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Troponin in both Smooth and Striated Muscles of Ascidian *Ciona intestinalis* Functions as a Ca^{2+}-Dependent Accelerator of Actin−Myosin Interaction[†]

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ABSTRACT: Troponin, a Ca^{2+}-dependent regulator of muscle contraction, acts as an inhibitor of the actin−myosin interaction in the absence of Ca^{2+} during contraction in vertebrate striated muscle. However, variation has been observed in the mode of troponin-dependent regulation among the animals belonging to Protochordata, the taxon most closely related to Vertebrata. Although troponin in striated muscle of a cephalochordate amphioxus functions as an inhibitor in the absence of Ca^{2+} as in vertebrates [Dennisson, J. G., et al. (2010) *Zool. Sci. 27*, 461−469], troponin in the smooth muscle of a urochordate ascidian (*Halocynthia roretzi*) regulates actin−myosin interaction as an activator in the presence of Ca^{2+} and not an inhibitor in the absence of Ca^{2+} as in vertebrates [Endo, T., and Obinata, T. (1981) *J. Biochem. 89*, 1599−1608]. In this study, to further clarify the functional diversity of troponin, we examined the role of troponin in Ca^{2+}-dependent regulation of the actin−myosin interaction in striated and smooth muscles in another member of Ascidiacea (*Ciona inetestinalis*) using three recombinant troponin components, TnT, TnI, and TnC, produced using an *Escherichia coli* expression system. On the basis of actomyosin ATPase assays, we show here that troponins in both smooth and striated muscles of ascidian function as a Ca^{2+}-dependent activator of the actin−myosin interaction and TnT is the component responsible for this activation. These results indicate that troponin of ascidian has evolved in a manner different from that of amphioxus and vertebrates in terms of function.

Ca^{2+}-dependent regulation of striated muscle is mediated by troponin, in combination with tropomyosin (*1*). Troponin is a complex of three components: troponin T (TnT),[1] a tropomyosin-binding component; troponin I (TnI), an inhibitory component of the actin−myosin interaction; and troponin C (TnC), a Ca^{2+}-binding component (*1*). Troponin of vertebrate striated muscle is known to function as an inhibitor (or a brake) of the actin−myosin interaction (actomyosin Mg^{2+}-ATPase activity) in the absence of Ca^{2+}. Binding of Ca^{2+} to TnC induces a structural change in the troponin complex, specifically in troponin I within the complex. Subsequently, the inhibitory action of troponin is removed, enabling myosin to interact with actin (*2*). This troponin−tropomyosin regulatory system also exists in a variety of invertebrate striated and obliquely striated muscle (*3*). In most cases, troponin of invertebrate muscle also inhibits the actin−myosin interaction in the absence of Ca^{2+} in a manner similar to that of vertebrate striated muscle troponin.

Protochordata, comprising subphyla Cephalochordata and Urochordata, are the closest to Vertebrata. A recent study on chordate evolution has shown that Urochordata and Vertebrata diverged from an ancestor of subphylum Cephalochordata (*4*). Phylogenic analysis of troponin components based on their amino acid sequences across Chordata has demonstrated that chordate troponin can be classified into three different monophyletic groups, cephalochordate (amphioxus) troponin, urochordate (ascidian) troponin, and vertebrate troponin (*5*−*7*). Cephalochordate (amphioxus) troponin seems to have diverged first from a chordate troponin ancestor.

Functional analysis of troponin showed that troponin in amphioxus striated muscle inhibits the actin−myosin interaction in the absence of Ca^{2+} in a manner similar to that of vertebrate striated muscle troponin (*7*). Interestingly, however, troponin isolated from giant multinucleated smooth muscle that constitutes the body wall of a urochordate ascidian (*Halocynthia roretzi*) (*8*) scarcely inhibits the actin−myosin interaction in the absence of Ca^{2+} but enhances it remarkably in the presence of Ca^{2+} (*9*). Although vertebrate striated muscle troponin can enhance the actin−myosin interaction in the presence of Ca^{2+} (*10*, *11*), the level of enhancement is much higher in the case of ascidian smooth muscle troponin. This unique property of ascidian troponin has been attributed to TnI and TnT; that is, isolated ascidian TnT alone remarkably promotes actomyosin Mg^{2+}-ATPase activity, regardless of Ca^{2+} ion concentration, while isolated ascidian TnI alone scarcely inhibits this activity (*9*). On the basis of these functional characteristics, ascidian (*H. roretzi*) smooth muscle troponin has been regarded as "accelerator-type" troponin, a Ca^{2+}-dependent accelerator, compared with vertebrate striated muscle troponin that acts like a "brake-type" troponin, a Ca^{2+}-sensitive inhibitor (*7*, *12*). The ascidian smooth muscle troponin might have acquired the unique property of a Ca^{2+}-dependent accelerator during evolution, while amphioxus and vertebrate striated muscle troponin may have inherited the brake property from the ancestor troponin.

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[1]Abbreviations: TN, troponin complex with the three components; TnT, troponin T; TnI, troponin I; TnC, troponin C.

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The smooth muscle cells of *H. roretzi* are unique, as they are very long and multinucleated like vertebrate skeletal muscle cells but lack striated contractile apparatus like vertebrate smooth muscle cells. Myosin-linked regulation of the actin−myosin interaction as observed in vertebrate smooth muscle has not been detected in the *H. roretzi* smooth muscle (*13*). It is not known whether the property of the troponin in this unique smooth muscle is common to the smooth muscle of other protochordate animals.

