Troponin T Isoform Regulation and Structure-Function Relationships

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Abstract

Three fiber type-specific genes have evolved in vertebrates to encode cardiac, slow and fast skeletal muscle troponin Ts (TnT). From the transcript of each gene, alternative RNA splicing produces multiple TnT isoforms with structural differences in three variable regions of the polypeptide chain. The expression of TnT isoform is regulated during heart and muscle development and adaptation. Muscles containing different TnT isoforms show differences in their sensitivity to Ca^{2+}-activation and cooperativity of contraction. The NH_2-terminal region of TnT is hypervariable among isoforms and may play a role in modulating the contractility corresponding to the cellular environment and functional state of the muscle. Molecular evolution of the NH_2-terminal variable region of TnT indicates its tolerance to structural variation and role in the functional divergence of striated muscle. Physical property of the NH_2-terminal domain of TnT modulates conformation and function of other regions of TnT, explaining the functional significance of TnT isoform regulation. Species-specific timing of cardiac TnT isoform switching is synchronized in the heart and skeletal muscle, suggesting control by a genetic program. Therefore, the regulation of TnT isoform expression is not only a response to functional demands but may contribute to both physiological adaptation and pathogenesis.

Key words: alternative splicing, development, gene regulation, muscle, protein conformation.

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Troponin T (TnT), the tropomyosin (Tm)-binding subunit of the troponin complex, is a central component in the thin filament-based Ca^{2+}-regulatory system of striated muscles [21, 34, 40, 47]. Three genes have evolved to encode the cardiac (TNNT2), slow (TNNT1) and fast (TNNT3) skeletal muscle TnTs. By transcriptional regulation, the three TnT genes are specifically expressed in cardiac, slow and fast skeletal muscles, respectively [3, 6, 11, 18].

From the transcript of the TnT genes, alternative RNA splicing further generates multiple protein isoforms [3, 4, 6, 16, 18, 20, 36, 42, 43]. The primary structure of cardiac, slow and fast skeletal muscle TnT isoforms from various species showed that a major portion of the TnT primary structure is highly conserved and encoded by constitutively expressed exons. There are three alternatively spliced regions in the TnT polypeptide chain: 1) a fast skeletal muscle TnT-specific COOH-terminal segment encoded by a pair of mutually exclusive exons (exons 16 and 17); 2) a cardiac TnT-specific central segment, and 3) an NH_2-terminal variable region found in all three TnTs. The amino acid sequence and alternative splicing pattern of the NH_2-terminal region is highly variable both phylogenically and among the three muscle type-specific TnTs [16].

Alternative splicing of an NH_2-terminal acidic segment is responsible for a cardiac TnT isoform switch during avian and mammalian heart development [6, 13, 20]. A large to small, acidic to basic isoform transition of fast skeletal muscle TnT is also found during skeletal muscle development, involving alternative splicing of multiple exons encoding the NH_2-terminal variable region [43]. Altered cardiac TnT isoform expression has been observed in myocardial disorders [1, 2].

Structure-function relationship studies using TnT fragments have identified the functional domains of TnT. Experiments using rabbit fast skeletal muscle TnT dissected the protein into two major functional fragments. The COOH-terminal fragment T2 (residues 159-259) binds to the central region of Tm [23, 27] and interacts with troponin I (TnI), troponin C (TnC) and F-actin [8, 33, 37]. The NH_2-terminal fragment T1 (residues 1-158) interacts with the carboxyl terminus of Tm, extending to include the amino terminus of the adjacent

Tm molecule in the head-to-tail overlap structure on the muscle thin filament assembly [32, 46]. The alternatively spliced central segment in cardiac TnT locates in between the T1 and T2 functional fragments [20] and may represent a linker segment connecting the NH_2- and COOH-terminal structure-function domains of TnT. The alternatively spliced NH_2-terminal variable region of TnT (e.g., the CB3 fragment representing residues 1-69 in the rabbit fast skeletal muscle TnT) does not directly bind to any other known proteins in the thin filament of muscle. TnT molecules with a deletion of the NH_2-terminal variable region (e.g., deletion of the first 45 residues and the 25-kDa NH_2-terminal truncation of the rabbit fast skeletal muscle TnT) retained activity in the Ca^{2+} activation of actomyosin ATPase [7, 31]. Therefore, the alternatively spliced NH_2-terminal variable region is likely under low evolutionary pressure and behaves as a non-essential structure for TnT's core activity in the Ca^{2+}-regulation of muscle contraction.

Nevertheless, when intact TnT was investigated, the results have shown functional differences between TnT isoforms differing in the alternatively spliced NH_2-terminal variable region. For example, two bovine adult cardiac TnT's with a difference in the inclusion or exclusion of an acidic NH_2-terminal pentapeptide produced different overall Ca^{2+} response in reconstituted thin filaments [41]. Studies have demonstrated that the more acidic embryonic isoform of cardiac TnT contributes to the higher tolerance of myofibril performance to acidosis [39]. Using reconstituted systems, it was shown that the NH_2-terminal segment of TnT increases the actomyosin ATPase activity in the presence of Tm [24]. Therefore, when residing in the TnT molecule, the "non-essential" NH_2-terminal segment may play a structural and functional role. To understand the physiological and pathological significance of the TnT isoform regulation during muscle development and adaptation, it is essential to understand the structure-function relationship of TnT and the alternatively spliced variable regions.

