methods

2. 1. Construction of expression vectors

2. 1. 1. Construction of the N-terminal, C-terminal and C-terminal extension-deletions of the TNI expression vectors

Expression vectors of the N- and C-terminal fragments of TNI-2, pCTNI-$2_{1–117}$ and pCTNI-$2_{118–242}$ were constructed by inserting *Bam*HI restriction fragments corresponding to a 354 bp 5'-region and a 378 bp 3'-region of the *tni-2* cDNA [14] into the *Bam*HI site of pET-28a(+) vector, respectively. These restriction fragments were prepared by PCR amplification using *tni-2* cDNA as a template with the following sets of primers followed by *Bam*HI digestion; TNI-2N-s (5'-AAG GAT CCA TGA GTG AAG AAG CCG GAG A-3') and TNI-2N-as (5'-AAG GAT CCG TTG ATG TCG TAC TTC TCC TCC-3') primers for pCTNI-$2_{1–117}$, and TNI-2C-s (5'-AAG GAT CCA TCA ACT ACG TCG TCT CCC AGA-3') and TNI-2C-as (5'-AAG GAT CCT GGA TTT AGG AGT TTA CTC CTC-3') primers for pCTNI-$2_{118–242}$. pCTNI-$2_{94–242}$ was constructed by inserting a *Xho*I-*Hin*dIII restriction fragment corresponding to the 453 bp 3-region of the *tni-2* cDNA into the *Xho*I-*Hin*dIII site of pET-28c(+) vector. The C-terminal extension-deletion construct of TNI-2 expression vector pCTNI-2_{1-220} was constructed by inserting a *Bam*HI restriction fragment corresponding to the 5'-region of the *tni-2* cDNA, which is derived from the subcloned vector pGEX-CTNI-2 [14], into the *Bam*HI site of the pET-28a(+) vector. This restriction fragment was prepared by PCR using pGEX-CTNI-2 as a template with pGEX5' (5'-GGG CTG GCA AGC CAC GTT TGG TG-3') and UNC-27-P2 (5'-TTG GAT CCT TAC TCA GCT TCT GGA GCA ACG-3') primers, followed by *Bam*HI digestion. pCTNI-4_{1-166} was constructed by inserting an *Eco*RI restriction fragment corresponding to the 530 bp 5'-half region of the *tni-4* cDNA from yk328a10 into the *Eco*RI site of the pET-28b(+) vector. This restriction fragment was prepared by PCR using yk328a10 as a template with M13 (–21) and TNI-4-s-*Eco*-pETb (5'-GCG AAT TCA ACC ATG AGT GAC GTT GAC G-3') primers. Note that the TNI-4-s-*Eco*-pETb primer altered the stop codon in the 5' untranslated region (UTR) of the *tni-4* cDNA. pCTNI-$4_{91–194}$ was constructed by inserting an *Xho*I restriction fragment corresponding to the 519 bp 3'-region of the *tni-4* cDNA from yk328a10 into the *Xho*I site of the pET-28b(+) vector.

Expression vectors of other TNI-1/-3 isoforms were also constructed briefly as follows. pCTNI-1_{1-126} was constructed by inserting an *Eco*RI restriction fragment of *tni-1* cDNA from

yk103h4 into the *Eco*RI site of pET-28b(+) vector. pCTNI-$1_{106-250}$ was constructed by inserting the *Xho*I restriction fragment of *tni-1* cDNA from yk103h4 into the *Xho*I site of pET-28b(+) vector. pCTNI-3_{1-125} was constructed by inserting an *Eco*RI restriction fragment of *tni-3* cDNA from yk338a12 into *Eco*RI site of pET-28a(+) vector. pCTNI-$3_{118-260}$ was constructed by inserting the *Bgl*II-*Xho*I restriction fragment of *tni-3* cDNA from yk338a12 into *Bgl*II-*Xho*I site of pET-28c(+) vector. In summary, the N-terminal, C-terminal and C-terminal extension-deletion constructs of the TNI isoforms, as shown by the bars in Fig. 1, were cloned into the pET-28a(+), pET-28b(+) and pET-28c(+) vectors (Novagen), respectively and each of fragments were produced in *E. coli* strain BL21(DE3).

2. 1. 2. Construction of the TNT-1, TNT-4, TNC and TM expression vectors

pCTNT-1 was constructed by inserting an *Eco*RI restriction fragment corresponding to the 1215-bp full-length *tnt-1/mup-2* cDNA from yk1354g3 into the *Eco*RI site of the pET-28a(+) vector. This restriction fragment was prepared by PCR using yk1354g3 as a template with TNT-1-s (5'-TTG AAT TCA TGT CCG ACG AGG AGG AGG TAT AC-3') and TNT-1-as (5'-TTG AAT TCA TTC GAC AAC GAC CTC TTC TCC-3') primers, followed by *Eco*RI digestion. pCTNT-4 was constructed by inserting a *Bam*HI restriction fragment corresponding to the 1044-bp full-length *tnt-4* cDNA from yk1174g10 into the *Bam*HI site of the pET-28a(+) vector. This restriction fragment was prepared by PCR using yk1174g10 as a template with TNT-4-s (5'-CCT GGA TCC TCG ATG AAC ATG TCT GAC GAG GAA TAC TCC G-3') and TNT-4-as (5'-TCT GGA TCC TTA ATA GTC TTC CTC TTC CTC GGC-3') primers. The plasmid vectors for the bacterial expression of TNC-1 and TNC-2 have been described previously [14, 15].

Some expression vectors of TNC, TNI and tropomyosin of *C. elegans* were used as reported previously (Table 1) [14-16]. TMI and TMIII expression vectors and the cDNA clones of CeTMI and CeTMIII were used as described previously [16]. All sub-cloned constructs were verified by DNA sequencing.

2. 2. Western blot analysis and protein overlay assay

Western blot analysis was performed using the ECL detection system (Amersham, now GE Healthcare Bio-Sciences) by using the following specific antibodies: anti-CeTMIII [16], anti-CeTNC-1/-2 [15] and anti-CeTNI-2/-4 [14]. Table 1 also describes the antibodies used in this study. Anti-CeTNC-1 and anti-CeTNC-2 were tissue-specific as they detected only body wall

TNC-1 and pharyngeal TNC-2, respectively (Fig. 2c) [14]. Anti-CeTMIII detected both the body wall TMI and the pharyngeal TMIII isoforms (Fig. 2d). Cross-reactivity of the TMI isoform with anti-CeTMIII, which was raised to detect the pharyngeal tropomyosin, TMIII isoforms, is due to the sharing of some amino acid residues from common exons [16, 17].