Another ascidian species, *Ciona intestinalis*, belongs to Urochordata as *H. roretzi*, but they are phylogenetically categorized in different orders, Enterogona and Pleurogona, respectively. Between the two species, distinct differences are present in the number of genes encoding troponin components and the troponin isoforms generated. Namely, in the case of *H. roretzi*, two genes and two protein isoforms for TnT (*14*), four genes and five protein isoforms for TnI (*15*), and one gene and two protein isoforms for TnC (*16*) are present, while in the case of *C. intestinalis*, there are one gene and three protein isoforms for TnT (*17*), one gene and two protein isoforms for TnI (*5*, *18*), and three genes and four protein isoforms for TnC (*17*). Adult smooth muscle and larval striated muscles of *C. intestinalis* use the same TnI isoform but different TnT and TnC isoforms, while smooth and striated muscles of *H. roretzi* use different TnT, TnC, and TnI isoforms.

In this study, with such diversity in troponin components among ascidians in mind, we focused on troponin in the striated muscle and smooth muscle of the ascidian *C. intestinalis*. The major aim of this study was to determine (i) whether accelerator-type troponin is also present in striated muscle of ascidians and (ii) whether accelerator-type troponin is common to smooth muscle of ascidians. Troponin components were isolated using reverse transcription polymerase chain reaction (RT-PCR) and an *Escherichia coli* expression system to analyze Ca^{2+}-dependent regulation of actomyosin Mg^{2+}-ATPase activity. The results indicate that troponin in both smooth and striated muscles of ascidian functions as a Ca^{2+}-dependent accelerator of the actin−myosin interaction.

MATERIALS AND METHODS

Vector Construction of Ciona Troponin. *C. intestinalis* TnI cDNA (TnI, clone ID citb005n24 in Ghost Database) and TnC cDNA (TnC, clone ID ciad080j01) were provided by *C. intestinalis* genomic and cDNA resources (Kyoto University, Kyoto, Japan). The coding regions of TnI and TnC cDNA were obtained by PCR using the following primers: TnI, 5′-AAACTCGAGCATATGTCGGAAGAGAGCGGAGTTCGC-3′ and 5′-AAGAATTCTTGAGCTCCCTCAAAAACTGCCTTTTTTC-3′; TnC, 5′-AAACTCGAGCATATGGAGATCAACCTGTCAGATGAAC-3′ and 5′-AAAGATATCCTGGACAAACTTCATCATGTTGAGG-3′. The *Xho*I−*Eco*RI fragment of the TnI PCR product and the *Xho*I−*Eco*RV fragment of the TnC PCR product were inserted into pBluescript SK(−) (Stratagene, Cedar Creek, TX), containing synthesized nucleotides encoding eight histidine residues following a termination codon between the *Eco*RI and *Pst*I sites. The *Nde*I−*Bam*HI fragments of TnI and TnC carrying a C-terminal His tag coding sequence were cloned into expression vector pET3a (Novagen, Darmstadt, Germany).

On the basis of the nucleotide sequence of *C. intestinalis* TnT cDNA (TnT, cluster ID 03288), TnTb and TnTc, which are termed stTnT and smTnT, respectively, in this report, were cloned from *C. intestinalis* body-wall muscle RNA by RT-PCR using the following primers: 5′-CCGTTTGCTCAAAAATGTCGGACTCAG-3′ and 5′-GTGATCACTTGAAGTTAGATGCGGCTGC-3′. The coding regions of stTnT and smTnT were obtained by PCR using the following primers: 5′-AAAGAATTCATGTCGGACTCAGAAGAATACAGC-3′ and 5′-AAACTCGAGTCACTTGAAGTTAGATGCGGCTGC-3′. smTnT1−206, smTnT1−133, and smTnT54−176 encoding smTnT fragments were obtained by PCR using the following primers: smTnT1−206, 5′-AAAGAATTCATGTCGGACTCAGAAGAATACAGC-3′ and 5′-AACTCGAGTCAAATTCTTTGCTCGTGATCGTATTTCTC-3′; smTnT1−133, 5′-AAAGAATTCATGTCGGACTCAGAAGAATACAGC-3′ and 5′-AACTCGAGTCAGTTGGCGATCGCTGCCTTCTTTC-3′; smTnT54−176, 5′-AAGAATTCGAACTGCAGGGGTTGATCAACGC-3′ and 5′-AACTCGAGTCAAAGATGATCAATGTTGAGTGGCTTCC-3′. The *Eco*RI−*Xho*I fragments of the PCR products were then inserted into pBluescript KS(−), containing synthesized nucleotides encoding eight histidine residues followed by a *Nde*I site between the *Bam*HI and *Eco*RI sites. These *Nde*I−*Bam*HI cDNA fragments carrying an N-terminal His tag coding sequence were cloned into expression vector pET3a. All troponin expression vectors were transformed into *E. coli* strain BL21(DE3) pLysS (Novagen).