Materials and Methods

Western blotting examination of TnT isoform expression in muscle tissues

Muscle samples were homogenized in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 1% SDS and heated at 80°C for five minutes. The total muscle protein extracts were clarified by centrifugation prior to SDS-PAGE [29]. The SDS-PAGE resolved protein bands were transferred to nitrocellulose membranes as previously described [28]. The nitrocellulose membranes were incubated with a rabbit polyclonal antiserum (RATnT) recognizing all TnT isoforms across species [44] or an anti- TnT mAb CT3 recognizing cardiac and slow, but not fast, TnT [16]. The subsequent washing, incubation with alkaline phosphatase-labeled anti-rabbit or anti-mouse IgG second antibodies, and 5-bromo-4-chloro-3-indolylphosphate - nitro blue tetrazolium substrate color development were performed as previously described [29].

Molecular cloning and DNA sequence analysis

The cloning and sequence analysis of cDNAs encoding TnT isoforms from various species and genomic DNAs containing the TnT genes have been described previously [11, 18, 20, 43]. Both reverse transcription-coupled polymerase chain reaction (RT-PCR) and library screening were used for the cDNA cloning. For the RT-PCR, total RNA was extracted from the muscle tissues. Oligonucleotide primers were synthesized according to sequences deduced from the conserved coding regions of TnTs from closely related species with their 3'-end bases placed at the first or second base of a codon to avoid wobble mismatches. The TnT cDNA synthesized by RT-PCR from the RNA template was cloned into plasmid vectors. After transformation of *E.coli*, ampicillin-resistant colonies containing the recombinant plasmids were verified by PCR for inserts with expected size. For the cDNA library screening, cardiac or skeletal muscle cDNA libraries were constructed in λ phage vectors as described previously [20, 43]. TnT cDNA probes or anti-TnT antibodies was used to screen the cDNA libraries. The positive phages identified by hybridization or antibody detection were plaque-purified. The cDNAs cloned by the two approaches were sequenced using the dideoxy chain-termination method.

By screening using [^{32}P]-labeled TnT cDNA probes, genomic DNA clones containing TnT gene were isolated from a mouse genomic library constructed in the λDASHII phage vector (Stratagene) as described previously [18]. DNA was purified from the positive phages and the insert was subcloned into plasmid vectors for sequencing characterization. By constructing exonuclease III serial deletion subclones using the Erase-A-Base kit from Promega [18], restriction endonuclease deletion subclones, and using synthetic oligonucleotide primers, overlapped sequencing reactions were carried out as above.

Two-dimensional gel electrophoresis analysis of cardiac TnT isoform switching

Two-dimensional gel electrophoresis on developing rat and chicken cardiac muscle was performed as described previously [13]. The first dimension in tube gels was isoelectric focusing in ampholine (Pharmacia) with a pH range of 4 to 6 to separate the muscle proteins according to their charge differences. The second dimension was SDS-PAGE to further separate the proteins by size. After the two-dimensional gel electrophoresis, the resolved protein bands were transferred to nitrocellulose for Western blotting with the anti-cardiac TnT mAb CT3 as described above. ^{125}I-radioactively labeled sheep anti-mouse IgG was used as the second antibody. The

nitrocellulose membranes were washed to remove excess and nonspecifically bound first and second antibodies and the cardiac TnT isoforms were visualized by autoradiography at -70°C using an intensifying screen.

Functional characterization of TnT isoforms

Conformational analysis

An enzyme-linked immunosorbant assay (ELISA)-based protein epitope analysis was developed to detect conformational changes in chicken breast muscle TnT induced by the binding of metal ions or mAbs to a cluster of His pairs in the NH_2-terminal variable region [44]. The conformational changes in other regions of the TnT molecule were monitored by antibodies against specific epitopes.

Protein binding assays

An ELISA-mediated protein binding assay was developed to characterize the effect of TnT conformational changes on the interaction with TnI, TnC and Tm [17, 28, 44]. Microtiter plates were coated with purified TnT. After washes to remove the excess TnT and blocking the remaining plastic surface, the plates were incubated with serial dilutions of TnI, TnC or Tm. The plates were then washed to remove unbound TnI, TnC and Tm before incubation with an anti-TnI, TnC or Tm antibody. Following washes, horse radish peroxidase-conjugated anti-mouse immunoglobulin second antibody was incubated with the plate at room temperature for 40 min followed by washes and H_2O_2-2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate color reaction. The A_{405nm} of triplicate assay wells was monitored at a series of time points and the data within the linear course of the color development were used to plot the protein binding curves.

Contractility characterization

Chicken muscle fiber bundles were skinned in a relaxing solution containing 1% (w/v) Triton X-100 [30]. The bundles were then washed with the relaxing solution in 50% glycerol. Contractility measurement was performed at 22°C on fibers containing different TnT isoforms. Force results were normalized against the cross-sectional area calculated from their measured maximal and minimal diameters. The pCa of the perfusing solution was varied by mixing relaxing solution with varying amounts of activating solution that contained 10 mM Ca^{2+}.