The body wall TNI-2 and TMI, and the pharyngeal TNI-4 and TMIII proteins of *C. elegans* were produced in *E. coli* strain JM109, which were transformed with each of cDNA expression vectors. cDNA clones of TNI-2/-4 and TMI/III were cloned into pGEX-4T series and pUR289, respectively [14,16]. The TNC-1 and TNC-2 proteins were produced in *E. coli* strain BL21 (DE3) transformed with the pCTNC-1 and pCTNC-2 vectors, respectively [15]. Note that the expressed proteins had no tag. TNT-1 and TNT-4 proteins were produced in *E. coli* BL21 (DE3) transformed with the pCTNT-1 and pCTNT-4 vectors, which expressed His-tagged TNT-1, the body wall isoform, and TNT-4, the pharyngeal TNT isoform of *C. elegans*. Protein samples were prepared according to the standard procedure in which 0.05 µg/ml TNI, 0.02 µg/ml TNC, 0.04 µg/ml tropomyosin and 0.05 µg/ml TNT were run on SDS-PAGE and blotted onto a nylon membrane. The processed membranes were immersed in 20 µg/ml TNI, 30 µg/ml TNC, 25 µg/ml tropomyosin and 50 mM Tris-HCl (pH 6.8) solution with 1 mM $CaCl_2$ or 2 mM EGTA at 4 °C for 12 hours. After washing, Western blot analysis was performed using 0.05 mg/ml rabbit polyclonal anti-CeTNI-2, anti-CeTNI-4, anti-CeTNC-1 and anti-CeTNC-2 antibodies, anti-CeTMIII anti-serum were used as the primary antibody, and 0.2 mg/ml of horseradish anti-rabbit IgG (Amersham Biosciences) was used as the secondary antibody; the samples were incubated at room temperature for 1 hour and analyzed by using the ECL detection system.

3. Results

3. 1. Characterization of the fragments of the TNI, TNT and TM isoforms and their antigenic characters

In *C. elegans* all isoforms of any muscle protein are identified and are available from the database, WormBase. We expressed several muscle thin filament proteins in *E. coli* by constructing expression vectors that are summarized in Table 1. We also constructed expression vectors to produce the N- and C-terminal fragments of three body wall isoforms (TNI-1, TNI-2 and TNI-3) and one pharyngeal isoform (TNI-4) (see Materials and methods; Fig. 1 and Table 1). The fragments consisted of two parts: the N-terminal fragment containing the TNC-binding site and the C-terminal fragment containing the actin/TNC-binding site. For example, deletion of the C-terminal extension fragment of TNI-2 was designated as TNI-$2_{1–220}$. The molecular sizes of the N-terminal TNI-$2_{1–117}$, C-terminal TNI-$2_{94–242}$, TNI-$2_{118–242}$ and C-terminal extension- deletion TNI-$2_{1–220}$ fragments of the TNI-2 isoform were confirmed by SDS-PAGE (data not shown) and are summarized in Table 1. The products of these fragments were detected by Western blot analysis by using the anti-CeTNI-2 antibody that stained all four TNI isoforms of *C. elegans* (Fig. 2a) [14]. Size differences were observed in the fragments of the TNI isoforms due to the design of the expression vectors. We focused on the body wall TNI-2 isoform, a major known TNI component of the body wall muscle and the product of the *tni-2/unc-27* gene [14].

The expression vectors pCTNT-1 and pCTNT-4 expressed body wall TNT-1, a product of *mup-2* [18] and pharyngeal TNT-4, a product of *tnt-4*, respectively. The molecular sizes of these recombinant proteins are summarized in Table 1. These proteins and other thin filament components were used in the molecular interaction experiments. The anti-CeTNI-2 antibody, which was raised against the major body wall type, cross-reacted with all TNI isoforms, three body wall types and one pharynx type (Fig. 2a). This antibody also detected the N- and C-terminal fragments and the full length of all TNI isoforms; which suggested the presence of more than one recognition site in the TNI-2 isoform (Fig. 2a). As shown in Fig. 2b, the anti-CeTNI-4 antibody specifically detected the pharyngeal isoform, TNI-4. Fig. 2c shows that antisera against each of both the body wall type TNC-1 and the pharynx type TNC-2 isoforms had tissue specificity [14, 15]. The recombinant proteins and antibodies produced in this study are summarized in Table 1 and Materials and methods.

3. 2. Molecular interactions of body wall and pharyngeal TNI isoforms with other thin filament components

3. 2. 1. Interaction between TNI and TNC isoforms

Both the TNC-1 and TNC-2 isoforms were overlaid on blotted N-terminal TNI-$2_{1–117}$, C-terminal TNI-$2_{94–242}$ and TNI-$2_{118–242}$, and the C-terminal extension-deleted TNI-$2_{1–220}$ fragments in the presence or absence of Ca^{2+}. Similar overlay experiments were also performed with the TNC isoforms and the pharyngeal TNI-$4_{1–166}$ and TNI-$4_{91–194}$ fragments. Each of the overlaid TNC isoforms was detected by using the anti-CeTNC-1 or anti-CeTNC-2 antibody (Fig. 3a-b). These results indicated that body wall TNC-1 bound specifically with the N-terminal body wall TNI-$2_{1–117}$ fragment, but not with the pharyngeal TNI-4 isoform (Fig. 3a, upper and lower panels) [14]. Pharyngeal TNC-2 interacted with both the N-terminal TNI-$2_{1–117}$ and TNI-$4_{1–166}$ fragments and also the full-length TNI-2 and TNI-4 isoforms (Fig. 3b, upper and lower panels) [14]. The C-terminal extension-deleted TNI-$2_{1–220}$ fragment of body wall TNI-2 isoform was able to bind with both the TNC isoforms but both the C-terminal body wall TNI-$2_{94–242}$, TNI-$2_{118–242}$ and pharyngeal TNI-$4_{91–194}$ fragments did not bind with either the body wall TNC-1 or pharyngeal TNC-2 isoforms, respectively (Fig. 3a-b). All interaction profiles between each pair of TNI and TNC isoforms are summarized in Fig. 3c. We concluded that the N-terminal body wall TNI-2 interacted with both the body wall and pharyngeal TNC isoforms, but the N-terminal pharyngeal TNI-4 interacted only with pharyngeal TNC-2.

The extent of the interaction varied among the TNI isoforms and the N-terminal fragment used depending on the presence or absence of Ca^{2+}. However, it was difficult to determine whether these results reflect quantitative differences in these interactions. We hereafter focus on the TNI-2 isoform to study the molecular interactions with other thin filament components from both the body wall and the pharynx.