Purification of Recombinant TnI, TnC, and TnT of C. intestinalis. For the purification of recombinant *Ciona* TnI and TnC, the soluble fraction of bacterial lysates was applied to a HisTrap affinity column (GE Healthcare UK Ltd.) in His buffer [10 mM imidazole, 20 mM phosphate buffer, and 0.5 M NaCl (pH 7.4)]. Proteins were eluted using a gradient from 50 to 500 mM imidazole in 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl. TnC was further dialyzed against Q buffer [20 mM NaCl, 25 mM Tris-HCl, and 1 mM DTT (pH 8.0)] and then applied to an anion exchange Econo-Pac Q cartridge (Bio-Rad Laboratories). The purified TnC was obtained with a gradient from 40 to 500 mM NaCl.

For the purification of recombinant stTnT, smTnT, and the deletion fragments, the insoluble fraction of bacterial lysates was dissolved in His buffer containing 6 M urea and then dialyzed against the same buffer without urea. The solubilized proteins were subjected to HisTrap affinity chromatography in His buffer. Purified proteins were eluted using a gradient from 10 to 500 mM imidazole in 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl.

Analysis of Ciona TnT mRNA by RT-PCR. Total RNA was isolated from adult body-wall and cardiac muscles and larval whole tissues from *C. intestinalis* with the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was conducted with 50 ng of total RNA extract, using the Sensiscript RT Kit (Qiagen) with the oligo-dT primer. The following primers were used for PCR: TnT, 5′-CCGTTTGCTCAAAAATGTCGGACTCAG-3′ and 5′-CGCACTCTGAGCTCCTCAATCTCC-3′; β-actin (AJ297725), 5′-CAGTGGTGATGGTGTTTCCCACACC-3′ and 5′-CGGCGATTCCAGGGAACATAGTGG-3′.

Association of Troponin Components with Actin Filaments. Rabbit actin (7 μM), chicken tropomyosin (1 μM), and the *Ciona* troponin complex (1 μM stTnT, 1 μM TnI, and 1 μM TnC), or tropomyosin and the *Ciona* troponin complex were combined in a 20 mM Tris-HCl buffer solution (pH 7.5) containing 0.1 M KCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ for 1 h on ice. In some experiments, actin alone was incubated in the solution. Rabbit myosin (1.77 μM) was then combined with the solution and incubated for 20 min on ice. After incubation, the mixture

was centrifuged at 20630*g* for 30 min at 4 °C. Fractions were examined in the presence of 0.5 mM EGTA by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Cosedimentation Assay of TnT Fragments with Actin. Rabbit actin (7 μM) with the *Ciona* TnT fragment (3 μM) or TnT fragment alone was incubated in a 20 mM Tris-HCl buffer solution (pH 7.5) containing 0.1 M KCl, 1 mM $MgCl_2$, 0.2 mM ATP, and 1 mM 2-mercaptoethanol for 1 h on ice. In some experiments, chicken tropomyosin (1 μM) was included in the mixture. The mixtures were then centrifuged at 386000*g* for 20 min.

Pull-Down Assay of TnT Fragments with Tropomyosin. A mixture of chicken tropomyosin (1.23 μM) and the *Ciona* TnT fragment (smTnT1−206 or smTnT54−176, 1.23 μM) or either tropomyosin or the *Ciona* TnT fragment alone was incubated in binding buffer [0.1 M KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM ATP, and 20 mM imidazole-HCl (pH 7.5)] for 1 h on ice. Profinity IMAC Ni-Charged Resins (Bio-Rad Laboratories) were then added to the solution, and the mixture was incubated for 1 h at 4 °C, followed by centrifugation at 830*g* for 15 s.

Preparation of Other Proteins. All other proteins were prepared as previously reported: myosin from rabbit back muscle (*19*), actin from acetone-dried powder of rabbit back muscle (*20*), and tropomyosin from chick skeletal muscle (*21*).

Protein Concentration. Protein concentrations were determined photometrically at 310 nm using a microbiuret reaction (*22*) or BCA protein assay kit (Pierce, Rockford, IL).

Gel Electrophoresis. SDS−PAGE was conducted using a 13.5% polyacrylamide gel and a discontinuous Tris-glycine buffer system (*23*). The Ca^{2+}-dependent mobility shift of TnC on SDS−PAGE was examined in the presence of 2 mM EGTA or 2 mM $CaCl_2$ as described previously (*24*). For analysis of the Ca^{2+}-dependent interaction of the troponin components, alkaline urea−PAGE was conducted on 8% polyacrylamide gels with 8 M urea in the presence of either 10 mM EGTA or 2 mM $CaCl_2$ using a Tris-glycine buffer (pH 8.3) system (*25*).

Actomyosin ATPase Measurements. ATPase assays were conducted according to our previously reported method (*26*). Breifly, myosin (0.4 μM), actin (1.6 μM), tropomyosin (0.4 μM), and the troponin components (0.4 μM TnT, 0.4 μM TnI, and 0.4 μM TnC) were preincubated in binding buffer [50 mM KCl, 1 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5) with 0.1 mM $CaCl_2$ or 1 mM EGTA] for 5 min at 25 °C, and then ATP (final concentration of 1 mM) was added to the mixture, before incubation for 10 min at 25 °C. The actin:tropomyosin:troponin molar ratio in the mixtures was adjusted to 4:1:1. In some experiments, myosin (0.4 μM), actin (1.6 μM), tropomyosin (0.23 μM), and the troponin components (0.23 μM) were preincubated in binding buffer, in specific combinations as shown in Figures 6 and 8. Consequently, the actin:tropomyosin:troponin molar ratio in the mixtures was adjusted to 7:1:1. Alternatively, the amount of TnT was doubled; in this case, the ratio was 4:1:1 [stT(4) and smT(4), Figure 6A]. The concentration of liberated P_i was measured according to the method of Taussky and Shorr (*27*). The data obtained from at least three independent experiments were analyzed by one-way analysis of variance (ANOVA) followed by the multiple-comparison analysis with the test of Ryan (*28*, *29*).