Results and Discussion

Muscle type-specific expression of cardiac, slow and fast skeletal muscle TnTs

Multiple isoforms of cardiac, slow and fast skeletal muscle TnT are shown by the Western blots of neonatal mouse cardiac and skeletal muscles. Figure 1 demonstrates that the molecular weight of cardiac TnT is higher than that of the skeletal muscle TnTs. The sizes of slow and fast skeletal muscle TnTs overlap but the CT3 mAb specifically identifies the slow TnT. Similar Western blots on adult muscles showed that slow TnT is specifically expressed in slow fibers and may be used as a marker to identify muscle fiber types [16] and to monitor TnT isoform switches [9]. While cardiac TnT is expressed in embryonic and neonatal skeletal muscles (Figure 1), the two skeletal muscle TnTs are not detected in cardiac muscle at any of the developmental stages examined [9]. Protein structure similarity and gene homology analysis demonstrated that each muscle type-specific TnT is well conserved in the vertebrate phyla while the three TnTs have significantly diverged [16], suggesting an adaptation to the functional characteristics of the muscle.

Alternative RNA splicing-generated TnT isoforms

Sequencing data up to date show that the size of vertebrate TnTs ranges from 223 to 305 amino acids. This size variation is almost solely due to the variable length of the NH_2-terminal segment. This segment is encoded by different combinations of multiple alternative exons and is highly diverged in sequence (Figure 2). This region is the least conserved among the three TnT genes or in any one TnT gene from different species [16]. In contrast, the central and COOH-terminal regions of the TnT polypeptide chain are conserved among the three muscle type-specific TnT genes and across species.

Our sequencing data from a large sample (fifty-two) of original mouse cardiac TnT cDNAs identified two alternatively-spliced coding exons (exons 3a and 4) responsible for the expression of four protein isoforms with primary structure differences in the NH_2-terminal variable region [20]. We have found three slow TnT isoforms generated by alternative splicing of exons 5 and 6 in the NH_2-terminal region [16]. Genomic sequence

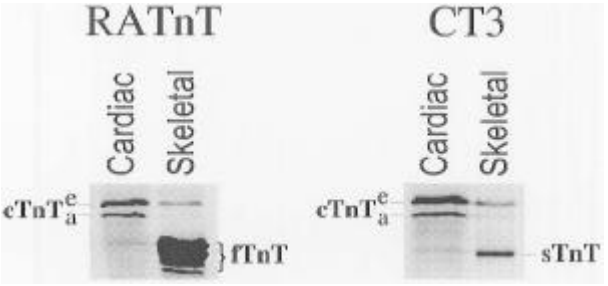

Figure 1. Cardiac, slow and fast skeletal muscle TnT isoforms. The TnT isoforms expressed in neonatal mouse cardiac and skeletal muscles were examined by Western blotting. All TnT isoforms are detected by the RATnT polyclonal antibody. The anti-cardiac and slow skeletal muscle TnT mAb CT3 blots show that only cardiac TnT (cTnT) isoforms (e, embryonic; a, adult) are expressed in the heart. Multiple fast skeletal muscle TnT (fTnT) isoforms are expressed in the skeletal muscles together with slow skeletal muscle TnT (sTnT). Cardiac TnT is also expressed in the neonatal (and embryonic, data not shown) skeletal muscles.

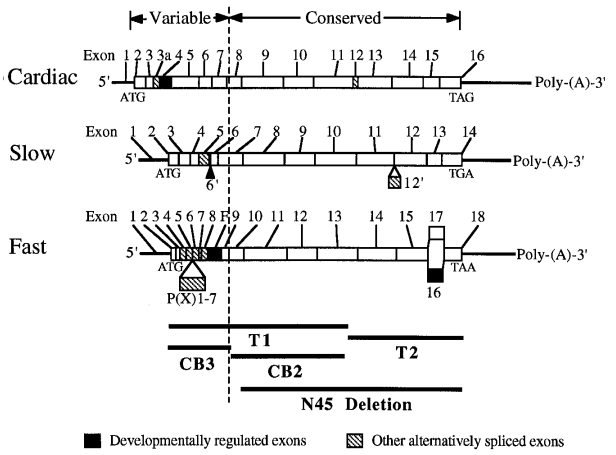

Figure 2. Structural comparison of cardiac, slow and fast skeletal muscle TnTs and the alternatively spliced variable regions. cDNA maps of mouse cardiac, slow and fast skeletal muscle TnTs are shown with the coding region as a wider bar. The segments encoded by each exons are indicated. The alternatively spliced exons are illustrated by the shaded boxes with the developmentally regulated exons in solid black. The coding region is aligned with the characterized functional fragments of TnT (see the text for details). In additional to the size differences generated by alternative inclusion or exclusion of multiple exons, the NH_2-terminal region of TnTs is also highly variable in amino acid sequences.

indicates that an alternatively spliced central segment found in human slow skeletal muscle TnT cDNA is due to the inclusion of a portion of the 3'-region of intron 11 into the mRNA (exon 12', ref. 11). The fast skeletal muscle TnT isoform expression is more complex involving alternative splicing of a pair of mutually exclusive COOH-terminal exons and more than seven exons encoding the NH_2-terminal region: exon 4-8, a mammalian fetal exon [3, 4, 43], the w, y, and up to 7 P (X) exons in birds [5, 26, 36, 38]. Through many combinations of splicing pathways, a large number of TnT isoforms may be generated from the transcript of the fast TnT gene. Indeed, we have identified 13 mouse [43] and 11 chicken fast skeletal muscle TnT isoforms [29]. The structural organization and alternatively spliced exons in the three TnT genes are summarized in Figure 2.