3. 2. 2. Interaction between TNI and TNT isoforms

We performed protein overlay assays on full-length and fragmented body wall TNI isoforms with both body wall and pharyngeal TNT isoforms (Fig. 4). Full length TNI-2, the N-terminal TNI-$2_{1–117}$, the C-terminal TNI-$2_{94–242}$ and the C-terminal extension-deleted TNI-$2_{1–220}$ fragments interacted with both the TNT isoforms (Fig. 4a, b, d and e) but the C-terminal TNI-$2_{118–242}$ fragment did not (Fig. 4c).

The full length TNI-4 and two fragments TNI-$4_{1–166}$ and TNI-$4_{91–194}$ bound only the

pharyngeal TNT-4 isoform (Fig. 4f–h). The results of the interaction of the TNI isoforms with the TNT isoforms in the presence or absence of Ca^{2+} were almost similar (data not shown). The interaction profiles between the TNI and TNT isoforms are summarized in Fig. 4i. In conclusion, the body wall TNI isoforms interacted with both the body wall TNT-1 and the pharyngeal TNT-4 isoforms, but the pharyngeal TNI-4 only bound the pharyngeal TNT-4. The TNT interaction sites of four TNI isoforms in Fig. 1 were presented more details in Fig. 6a (see Discussion).

3. 2. 3. Interaction between TNI and TM isoforms

We further determined which of the TNI terminal regions interact with the tropomyosin isoforms of *C. elegans*. The pharyngeal TNI-4 isoform bound only to the pharyngeal tropomyosin, TMIII (Fig. 5b). Interestingly, the TNI-$2_{1–117}$, TNI-$2_{94–242}$, TNI-$2_{118–242}$, TNI-$4_{1–166}$ and TNI-$4_{91–194}$ fragments had lost the ability of the body wall and pharyngeal TNI isoforms to interact with tropomyosin isoforms (Fig. 5a-b). We conclude that body wall TNI-2 bound TNC/T and TM components from both tissues but pharyngeal TNI-4 interacted only with the pharyngeal components (Fig. 1). All the tissue-specific interactions of the TNI isoforms with other thin filament components are summarized in Fig. 1 (right panel) and are schematically shown in Fig. 6b (see Discussion).

4. Discussion

By performing the protein overlay assays using antibodies as probes we have determined the molecular interactions of the body wall TNI isoforms with other thin filament components, TNC, TNT and TM. We obtained evidence that the pharyngeal TNI-4 isoform bound tissue-specifically with other thin filament components but that the body wall TNI isoforms interacted with these components from both tissues. The functional significance of the C-terminal extension, which is unique to the body wall TNI isoforms of *C. elegans*, was not found by this approach.

4. 1. Molecular interactions among troponin components and tropomyosin

4. 1. 1. Function of the C-terminal extension in the body wall TNI-2 isoforms

In this study, we found that the N-terminal TNI-$2_{1–117}$, the C-terminal TNI-$2_{94–242}$, C-terminal extension-deleted TNI-$2_{1–220}$ and full-length TNI-2 all bound in a similar manner with both the body wall and pharyngeal TNT isoforms (Fig. 4a, b, d and e). The evidence obtained in this in vitro study confirmed that there was no difference in the interactions of body wall TNI isoforms with or without the C-terminal extension (Fig. 4d and e) and in the presence or absence of Ca^{2+} (data not shown). Similarly, the overlay assay results of TNC and TM isoforms also show that the C-terminal extension-deleted TNI-$2_{1–220}$ fragment is able to bind to both TNC and TM isoforms (Fig. 3a-b and Fig. 5a-b). Therefore, these results imply that the C-terminal extension has no direct role on binding to TNC, TNT and TM isoforms in *C. elegans*. Although we did not study the molecular interactions of the C-terminal TNI extension deletions of the two other body wall isoforms, the sequence similarity of the three TNI isoforms suggests that the C-terminal extension could have functions that were not detected by in vitro overlay assay. As the atomic structure of the core component of the human cardiac troponin complex does not include the TNI C-terminal region of this end of TNI [4], it could still have an important role in actin-myosin interactions. Tanaka et al. [19] reported that the ATNI$_{232-292}$ C-terminal fragment of Akazara scallop TNI inhibited actomyosin-tropomyosin Mg-ATPase activity. Al-Hillawi et al. [20] also reported that the recombinant human cardiac TNI inhibited the acto-S1 Mg-ATPase activity. This inhibition was potentiated by the presence of tropomyosin and was reversed by the addition of the TNC to the system. Although we have not done experiments on the C-terminal extension to determine functional effects on ATPase activity, we assume that the C-terminal extension of the three body

wall TNI isoforms of *C. elegans* has some biological function.

In muscle studies for *C. elegans* system [21], in vivo experiments could be used to determine the functional importance of the C-terminal extension. This might be achieved by studying whether the pharyngeal or C-terminal extension-deleted body wall TNI isoform could rescue the mutant phenotype of *tni-2/unc-27* animals that have a mutation in the major body wall isoform gene, *tni-2* [14].

4. 1. 2. Molecular interactions between the TNI and TNT isoforms

Immunoblotting, cross-linking and competitive labeling studies on the rabbit fast skeletal TNI and TNT suggest that the TNI 58-107 segment bound to TNT [22-24]. The atomic structure of the human cardiac troponin core complex shows that the helical part of H2 (I) interacted to H2 (T2) [4]. This region has been designated to the IT-arm [4]. We have focused on the IT-arm region, residue F90-R136 in human cardiac, of TNI in comparing residues among the four TNI isoforms of *C. elegans* and other animals (Fig. 6a) [14]. Our overlay assay results of the N-terminal TNI-2_{1-117} and the C-terminal TNI-2_{94-242} fragments of TNI-2 isoform suggest that TNI and TNT interaction sites are located in residues 94-117 (Fig. 1, Fig. 6a). The sequence alignment for the interaction sites in the four TNI isoforms of *C. elegans* (shaded in Fig. 6a) show that the interaction sites located in the N-terminal part of the IT-arms consist of three helical surfaces, three a and d surface of the heptad. In contrast to that, the pharyngeal TNI-4 isoform bound only with the pharyngeal TNT-4 isoform (Fig. 4f-h), whereas the body wall TNI-2 interacted with both tissue TNT isoforms (Fig. 4a, b, d, e and i). This suggests that the sites at which TNI and TNT interaction occurred contain some differences. From these results we assume that functional differences between the body wall and pharyngeal TNI isoforms came from deleting three amino acid residues in the pharyngeal isoform, TNI-4. The sequence alignment and the secondary structure prediction algorithm also suggest that this region can form a helical configuration. In the outside of the N-terminal of the helix, one proline residue is present in both TNI-1 and TNI-2 but two proline residues occur in TNI-3 and TNI-4. These will cause tilting of the helical rod of the IT-arm. Compared to the actin/TNC-binding sites of these TNI isoforms, the identity of TNT-binding sites among animals does not have a high sequence homology but there are some sequences homologies for forming helical conformations. Functional differences in the TNT-binding site between these animals may arise from fine-tuning the conformation of these regions between TNI and TNT. More detailed experimental studies on functional differences will be needed to investigate this further.