Phylogenetic Analysis. TnT amino acid sequences were aligned using Clustal X version 2.0 (*30*). A phylogenetic tree was constructed with the aligned sequences by the neighbor-joining method (*31*), and the degree of support for internal branches was assessed in 1000 bootstrap replicates (*32*).

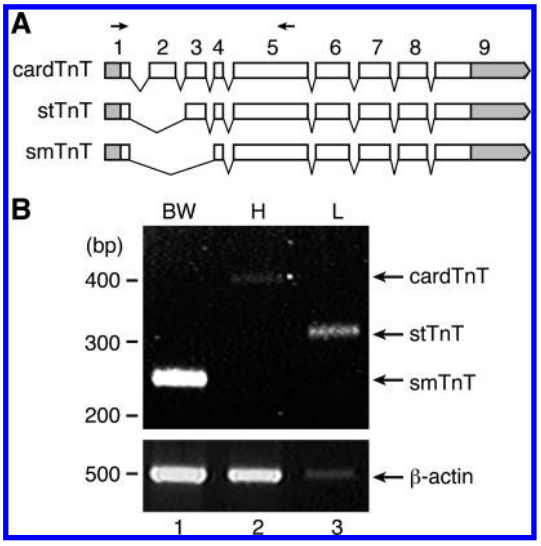

FIGURE 1: TnT isoforms in ascidian *C. intestinalis* and their expression. (A) Scheme of the structures of three TnT isoforms, cardTnT (TnTa, KH.C4.57.V1 in Ghost Database), stTnT (TnTb, KH.C4.57.V3), and smTnT (TnTc, KH.C4.57.V4), according to http://hoya.zool.kyoto-u.ac.jp/SearchGenomekh.html#CDNA. The TnT gene is constituted of nine exons as shown by boxes. White and gray boxes denote coding and noncoding regions, respectively. cardTnT, stTnT, and smTnT isoforms are mainly generated in cardiac muscle, larval striated muscle, and adult body-wall smooth muscle, respectively, by alternative RNA splicing. The three isoforms differ with or without the sequences encoded by exons 2 and 3. Arrows indicate the location of primers for RT-PCR. (B) RT-PCR analysis of TnT mRNA transcribed in ascidian (*C. intestinalis*) tissues. RNA isolated from body-wall (lane 1, BW), heart (lane 2, H), and larval whole tissues (lane 3, L) was analyzed by RT-PCR using the sequences in exon 1 and the sequence in exon 5 as forward and reverse primers, respectively (panel A), to detect the messages encoding three TnT isoforms and to detect the β-actin message as an internal standard. The PCR products of ~250, ~400, and ~300 bp corresponding to smTnT, cardTnT, and stTnT, respectively, were generated with body-wall RNA, heart RNA, and larval RNA, respectively.

RESULTS

Differential Expression of TnT Isoforms in Ascidian C. intestinalis Tissues. A previous report demonstrated that three TnT isoforms, TnTa, TnTb, and TnTc, are present in *C. intestinalis* (*17*). They are generated from a single gene by alternative RNA splicing. We cloned the cDNAs encoding these isoforms and confirmed by sequencing that they differ only in the sequence of the N-terminal side as shown schematically in Figure 1A. Considering their tissue-specific expression as shown below, TnTa, TnTb, and TnTc are termed cardTnT, stTnT, and smTnT, respectively, implying cardiac-type TnT, striated muscle-type TnT, and smooth muscle-type TnT, respectively.

The expression of the three isoforms was examined by RT-PCR. As shown in Figure 1B, smTnT is the isoform that is expressed mainly in adult body-wall smooth muscle (lane 1), whereas stTnT is the isoform that characterizes larval striated muscle (lane 3). cardTnT seems to be characteristic of heart, although the detection level of this isoform is much lower than that of the others (lane 2). The pattern of the tissue-specific transcription of the three isoforms is consistent with the previous description based on EST counts (*17*). In comparison to the amount of actin expression as the internal control, the level of stTnT expression in larval whole tissue is considerably high. As judged by the expression characteristics, we designated smTnT as an adult smooth muscle-type isoform and stTnT as a larval striated muscle-type isoform.

Generation and Purification of Recombinant Troponin Components. To examine the functional properties of ascidian troponin, troponin components of *C. intestinalis*, stTnT, smTnT, TnI, and TnC, were generated in an *E. coli* expression system and purified as described in Materials and Methods. SDS−PAGE patterns of the purified proteins are shown in Figure 2. The purity of the preparations was satisfactory for the biochemical functional assay.