An acidic to basic TnT isoform switch during heart and skeletal muscle development

A regulated cardiac TnT isoform switch occurs during heart development. This perinatal cardiac TnT isoform switch is a large to small, acidic to basic transition of the TnT molecule (Figure 3). Immunoprecipitation using anti-TnT antibody also detected the two cardiac TnT isoforms in the *in vitro* translation products of $polyA^+$ RNA isolated from developing rat hearts [13]. This result indi-

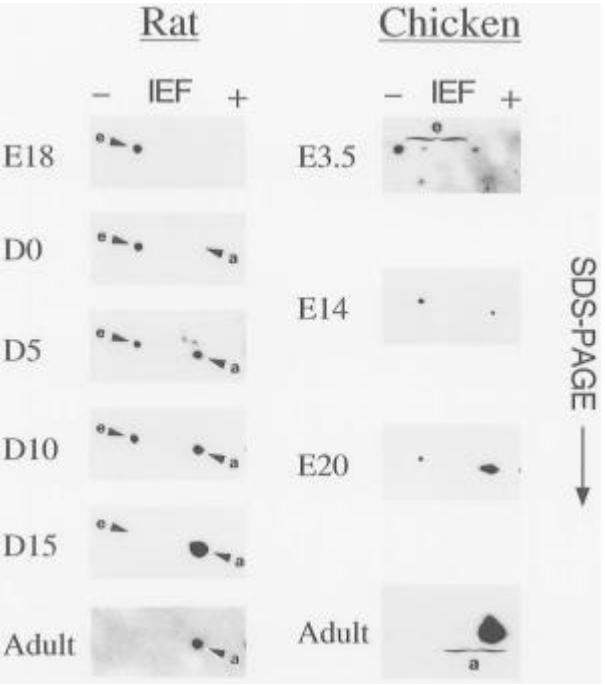

Figure 3. Alternative mRNA splicing-generated cardiac TnT isoform switching during heart development. Total protein extracts from developing rat and chicken hearts were resolved by two-dimensional gel electrophoresis (isoelectric focusing, IEF, followed by SDS-PAGE) and transferred to nitrocellulose membrane. Western blotting was performed using the anti-cardiac TnT mAb CT3 and ^{125}I-labeled second antibody. The autoradiograph demonstrates a switching of cardiac TnT from the high Mr, acidic embryonic isoform (e) to the low Mr, basic adult isoform (a) during both mammalian and avian heart development.

cates that two species of mRNA, other than post-translational modification of one polypeptide, are responsible for the embryonic and adult cardiac TnT isoforms. cDNA and genomic sequence data have revealed that the two cardiac TnT isoforms are generated by the inclusion or exclusion of an embryonic-specific exon of the cardiac TnT gene encoding 10 mainly acidic amino acids in the NH_2-terminal variable region [6, 18, 20].

Similar to the cardiac TnT isoform switch, the expression of fast skeletal muscle TnT isoforms during perinatal muscle development also undergoes a high Mr to low Mr isoform switch. Inclusion or exclusion of multiple NH_2-terminal coding exons in various combinations is responsible for this transition of fast skeletal muscle TnT isoforms [43]. Since the NH_2-terminal domain of TnT mainly contains acidic amino acid residues, a significant correlation is found between the size and isoelectrical point (pI) of TnT isoforms from various species (Figure 4). Accordingly, the length of the NH_2-terminal

variable region determines not only the size but also the NH_2-terminal charge of the TnT isoforms.

The alternative splicing-generated TnT NH_2-terminal variation involves three parameters: sequence, size and charge. The NH_2-terminal sequences of TnT are highly variable and no underlying pattern could be identified between the embryonic and adult isoforms. The sizes of the NH_2-terminal region often overlap between embryonic and adult TnT's. In contrast, the developmental NH_2-terminal charge transition shows a unique feature. The pI of fast skeletal muscle TnT isoforms has a wide range from 5 to 10. We have found that the pIs of 13 different randomly isolated embryonic and adult mouse fast skeletal TnT isoforms fall into two non-overlapping classes. The acidic isoforms have pIs ranging from 5.04 to 6.11 and the basic isoforms have pIs ranging from 8.85 to 10.06 [43]. The significantly different average pIs of the acidic and basic isoforms (5.47 vs. 9.50) are solely due to the charge differences between their alternatively spliced NH_2-terminal regions. Despite the complex splicing patterns of the 6 NH_2-terminal alternative exons and their overlapping sizes (Figure 4), there are two distinct charge groups of fast TnT isoforms. From the large number (sixty) of original cDNAs sequenced, we did not identify a TnT isoform with a pI intermediate to these two charge groups (Figure 5A). It is important to note that the acidic isoforms are only found in neonatal muscles whereas all of the adult fast TnT are basic isoforms (Figure 5B and C). Therefore, the NH_2-terminal charge may be a key parameter underlying the functional significance of the TnT isoform regulation.