4. 1. 3. Molecular interactions of body wall TNI-2 with other thin filament components

Our results that the pharyngeal TNI-4 only bound with the pharyngeal TNC-2 isoform but not with body wall TNC-1 (Fig. 3a-b) are similar to those in a previous report that the nature of the TNI and TNC interaction is very different in different organisms and tissues [19]. The result of our overlay assays of TNC binding to TNI isoforms implies that the N-terminal regions of both the TNI-2 and TNI-4 isoforms interacted with pharyngeal TNC-2 (Fig. 3a-b). Farah et al. [25] also reported that the N-terminal region of chicken skeletal muscle TNI bound to the C domain of TNC. The NMR structure of cardiac TNI reveals that the N-terminal fragments of cardiac TNI interact with the C-terminal region of cardiac TNC [2]. Therefore, our results suggest that the importance of the N-terminal region of TNI in interactions with TNC isoforms could be common in both vertebrates and invertebrates. The reason why all C-terminal fragments of TNI did not to bind to TNC although they all contain actin/TNC-binding region could be the character of invertebrates. This was consistent to the case that molluskan Akazara scallop $TNI_{232\text{-}292}$, which contained the actin/TNC-binding region, did not interact with TNC even in the presence of Ca^{2+} [19]. It is of interest to study how molecular interactions among TN complex are developed during evolution.

Figure 5c summarizes the interactions between the two tissue-specific TNI and tropomyosin isoforms. These results suggest that the conformation of the TNI isoform altered after fragmentation, as the fragmented forms could not bind the tropomyosin isoforms (Fig. 5a-b). Similar results were observed in overlay assays of the tropomyosin and TNI isoforms in the absence of Ca^{2+} (data not shown). It has been reported that truncated troponin binds weakly to tropomyosin, regardless of Ca^{2+} presence [26]. Our results are consistent with this. It has also been reported that TNI binds weakly to tropomyosin in the presence or absence of Ca^{2+}, whereas the TNI/TNC complex shows no detectable interaction with tropomyosin under similar conditions [27]. Thus, the direct binding of TNI with tropomyosin could be important in the regulation of muscle contraction.

4. 2. Possible limitation of the protein overlay assay

Although the yeast two-hybrid system is a well-characterized method for analyzing protein-protein interaction [28], it can only identify potential binding partners for a protein of interest; no information is provided about whether an interaction actually occurs in vivo or what the consequences of binding might be. Pull-down approaches [29] can reveal protein associations that

occur in a cell of interest, but still cannot address the functional consequences of those interactions. Thus, it remains difficult to identify those specific protein interactions that are functionally relevant to a biological activity of interest. Here, we show that protein overlay assays can be used to study molecular interactions between TNI and other thin filament components. This approach is more convenient than column chromatography. It is quicker and uses less equipment and protein. The degree of protein refolding, the antibody used as probe, and the order of overlaid or blotted proteins are important factors for success. Helical structures especially are easily recovered in the original structure and can interact with other proteins under the experimental conditions. Our results on Western analyses show that isoform specificities of each fragmented TNI isoform are detected by the corresponding antisera (Fig. 2). Depending on solubility or the size of proteins the interaction can vary. We designed our experiments so that the soluble and smaller proteins were overlaid onto blots of the less soluble or larger protein. Unfortunately we could not detect differences in protein binding in the presence or absence of Ca^{2+}. It is difficult to control the quantities of the sample solution. Despite these limitations the overlay approach is a powerful means to determine the overall relationships between wide ranges of proteins from different sources. We have discussed in more details our use of this approach to study TNI isoforms and other thin filament components.

4. 3. Gene duplication of thin filament components

Based on the assay results of the interaction between TNI and TNC (Fig. 3), TNI and TNT (Fig. 4) and TNI and TM (Fig. 5) in this study, we summarized the molecular interactions between these proteins in two tissues (Fig. 6b). Further, we observed the important fact that pharyngeal TNI-4 interacted only with other pharyngeal thin filament proteins, and not with the body wall proteins (Fig. 6b). We assume that this was due to evolutionary and functional reasons: Thin filament proteins expressed in the pharynx interacted with each other, as was observed in the overlay assay between the pharyngeal isoform pairs (step 1). In evolution, the body wall TNI gene, probably *tni-3*, duplicated subsequently, from *tni-4* (step 2) [14]. Two other body wall TNI genes are likely duplicates of the original body wall TNI gene *tni-3*; this is also implied their tandem array on chromosome X. However, these genes do not differ considerably in their functions, although we have not determined this experimentally. These three body wall TNI isoforms can still interact with the pharyngeal isoforms (step 3). The body wall type genes of the thin filament components TNC-1, TNT-1 and tropomyosin TMI/II subsequently duplicated together with a mutation in the corresponding pharyngeal genes (step 4). The products of these genes could not interact with

pharyngeal TNI-4 because of the mutations that had occurred following gene duplication. Finally, the three body wall TNI isoforms could interact with the body wall TNC, TNT and TM isoforms (step-5). Troponin gene duplication during evolution and current isoform interactions can be explained in this manner. The sequence homology of the TNT-binding sites in the four TNI isoforms was lower than those of TNC- and actin/TNC-binding sites (Fig. 6a) [14]. Even with regard to the helical configuration of the TNI-TNT interaction site, the functional differences that depend on the type of amino acid residues of the isoforms could be important, although we have yet to determine any functional differences between these body wall isoforms. The hypothesis presented in this study will be useful to explore further the interactions within the troponin complex and other thin filament components in detail.

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Figure legends

Fig. 1. Schematic structures of four TNI isoforms and their constructed fragments and summary of their molecular interactions. The fragments containing the number of amino acids are shown in suffix. For example, the N-terminal fragment of TNI-1 containing amino acid residues 1-126 was TNI-$1_{1–126}$. Amino acid residue number follows to the previous report [14]. Highlighted boxes represent each location of the TNC-binding, TNT-binding, actin/TNC-binding and the C-terminal extension, respectively. More details for TNT-binding site are presented in Fig. 6a. The bar represents 50 amino acid residues. The right panel summarizes the molecular interactions of the constructed fragments of TNI isoforms with TNC, TNT and TM. BW, body wall; PHX, pharynx. "+" and "-" indicate a positive or negative interaction, respectively.