Ca^{2+} Responsiveness of TnC and Ca^{2+}-Dependent Interaction of TnC and TnI. To clarify whether recombinant troponin components function in a Ca^{2+}-dependent manner, an essential property of troponin, the Ca^{2+} responsiveness of the ascidian TnC and Ca^{2+}-dependent ascidian TnI−TnC interaction was examined by electrophoresis. The Ca^{2+}-dependent mobility shift of TnC on SDS gels was analyzed as previously reported (*24*). Analysis of mixtures of either EGTA (2 mM) or $CaCl_2$ (2 mM) with TnC revealed a single band by SDS−PAGE (Figure 3A); however, TnC in the presence of Ca^{2+} migrated faster on the gel (corresponding to a protein size of ~19 kDa) than in the absence of Ca^{2+} (~22 kDa). This phenomenon has been observed for TnC in other animals (*7*, *24*) and confirms that the purified ascidian TnC has Ca^{2+} binding ability even in the presence of SDS. This Ca^{2+} binding mobility shift can be attributed to the conformational change of ascidian TnC following the binding of Ca^{2+}, as demonstrated in vertebrate skeletal muscle TnC.

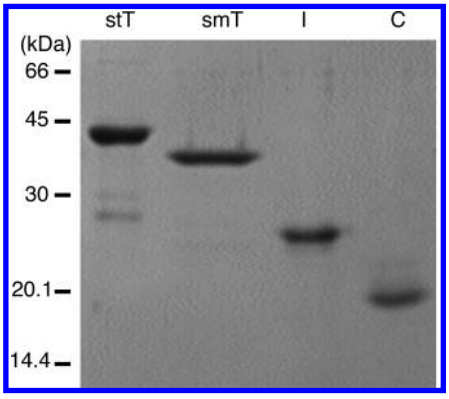

FIGURE 2: SDS−PAGE patterns of purified recombinant ascidin troponin components, stTnT (stT), smTnT (smT), TnI (I), and TnC (C). The molecular mass markers are shown at the left.

To analyze the Ca^{2+}-dependent interaction of TnI and TnC, we conducted alkaline urea−PAGE in the presence or absence of Ca^{2+} (Figure 3B). As seen in Figure 3B-c, in the presence of Ca^{2+} a slower migrating band was observed (left lane, with Ca^{2+}) and in the absence of Ca^{2+} a faster migrating band was observed (right lane, without Ca^{2+}). The slower migrating band corresponds to the recombinant TnI−TnC complex that was formed in a Ca^{2+}-dependent manner, and the faster migrating band is recombinant TnC alone (Figure 3B-a). Under these electrophoresis conditions, TnI alone was not resolved in a band in the presence or absence of Ca^{2+} (Figure 3B-b). The behavior of TnC and TnI was very similar to that of TnC and TnI in vertebrate and amphioxus as previously described (*7*, *25*). Therefore, we concluded that purified ascidian TnI and TnC can bind to each other in the characteristic manner of chordate troponin components.

Association of Ascidian Troponin Components with Actin and Tropomyosin. To confirm whether the three ascidian troponin components make a complex with actin, myosin, and tropomyosin from vertebrate striated muscle under a physiological condition, stTnT, TnI, and TnC were incubated with purified actin, myosin, and tropomyosin in a solution containing 0.1 M KCl at neutral pH for 1 h. Actomyosin, an actin−myosin complex, was then spun down by centrifugation at 20630*g* for 30 min. The proteins in the precipitate were examined by SDS−PAGE (Figure 4). Under this condition, troponin components and tropomyosin alone were scarcely precipitated (Figure 4, middle panel), but in the presence of actomyosin, they were precipitated together with actin and myosin (Figure 4, right panel), suggesting that the troponin components can associate with actin filaments together with tropomyosin. A combination of smTnT, TnI, and TnC was similarly coprecipitated with tropomyosin, actin, and myosin (data not shown).

Effects of the Ascidian Troponin Complex and Troponin Components on Actomyosin ATPase. Purified actin, tropomyosin, and the troponin complex (TnT, TnI, and TnC) were mixed to measure the effects of troponin on actomyosin Mg^{2+}-ATPase activity. After all the thin filament components had been combined, myosin was added in a 3:1 weight ratio to actin and allowed to incubate in a solution with or without Ca^{2+}. In the control (a mixture of myosin, actin, and tropomyosin without