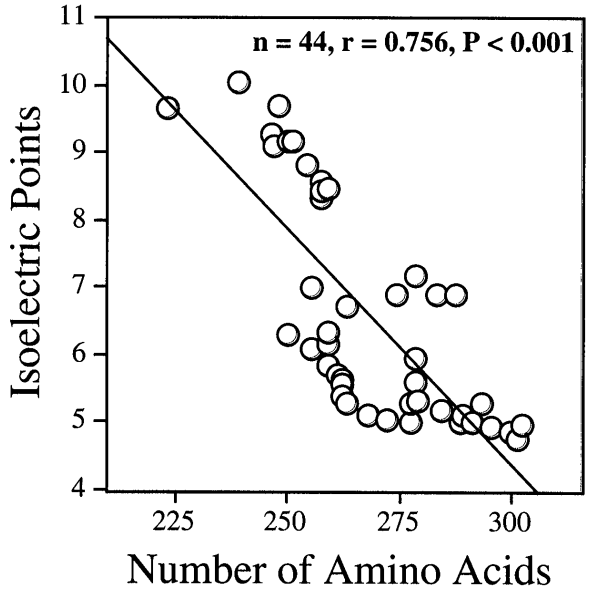

Figure 4. Correlation between the size and isoelectric point of TnT isoforms. Primary structural information of forty-four cardiac, slow and fast skeletal muscle TnT isoforms from various vertebrate species (fish, birds and mammals) was deduced from cDNA sequences. Isoelectric points of the TnT isoforms were calculated using the DNAStar computer program. The scatter plot produced using the Cricket Graph III program (Computer Association International, Inc., Islandia, NY) shows a significant correlation between the size (shown in the number of amino acids) that is almost solely determined by the length of the NH_2-terminal variable region and the isoelectric point reflecting the NH_2-terminal change.

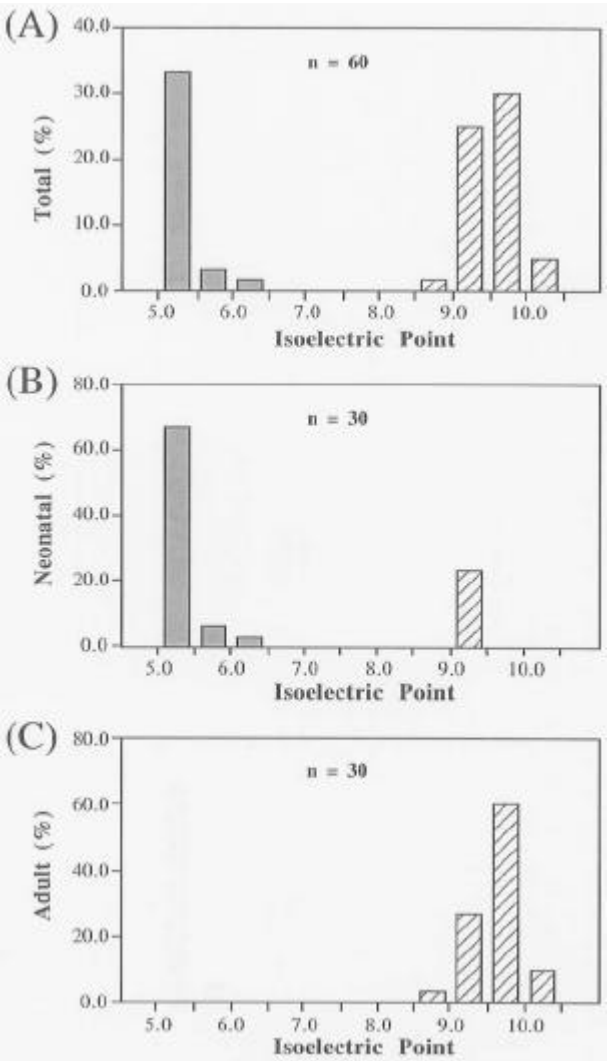

Figure 5. The acidic and basic TnT isoforms. (A) The histogram shows that a pool of 60 original embryonic and adult mouse fast skeletal muscle TnT clones corresponding to 13 isoforms generated by complex alternative splicing of multiple NH_2-terminal exons fall into two distinct charge groups. No intermediates were found in between the acidic and basic classes of fast TnT. (B) and (C) The acidic fast TnT is only found in the neonatal mouse muscle and the adult mouse muscle expresses only basic fast TnT.

Mature fast skeletal muscle-specific exon 16-encoded COOH-terminal segment

Comparing the mouse cardiac, slow and fast skeletal muscle TnT primary structures (Figure 2), it is interesting to note that the fast TnT exon 17 is similar to the counterpart exons of cardiac and slow skeletal muscle TnTs, suggesting a close evolutionary relationship. In contrast, the fast TnT exon 16 shows less similarity to its counterpart segments in slow and cardiac TnT as well as to the fast TnT exon 17. Inclusion of exon 16 is only found in adult and basic fast TnT isoforms [19, 43]. Therefore, the exon 16 structure and splicing pathway may have evolved in adaptation to the differentiation and functional demands of mature fast skeletal muscle.