Fig. 2. Specificity of the antiserum against TNI, TNC and TM of *C. elegans*. **(**a) Cross-reactivity of four TNI isoforms and their fragments with anti-CeTNI-2 antibody in Western blot analysis. The lanes contained the total protein of *E. coli* harboring the expression constructs containing the N-, C-terminal fragments of four TNI isoforms, C-terminal extension-deleted fragment of body wall TNI-2 and four full-length TNI isoforms (Table 1). (b) Cross-reactivity of four TNI isoforms with anti-CeTNI-4 antibody [14]. (c) Cross-reactivity of two TNC isoforms with the anti-CeTNC-1 and anti-CeTNC-2 antibodies [14]. (d) Cross-reactivity of the body wall tropomyosin TMI and the pharyngeal tropomyosin TMIII isoforms with anti-CeTMIII antibody in Western blot analysis [17]. The positions are indicated by the molecular size of marker proteins in kilo Daltons (kDa). Lane B contained the total proteins in *E. coli* BL21 (DE3).

Fig. 3. Protein overlay assay of the body wall TNC-1 and the pharyngeal TNC-2 isoforms with both body wall TNI-2 and pharyngeal TNI-4 isoforms in the presence or absence of Ca^{2+}. (a) Protein overlay assay of the TNC-1 isoform with the N- and C-terminal fragments of both TNI-2 and TNI-4 isoforms in the presence of Ca^{2+} (upper panel) or absence of Ca^{2+} (lower panel). The overlaid TNC-1 isoform was detected by using the anti-CeTNC-1 antibody. (b) A similar protein overlay assay between the TNC-2 isoform and TNI isoforms or their fragments. Overlaid TNC-2 was detected by using the anti-CeTNC-2 antibody. + Ca^{2+} and - Ca^{2+} indicates the presence and absence of Ca^{2+}, respectively. (c) Summary of the interactions between the TNI and TNC isoforms. It was noted that the body wall TNC-1 only interacted with the N- terminal containing TNI-$2_{1–117}$ fragment, C-terminal extension-deleted TNI-$2_{1–220}$ fragment and full-length TNI-2.

Fig. 4. Protein overlay assay between the body wall and pharyngeal TNI isoforms and both tissues TNT isoforms. Panels (a)-(h) demonstrate the results of the protein overlay assays; (a) TNI-$2_{1–117}$, (b) TNI-$2_{94–242}$, (c) TNI-$2_{118–242}$, (d) TNI-$2_{1–220}$, (e) TNI-2, (f) TNI-$4_{1–166}$, (g) TNI-$4_{91–194}$ and (h) TNI-4 isoforms with both the body wall and pharyngeal TNT isoforms. The arrowheads indicate a 52-kDa TNT-1 and a 45-kDa TNT-4. Asterisks indicate no interaction with respective TNT isoforms. The overlaid TNI-$2_{1–117}$, TNI-$2_{94–242}$, TNI-$2_{118–242}$, TNI-$2_{1–220}$ and TNI-2 fragments were detected by using the anti-CeTNI-2 antibody, and the overlaid TNI-$4_{1–166}$, TNI-$4_{91–194}$ and TNI-4 fragments were detected by using the anti-CeTNI-4 antibody. (i) Summary of interactions between the TNI and TNT isoforms.

Fig. 5. Protein overlay assay of body wall TMI and pharyngeal TMIII with both the body wall TNI-2 and pharyngeal TNI-4 isoforms. (a) Protein overlay assay of the TMI isoform with the N- and C-terminal fragments of both TNI-2 and TNI-4 isoforms and the C-terminal extension-deleted fragment TNI-$2_{1–220}$. (b) A similar assay of the TMIII isoform with those of TNI isoforms. Overlaid TMI and TMIII were detected by using the anti-CeTMIII antibody. (c) Summary of interactions between TNI and TM isoforms obtained from the results of (a) and (b).

Fig. 6. Alignment of the TNT-binding site of the TNI isoforms of invertebrates and vertebrates and the molecular interaction model. (a) Sequence comparison of the TNT-binding sites of four TNI isoforms of worms and those of Akazara scallop, crayfish, *Drosophila*, *Ciona* heart and body wall, rabbit cardiac, human cardiac, rabbit slow, human slow, rabbit fast and human fast skeletal muscles. The shade indicates the TNT-binding site of the TNI-2 isoform. The box indicates the IT-arm of the human cardiac TNI [4]. The black bars represent the (H2) TNI helix region [4] and TNT-binding site, respectively. Numbers in the TNT-, TNC- and actin/TNC-binding sites represent percentage homology. Data follows the previous report [14]. Hydrophobic amino acids locating at the ‘a’ and ‘d’ positions in the heptads repeat are located at the top of the sequence. The identity and similarity of the amino acid residues are indicated by (*) and (:/.), respectively. (b) The molecular interaction model to study the interactions between the TNI isoforms and those with the other thin filament components TNC, TNT and TM. Step 1 represents the interaction of the pharyngeal TNI-4 isoform with that of pharyngeal TNC-2, TNT-4 and TMIII; Step 2 represents the presumably gene duplication of pharyngeal TNI-4 to the body wall TNI isoforms; Step 3 represents the interaction of the body wall type TNI isoforms with other pharyngeal thin filament proteins (as mentioned in step

1); Step 4 represents presumably gene duplication with mutations of pharyngeal isoforms to their corresponding body wall isoforms; Step 5 represents the interaction of the body wall troponin and tropomyosin isoforms with the body wall TNI isoforms, respectively. The rectangles and circles indicate the body wall and pharynx specific isoforms, respectively.