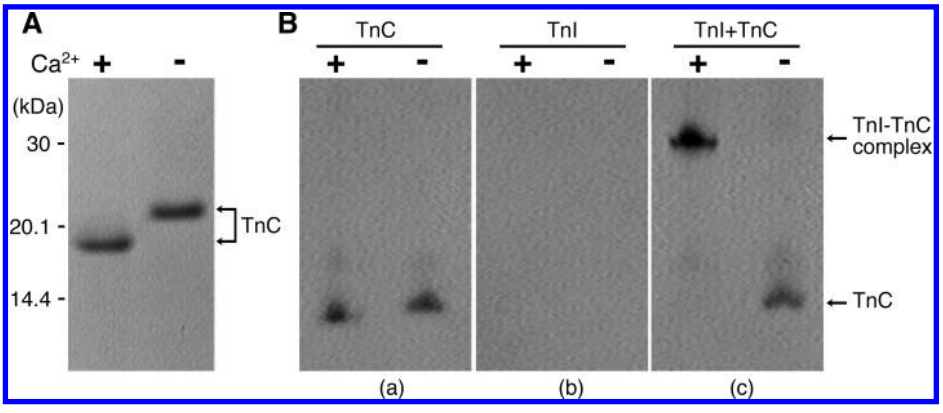

FIGURE 3: Ca^{2+} responsiveness of TnC and Ca^{2+}-dependent interaction of TnC and TnI. (A) Ca^{2+}-dependent mobility shift of TnC on SDS−PAGE. The difference in mobility of TnC was observed between the presence and absence of Ca^{2+} via SDS−PAGE. Purified TnC in SDS buffer solution with 2 mM $CaCl_2$ (left lane) showed a single band with a mobility faster than that of the same purified TnC in SDS buffer solution with 2 mM EGTA (right lane). The molecular mass markers are shown at the left. (B) Ca^{2+}-dependent interaction of TnI and TnC in alkaline urea−PAGE analysis. A binding assay with ascidian TnI (TnI) and ascidian TnC (TnC) was conducted in the presence of Ca^{2+} (2 mM $CaCl_2$, +) or in the absence of Ca^{2+} (10 mM EGTA, −): (a) TnC alone, (b) TnI alone, and (c) a mixture of TnI and TnC. The upper band corresponds to the TnI−TnC complex in the presence of Ca^{2+} and the lower band to TnC.

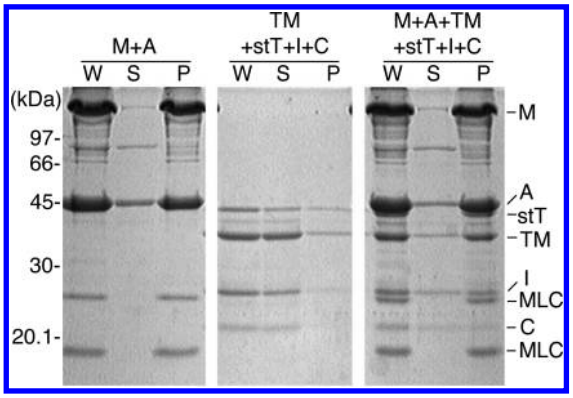

FIGURE 4: Association of ascidian troponin components with actin filaments. The mixtures of myosin with actin (left), tropomyosin with TN (complex of stTnT, TnI, and TnC) (middle), and myosin with actin, tropomyosin, and TN (the complex of stTnT, TnI, and TnC) (right) were subjected to centrifugation at 20630*g* for 30 min. The resultant supernatants (S), precipitates (P), and mixtures before centrifugation (W) were examined by SDS−PAGE: M, myosin heavy chain; A, actin; stT, stTnT; TM, tropomyosin; I, TnI; C, TnC; MLC, myosin light chain. The molecular mass markers are shown at the left.

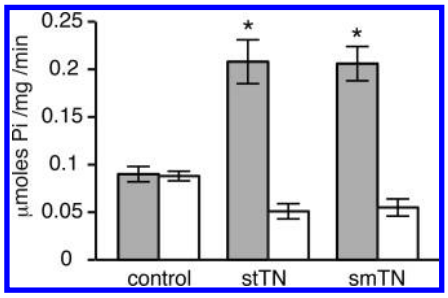

FIGURE 5: Effects of the ascidian troponin complex on the Mg^{2+}-ATPase activity of actomyosin. The effects of the ascidian troponin complex, combinations of striated muscle-type (stTN: stTnT, TnI, and TnC) and smooth muscle-type (smTN: smTnT, TnI, and TnC) forms, on actomyosin ATPase were examined. The actin, tropomyosin, TnT (stTnT or smTnT), TnI, and TnC were incubated in a molar ratio of 4:1:1:1:1 and then mixed with myosin. In the control mixture (control), actin, myosin, and tropomyosin were combined without any troponin components. The gray or white bar represents the reaction mixture with 0.1 mM $CaCl_2$ or 1 mM EGTA, respectively. The ordinate shows the values of ATPase activity. The standard error is indicated as an error bar ($n = 3$). $^*P < 0.01$ vs control with Ca^{2+}.

troponin), ATPase activity showed little difference between the condition with or without Ca^{2+}.

The effects of adding ascidian troponin, a combination of stTnT, TnI, and TnC (striated muscle-type troponin complex), or a combination of smTnT, TnI, and TnC (smooth muscle-type troponin complex) to the myosin−actin−tropomyosin complex (control) are summarized in Figure 5. With the addition of either combination (striated muscle-type or smooth muscle-type troponin), a significant increase in activity was observed in the presence of Ca^{2+} compared with the activity of the control without troponin; that is, both complexes showed a >2-fold activating effect in the presence of Ca^{2+}. In the absence of Ca^{2+}, ATPase activity was slightly decreased upon addition of troponin compared to the control without troponin, indicating that TnI in the troponin complex exerts a weak inhibitory effect in the absence of Ca^{2+}, although the inhibition is much weaker than that by vertebrate troponin.