Regulation of slow skeletal muscle TnT isoform expression

Alternative splicing of the NH_2-terminal region of slow skeletal muscle TnT also generates protein isoforms with differences in size and charge [16]. Different from the developmental cardiac and fast skeletal muscle TnT isoform switches, the pattern of slow TnT isoform expression seems not to be clearly related to development. However, the expression of slow TnT and its isoform regulation are more profound in large versus small mammals (Figure 6). In contrast to large mammals [16], only a few adult mouse skeletal muscles express sTnT. Knowing that the slow muscle fiber plays a differentiated role critical to the mobility of animals [35], the relative distribution of slow fibers in large and small animals may reflect mobility adaptations of different vertebrate muscles.

The alternative splicing-regulated NH_2-terminal charge difference between TnT isoforms contributes to the tolerance to acidosis

Solid-phase protein binding assay demonstrated that the adult muscle-specific inclusion of an acidic segment in the NH_2-terminal region of chicken pectoralis fast TnTs resulted in a higher tolerance to the effect of decreasing pH on TnT's interaction with TnI and Tm. While the interaction of acidic TnT isoforms with TnI was less sensitive to the decrease of pH than the basic fast TnT, the binding affinity of acidic TnT isoforms with Tm was little affected by the decreased pH in contrast to the basic TnT isoform [29]. Given that the majority of adult skeletal muscles express basic fast TnT isoforms, the switching between acidic and basic TnT isoforms may play a role in the functional adaptation of the contractile machinery to the muscle fiber environment. This result is in agreement with the observation that embryonic cardiac muscle expressing the more acidic TnT isoform has a higher tolerance to acidosis [39]. This observation is also consistent with the fact that both cardiac and slow skeletal muscle TnTs are more acidic in comparison with the normal adult fast skeletal muscle TnT, adding evidence for the functional role of the regulated NH_2-terminal region of TnT.

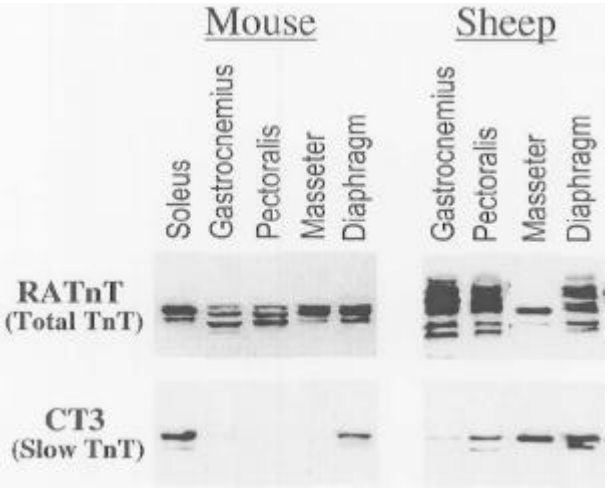

Figure 6. Fast and slow skeletal muscle TnT isoform expression in small and large mammals. Western blots were carried out on total protein extracts of adult mouse and sheep muscles using the RATnT polyclonal antibody (recognizing all TnT isoforms) and mAb CT3 (recognizing slow, but not fast, TnT isoforms). The results show three lines of information: a) the TnT isoform composition varies significantly among different muscle fibers; b) the muscles of large animals may simultaneously express more fast TnT isoforms in comparison to that in the small rodents; c) slow TnT expressing muscle fibers may be more widely present in the muscles of large animals.

The NH_2-terminal variable region-based conformational and functional modulation of TnT

To overcome difficulties due to the lack of crystal or NMR structural information for TnT, we applied an antibody epitope analysis to monitor molecular conformation of TnT. The binding between an antibody (immunoglobulin) to a specific antigenic epitope is a protein-protein interaction based on three-dimensional structure fits. Therefore, we may measure the binding affinity between the antibody paratope and the epitopes on TnT to monitor their conformational relationship [44]. A chicken fast skeletal muscle TnT containing a transition metal (Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+})-binding segment (Tx) in the NH_2-terminal variable region [15] provides an NH_2-terminal structural modulation model for these assays [28, 44]. We have demonstrated that Zn^{2+}-binding to the Tx site can alter the local conformation as reflected by decreases in the affinity for anti-Tx antibodies [28, 44]. ELISA epitope analyses further showed decreased binding affinity of specific anti-TnT antibodies against epitopes outside the Tx region, indicating metal-Tx binding-induced secondary conformational changes [44]. The secondary conformational changes induced by the binding of Zn^{2+} to the NH_2-terminal variable region of chicken breast muscle TnT are extensive as demon-

strated by the significantly decreased binding of a polyclonal antibody which recognizes multiple epitopes on the TnT molecule. The results suggest that a reconfiguration of the NH_2-terminal domain of TnT can modulate the overall conformation of the protein. This conformational modulation has also been observed by the binding of specific mAbs to the NH_2-terminus, indicating this conformational effect is not restricted to the metal-binding modulation [17, 44].