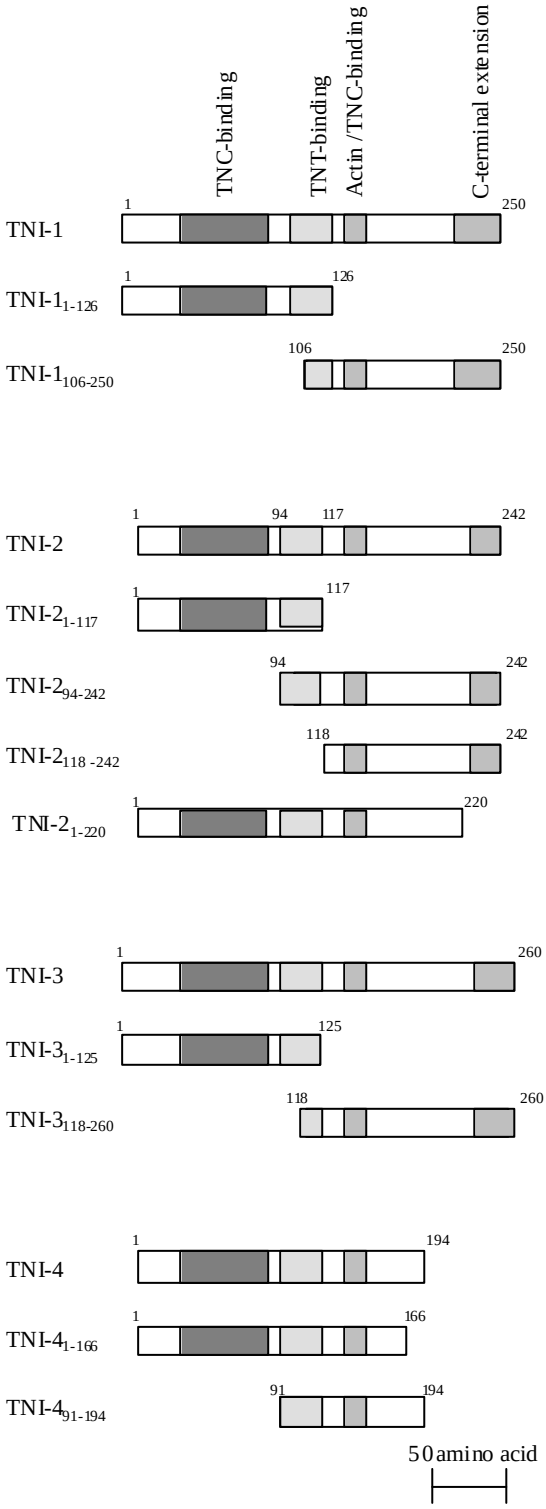
Table 1
Construction and expression of TNI, TNC, TNT and TM

Plasmid	Restriction sites	Product	Length (aa)	Molecular size[a] (kDa)	Expressed tissue
pCTNI-1	*Eco*RI-*Sma* I	TNI-1	250	50	BW[b]
pCTNI-1_{1-126}	*Eco*RI	TNI-1_{1-126}	126	23	ND
pCTNI-$1_{106-250}$	*Xho*I	TNI-$1_{106-250}$	144	22	ND
pCTNI-2	*Eco*RI	TNI-2[f]	242	50	BW[b]
pCTNI-2_{1-117}	*Bam*HI	TNI-2_{1-117}	117	18	ND
pCTNI-2_{94-242}	*Xho*I-*Hin*dIII	TNI-2_{94-242}	148	20	ND
pCTNI-$2_{118-242}$	*Bam*HI	TNI-$2_{118-242}$	124	20	ND
pCTNI-2_{1-220}	*Bam*HI	TNI-2_{1-220}	220	29	ND
pCTNI-3	*Eco*RI-*Sma* I	TNI-3	260	52	BW[b]
pCTNI-3_{1-125}	*Eco*RI	TNI-3_{1-125}	125	24	ND
pCTNI-$3_{118-260}$	*Bgl*II-*Xho*I	TNI-$3_{118-260}$	142	22	ND
pCTNI-4	*Bam*HI-*Sma* I	TNI-4[f]	194	48	PHX[b]
pCTNI-4_{1-166}	*Eco*RI	TNI-4_{1-166}	166	27	ND
pCTNI-4_{91-194}	*Xho*I	TNI-4_{91-194}	103	17	ND
pCTNC-1	*Eco*RI	TNC-1[f]	161	24	BW[c]
pCTNC-2	*Sal* I-*Eco*RI	TNC-2[f]	160	20	PHX[c]
pCTNT-1	*Eco*RI	TNT-1	405	52	BW[d]
pCTNT-4	*Bam*HI	TNT-4	347	45	PHX[d]
pCTMI	*Hin*d III	TMI	284	40	BW[e]
pCTMIII	*Pst* I-*Hin*d III	TMIII[f]	256	38	PHX[e]

BW, body wall; PHX, pharynx; ND, not determined; [a]molecular size on SDS-PAGE; [b]Ruksana *et al.*[14]; [c]Terami *et al*. [15]; [d]WormBase; [e]Kagawa *et al.*[16]; [f]Products are used for the production of antibody.

Fig. 1 Amin et al.

Schematic structure of four TNI isoforms and constructed fragments



Summary of molecular interactions

	TNC-1 (BW)	TNC-2 (PHX)	TNT-1 (BW)	TNT-4 (PHX)	TMI (BW)	TMIII (PHX)
TNI-1	+	+	+	+	+	+
TNI-1$_{1-126}$	+	+	+	+	-	-
TNI-1$_{106-250}$	-	-	-	-	-	-
TNI-2	+	+	+	+	+	+
TNI-2$_{1-117}$	+	+	+	+	-	-
TNI-2$_{94-242}$	-	-	+	+	-	-
TNI-2$_{118-242}$	-	-	-	-	-	-
TNI-2$_{1-220}$	+	+	+	+	+	+
TNI-3	+	+	+	+	+	+
TNI-3$_{1-125}$	+	+	+	+	-	-
TNI-3$_{118-260}$	-	-	-	-	-	-
TNI-4	-	+	-	+	-	+
TNI-4$_{1-166}$	-	+	-	+	-	-
TNI-4$_{91-194}$	-	-	-	+	-	-

Fig. 2 Amin et al.