These findings indicate that both smooth muscle and striated muscle troponins act as activators of actomyosin ATPase in the presence of Ca^{2+} and share the same functional accelerator-type

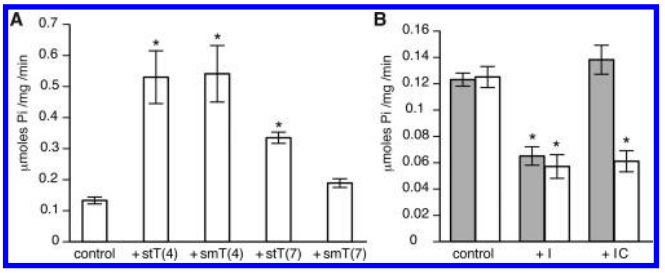

FIGURE 6: Effects of TnT (A) and TnI and TnC (B) on the ATPase activity of actomyosin. In panel A, actin, tropomyosin, and stTnT or smTnT were incubated in a molar ratio of 4:1:1 in stT(4) or smT(4), respectively, or alternatively 7:1:1 in stT(7) and smT(7), respectively, and then mixed with myosin. In the control mixture (control), myosin, actin, and tropomyosin were combined without TnT. The reaction mixture contained 1 mM EGTA. The standard error is indicated as an error bar ($n = 3$). $^*P < 0.01$ vs control. In panel B, actin, tropomyosin, and TnI and/or TnC were incubated at a molar ratio of 7:1:1 and then mixed with myosin. In the control mixture (control), myosin, actin, and tropomyosin were combined without any troponin components. Troponin components were added as indicated under each bar in the figure. The gray or white bar represents the reaction mixture with 0.1 mM $CaCl_2$ or 1 mM EGTA, respectively. The standard error is indicated as an error bar ($n = 3$). $^*P < 0.01$ vs control with Ca^{2+}. The ordinate shows the values of ATPase activity. stT, smT, I, and C indicate striated muscle stTnT, smooth muscle smTnT, TnI, and TnC, respectively.

property. Thus, ascidian troponin is functionally quite different from vertebrate (reviewed in ref *1*) and amphioxus (*7*) striated muscle troponin, known to have the brake-type property.

Because TnT and TnI in ascidian (*H. roretzi*) smooth muscle affect actomyosin ATPase in a manner distinct from that of the counterparts of vertebrate striated muscle (*9*), the effects of TnT and TnI of ascidian (*C. intestinalis*) striated and smooth muscles on actomyosin ATPase activity were further examined. As shown in Figure 6A, either stTnT or smTnT remarkably enhanced actomyosin ATPase activity. The enhancement was particularly marked when the amount of TnT was increased up to the actin:tropomyosin:TnT molar ratio of 4:1:1. The effects of TnT on actomyosin ATPase were Ca^{2+}-independent (data not shown). We stress that stTnT in striated muscle and smTnT in smooth muscle similarly enhanced actomyosin ATPase activity.

As shown in Figure 6B, the level of ATPase activity in the presence of *Ciona* TnI was roughly 50% of that in the absence of TnI (control). Thus, *Ciona* TnI is functionally similar to TnI in other animals rather than ascidian *H. roretzi* TnI, but with a much weaker inhibitory effect. When combined with TnC, *Ciona* TnI lost its inhibitory effect on actomyosin ATPase in the presence of Ca^{2+}, implying that TnC neutralizes the inhibitory action of TnI. These results are consistent with those reported for the vertebrate TnI−TnC complex (*1*).

Analysis of the Domain of the Ascidian TnT Molecule That Is Responsible for the Activation of Actomyosin ATPase Activity. In chicken TnT, a functional domain that is involved in the activation of actomyosin ATPase has been specified (*33*). In *Ciona* TnT, we detected a similar sequence of the functional domain of chicken TnT (Figure 7A). The amino acid sequences of the actin-binding region and tropomyosin-binding region in chicken TnT were 60 and 50% homologous with those of *Ciona* TnT, respectively. To clarify whether these regions of the *Ciona* TnT molecule are responsible for the activation of actomyosin ATPase, three deletion mutant fragments of smooth muscle TnT (smTnT), smTnT1−206, smTnT1−133, and smTnT54−176, were generated in an *E. coli* expression system,

as schematically shown in Figure 7A. His-tagged mutant and wild-type molecules were successfully purified with a HisTrap affinity column (Figure 7B).

The effects of the smTnT deletion mutants on actomyosin ATPase activity were examined. As shown in Figure 8A, two deletion mutants (smTnT fragments 1−206 and 54−176) significantly increased actomyosin ATPase activity in the presence of tropomyosin in a Ca^{2+}-independent manner as wild-type stTnT and smTnT, while fragment 1−133 failed to activate the ATPase activity. These results indicate that smTnT fragments including residues 54−176 are sufficient for the activation of actomyosin ATPase. This sequence comprises both the putative tropomyosin-binding and putative actin-binding regions, implying that binding of TnT to both tropomyosin and actin is required for the activation of the actin−myosin interaction (actomyosin ATPase). In fact, in the absence of tropomyosin, no activation of actomyosin ATPase by TnT or its deletion mutant was observed (Figure 8B).