By fluorescence spectral analysis, we have further characterized the effect of the NH_2-terminal variable region on the global conformation of TnT. A Tx-positive chicken fast skeletal muscle TnT isoform TnT8e16 titrated with Cu(II) showed changes in fluorescence intensity and anisotropy of the COOH-domain Trp residues (W_{234}, W_{236} and W_{285}) which demonstrated considerable environmental sensitivity in TnT denaturation studies. Nonlinear Stern-Volmer plots of Trp quenching indicated that the binding of metal ion to the NH_2-terminal region induced a conformational change in the COOH-terminal domain of TnT. Fluorescence anisotropy changes upon metal ion binding to the NH_2-terminal region indicated a decrease in the mobility of the Trp residues and an increase in the flexibility of fluorescein labeled Cys_{263} in the COOH-domain. These data support the model that the alternatively spliced NH_2-terminal variable region of TnT modulates the protein conformation and flexibility [14].

To characterize the effect of NH_2-terminal structure-induced conformational changes on TnT's function in the thin filament regulation of muscle contraction, we further examined the TnT-Tm, TnT-TnI and TnT-TnC interactions. Purified chicken breast muscle Tx-positive TnT or cloned TnT3 and TnT2 (NH_2-terminal Tx negative controls) were analyzed for interaction with Tm, TnI or TnC by the ELISA-mediated protein-binding assay [44]. The results show that the binding of Zn^{2+} induced significant decreases in the affinity of chicken breast muscle TnT to Tm, TnI and TnC. In contrast, Zn^{2+} had no effect on the interaction of Tx-negative TnTs with Tm as compared with the metal free controls. The Zn^{2+}-induced decreases in binding affinity of the Tx-positive TnT to TnI, TnC and Tm demonstrate that binding of Zn^{2+} to the Tx segment in the NH_2-terminal region can modulate the conformation of other domains of TnT to produce functional changes [17, 28, 44].

Unique TnT isoform regulation in avian pectoral muscles

The developmental regulation of fast skeletal muscle TnT isoform expression in avian pectoral muscles is unique in contrast to avian leg muscles or mammalian skeletal muscles. Western blots with the RATnT polyclonal antibody showed two classes of TnT isoforms in chicken pectoralis (Figure 7). The lower molecular weight fast TnTs are similar to that commonly seen in adult vertebrate skeletal muscles and the high molecular

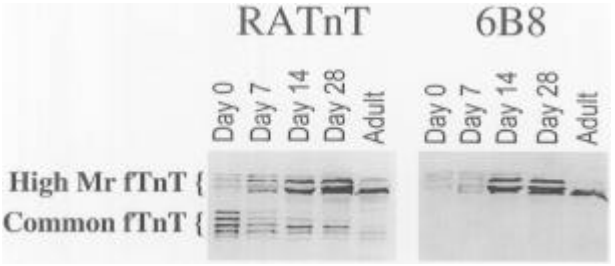

Figure 7. Two simultaneous transitions of the fast skeletal muscle TnT isoform switching in developing avian pectoral muscles. The expression of TnT isoforms in chicken pectoralis during post-hatch development was determined by Western blotting using anti-TnT polyclonal antibody RATnT (left panel). The results show two classes of TnT isoforms, the lower Mr fast TnTs that are normally seen in adult vertebrate muscles and the high Mr fast TnT specific to avian pectoral muscle. Both classes of the TnT isoforms undergo a high Mr to low Mr transition during post-hatch development. At mean time, the low Mr fast TnT isoforms cease expression after 28 days after hatch while the high Mr pectoral muscle-specific fast TnT isoforms increase expression to become the sole class of TnT in the adult muscle. The Western blots using the mAb 6B8 (right panel) show that the high Mr avian pectoral muscle TnT contains the newly evolved metal-binding segment in the NH_2-terminal variable region (see the text).

weight fast TnT is specific to the avian pectoral muscle. Similar to that observed during mammalian skeletal muscle and chicken leg muscle development, both classes of the TnT isoforms undergo a high molecular weight to low molecular weight transition during post-hatch development. The "normal" low molecular weight fast skeletal muscle TnT isoforms cease expression 28 days after hatching while the high molecular weight pectoral muscle-specific TnT increases expression to become the sole class of TnT in the adult muscle. The Western blots using mAb 6B8 (Fig. 7, right panel) show that the high molecular weight pectoral muscle TnT contains the unique metal-binding segment (Tx) in the NH_2-terminal variable region [30]. The higher to lower molecular weight transition of both classes of the avian fast TnT isoforms represent the developmental large to small, acidic to basic TnT isoform switch found in all muscles as indicated above. The up-regulation of the inclusion of the Tx segment in the NH_2-terminal variable region indicates an apparently independent process of the alternative RNA splicing regulation of TnT isoform expression. The two alternative splicing events both target the NH_2-terminal variable region although they may be under separate regulation. The composite result is that the adult avian pectoral muscles express more acidic TnTs in contrast to all other adult fast

skeletal muscles investigated. In other words, expression of the newly evolved Tx or Tx-like segment overrides the acidic to basic isoform switch to retain a more acidic NH_2-terminus in the adult avian pectoral muscle TnT. It is worth noting that together with the high molecular weight acidic fast TnT, the avian breast muscle, like all cardiac muscles, exclusively expresses α-Tm in contrast to the α/β-Tm in other adult muscles [30]. The more acidic NH_2-terminus of avian pectoral muscle fast TnT correlating to a higher sensitivity to the Ca^{2+} activation of contraction [30] may be an adaptation to the continuing muscle work during flight.