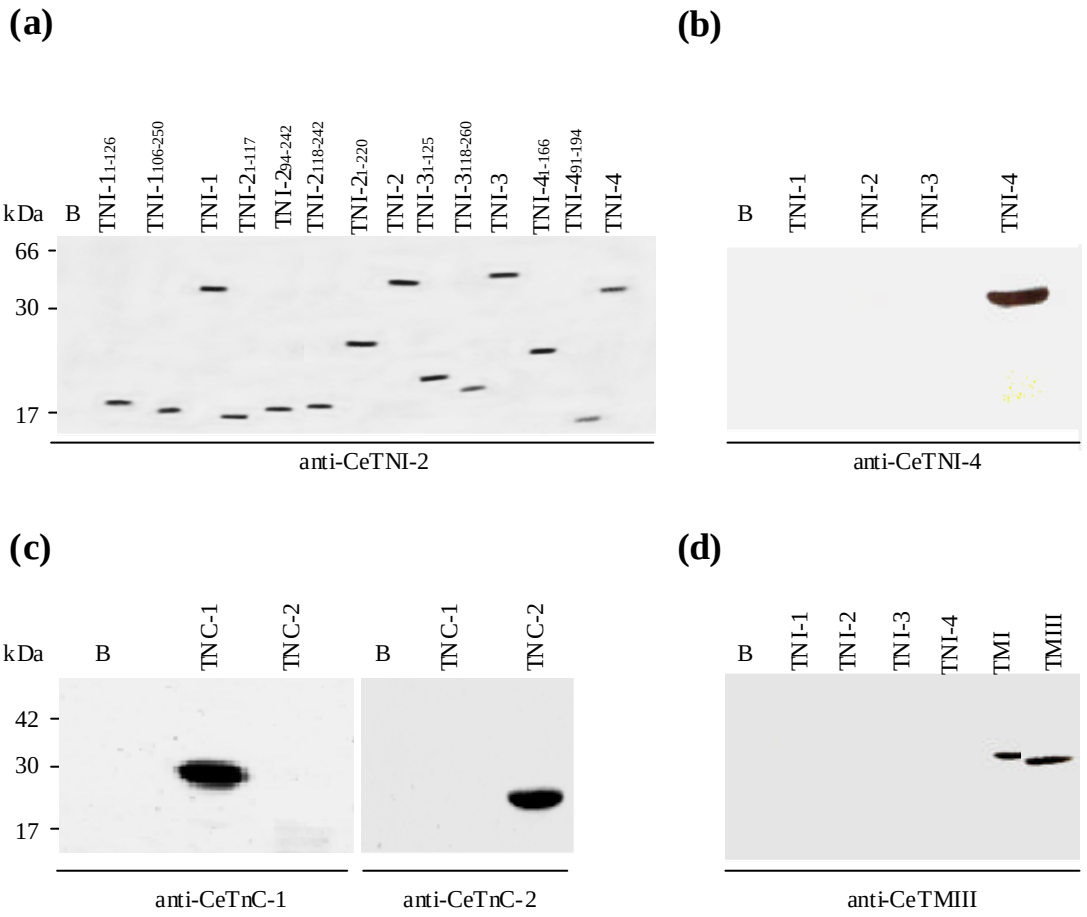
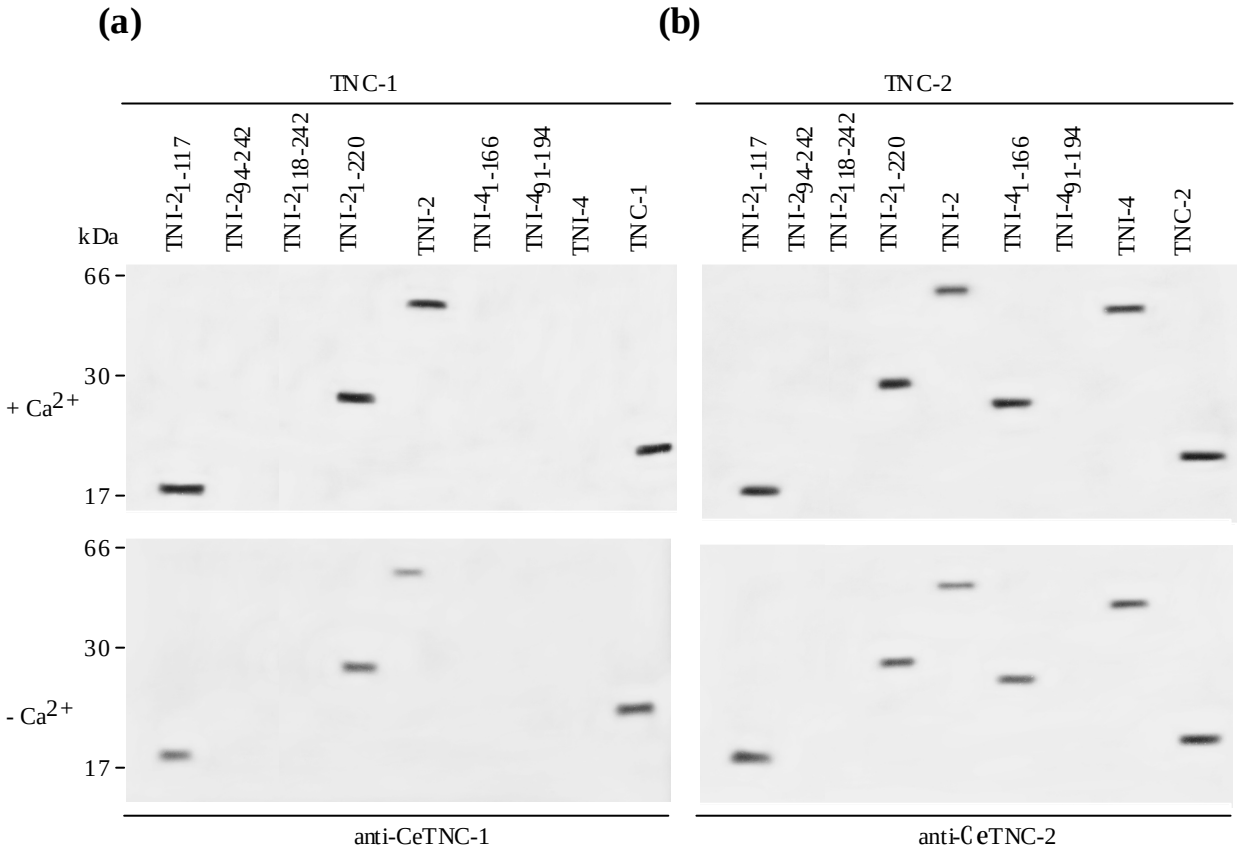


Fig. 3 Amin et al.



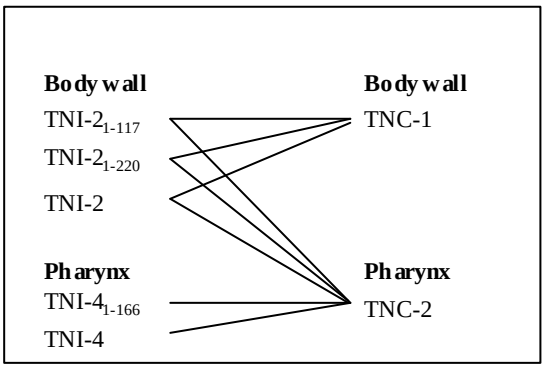
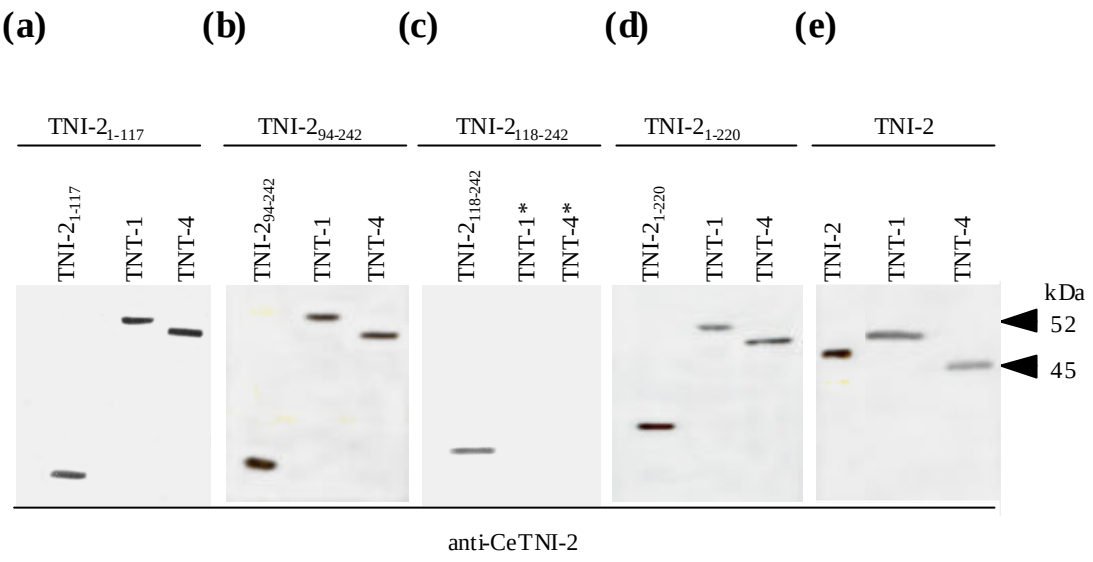


Fig. 4 Amin et al.



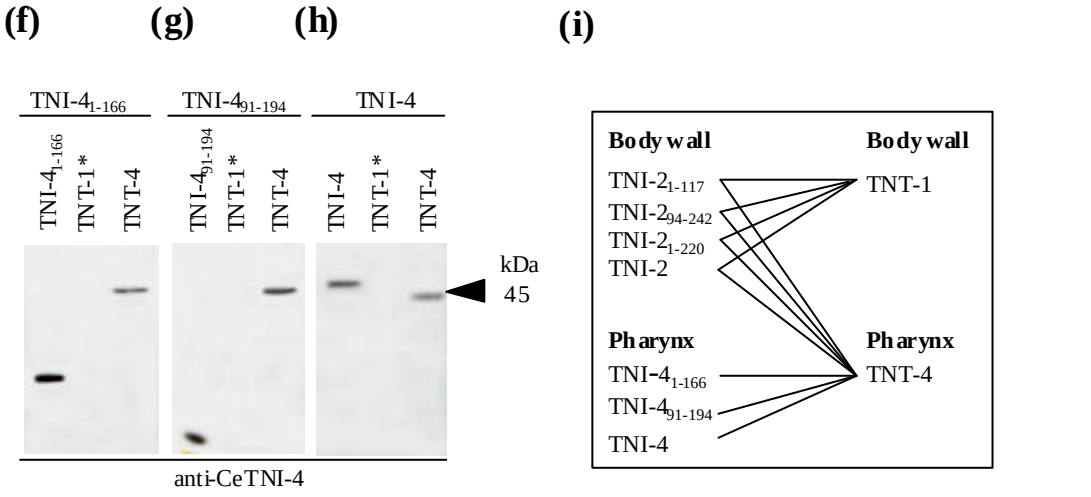
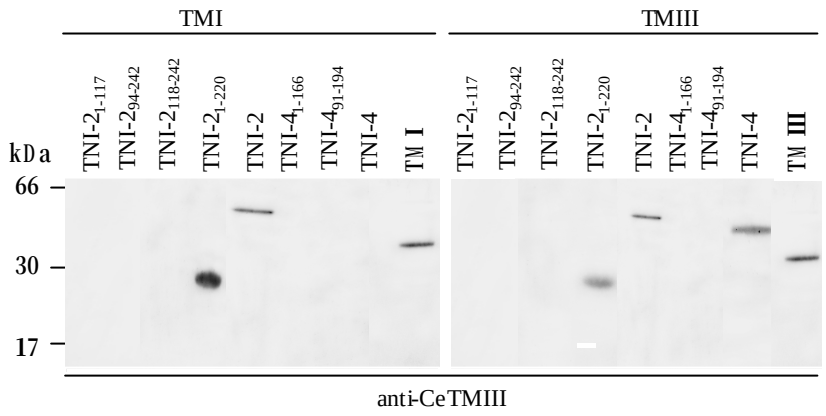


Fig. 5 Amin et al.

(a) **(b)**



(c)

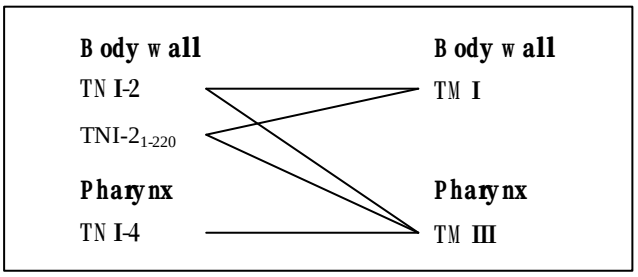
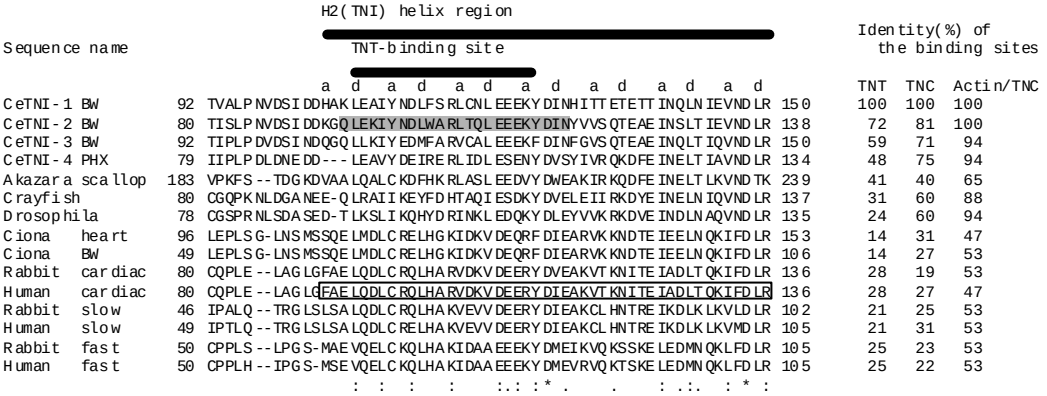


Fig. 6 Amin et al.

(a)



(b)