Evolution of the Tx and Tx-like element revealed two correlating components of the molecular evolution of avian pectoral muscle TnT: A larger, more acidic NH_2-terminal segment and a cluster of transition metal-binding sites; both may have functional significance for their observed selection value. The rapid evolution and expansion of the Tx segment in the NH_2-terminal variable region of avian fast TnT (J.-P. Jin et al., unpublished results) demonstrates a relationship between protein structural tolerance and molecular divergence, that may be essential to the diversity of TnT isoforms.

Physiological and pathological significance of TnT isoform regulation

The regulated TnT isoform switch during cardiac and skeletal muscle development suggests that the expression of TnT isoforms with structural differences in the alternatively spliced NH_2-terminal variable region may function in adjusting the contractility during adaptation to the cellular environment and functional state of the muscle. Re-expression of fetal cardiac TnT isoforms was observed in the hypertrophic hearts of aging spontaneous hypertensive rats [25], suggesting adaptive value in increasing Ca^{2+} sensitivity and/or tolerance to acidosis. Similarly, severely failing human hearts were found to switch the expression of TnT isoforms [2]. However, timing of the cardiac TnT isoform switching is synchronized in the perinatal developing cardiac and skeletal muscles in a species-determined manner. This intriguing finding indicates a determining role by genetic programming underlying the functional adaptation [12] and suggests that changes in TnT isoform expression are not simply an adaptive response to functional demands. In other words, when a hypertrophic response in the myocardium changes the gene expression profile, the TnT isoform switches may also contribute to pathogenesis.

Point mutations in the cardiac TnT gene are linked to human familial hypertrophic cardiomyopathies [45]. Scattered single amino acid substitutions or deletions (I79N, R92Q, A104Q, F110I, E160 deletion, E163K, E244D, and R278C) in cardiac TnT are found in chronic cardiomyopathy patients, indicating that various minor changes in TnT structure may cause a group of similar phenotypic disorders in myocardial function. It is worth noting that the I79N mutation locates in the NH_2-terminal "non-essential" region of cardiac TnT, since the "25K" TnT fragment with the corresponding region deleted showed no significant functional effect *in vitro* on the actomyosin activation [7]. However, this point mutation causes a severe cardiomyopathy phenotype [45] and has been demonstrated to result in a faster thin filament movement in *in vitro* motility assays [22]. This NH_2-terminal mutation of cardiac TnT provides evidence for the functional importance of the NH_2-terminal variable region. Nevertheless, the fact that scattered point mutations in various regions of the TnT polypeptide chain cause similar pathological phenotype in the cardiac muscle function suggests that it may be the protein conformational change rather than the mutant site *per se* that is important in the structure-function relationship of TnT mutants or isoforms. Contractility analysis of transgenic mouse cardiac muscle expressing a fast TnT isoform demonstrated that the structural and functional variation of TnT isoforms may be a factor determining the difference in the cooperativity between cardiac and skeletal muscles. The altered reactivity of the thin filament regulatory system by different TnT isoforms may cause a de-synchronization of the cardiac muscle, resulting in a cardiomyopathy phenotype [10].

Conclusions

Although studies using TnT fragments showed that the developmentally regulated NH_2-terminal variable region of TnT does not directly interact with other muscle contractile or regulatory proteins, functional differences are found between intact TnT isoforms differing in the NH_2-terminal region. The structural variations in the NH_2-terminal region of TnT can induce secondary conformational changes in other domains of TnT and modulate the interactions of TnT in the thin filament regulatory system. Structural evolution and functional characterization have led to a hypothesis that the alternatively spliced NH_2-

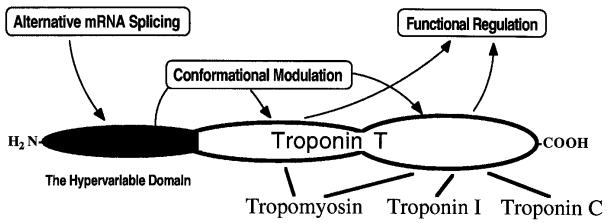

Figure 8. A hypothetical model for the functional role of the alternative splicing-regulated NH_2-terminal variable region of TnT. From functional interaction characterization of TnT fragments and intact TnT isoforms, a conformational modulation model is proposed for the functional significance of the NH_2-terminal hypervariable region of TnT. Although bearing no direct interaction with other thin filament regulatory proteins, structural configuration of the NH_2-terminal region plays an active role in tuning the molecular conformation and function of other domains of TnT.

terminal variable region of TnT plays a role by tuning the conformation and function of the thin filament regulatory system to contribute to the diversity, adaptation and pathogenesis of the muscle (Figure 8).

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