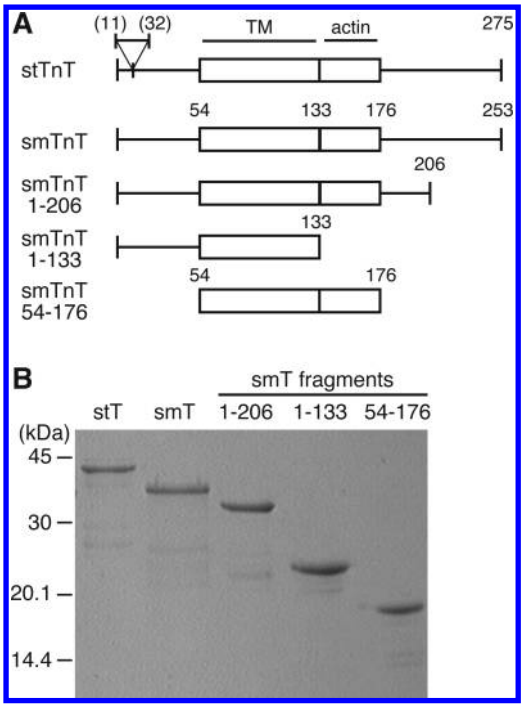

FIGURE 7: Generation and purification of deletion mutants of ascidian TnT. (A) Schematic representation of the wild-type molecules (stTnT and smTnT) and the mutated smTnT molecules generated. Each molecule has a His tag at the N-terminal end. On the basis of the homology of the sequence of ascidian TnT with that of vertebrate TnT, putative regions that may have the ability to bind to tropomyosin (TM) and actin are marked on the top of the scheme of stTnT. Amino acid numbers are also represented. (B) SDS−PAGE patterns of purified deletion mutant fragments and wild-type TnT: stT, stTnT; smT, smTnT; 1−206, smTnT1−206; 1−133, smTnT1−133; 54−176, smTnT54−176. The molecular mass markers are shown at the left.

Interaction of TnT Fragments with Actin and Tropomyosin. Binding of TnT fragments to F-actin was examined by a cosedimentation assay. Under the experimental conditions, the three TnT fragments were not precipitated in the absence of actin (Figure 9A−C), while actin without the TnT fragments was mostly precipitated (data not shown). When the TnT fragments were mixed with F-actin, both smTnT1−206 and smTnT54−176 were precipitated together with actin (Figure 9A,C, right lane), but smTnT1−133 was not precipitated at all (Figure 9B, middle lanes). However, smTnT1−133 was coprecipitated together with actin and tropomyosin when it was preincubated with actin and tropomyosin (Figure 9B, right lane). Interaction of smTnT1−206 and smTnT54−176 with F-actin was scarcely affected by the presence of tropomyosin (data not shown). Thus, smTnT1−206 and smTnT54−176 can bind to F-actin directly at the actin-binding region that is present in the sequence of residues 134−176. In contrast, smTnT1−133 is able to interact with tropomyosin that is associated with F-actin, so that actin, tropomyosin, and smTnT1−133 were coprecipitated.

Binding of the TnT fragments (smT54−176 and smT1−206) to tropomyosin was further examined by a pull-down assay using Ni-charged resins and a mixture of His-tagged TnT fragments and tropomyosin. Under these conditions, His-tagged smTnT54−176 and smTnT1−206 were completely precipitated by the resins (Figure 10). Tropomyosin alone was not precipitated by the resins, but when mixed with His-tagged smTnT54−176 or smTnT1−206, tropomyosin was pulled down by His-tagged TnT fragments.

The functional properties of smTnT and its fragments are summarized in Table 1. The results indicate that the sequence of residues 54−133 is responsible for tropomyosin binding of smTnT1−206, smTnT1−133, and smTnT54−176, while the sequence of residues 134−176 is responsible for actin binding.

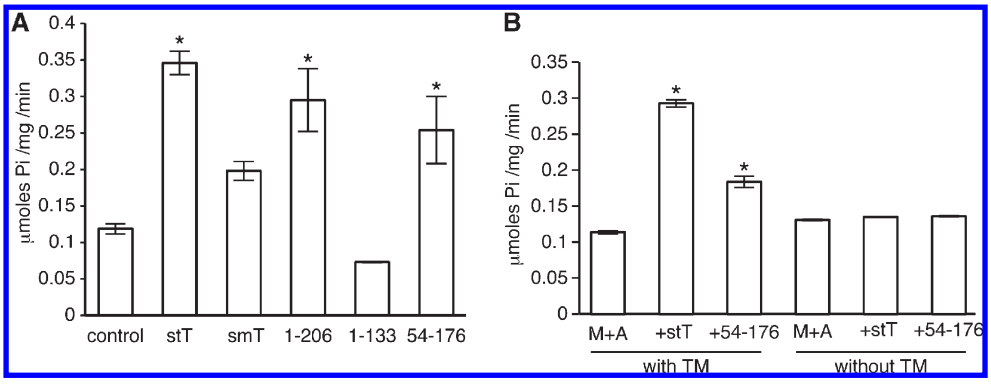

FIGURE 8: Effects of fragments of ascidian smTnT on the Mg^{2+}-ATPase activity of actomyosin. The effects of the fragments of ascidian smTnT, various deletion mutants as shown in Figure 7, on actomyosin ATPase were examined. (A) Actin, tropomyosin, whole molecules of TnT (stTnT or smTnT), or the deletion mutants (smT1−206, smT1−133, or smT54−176) were incubated in a molar ratio of 7:1:1 and then mixed with myosin. In the control mixture (control), myosin, actin, and tropomyosin were combined without any TnT molecules. The reaction mixture contained 1 mM EGTA. The ordinate shows the values of ATPase activity. stT, smT, 1−206, 1−133, and 54−176 denote stTnT, smTnT, smT1−206, smT1−133, and smT54−176, respectively. The standard error is indicated as an error bar ($n = 6$). *$P < 0.01$ vs control. (B) The effects of stTnT and the smTnT fragment (smT54−176) on actomyosin ATPase were examined with or without tropomyosin. The assay conditions are the same as in panel A. The standard error is indicated as an error bar ($n = 3$). *$P < 0.01$ vs mixture of myosin, actin, and tropomyosin (M+A with TM).

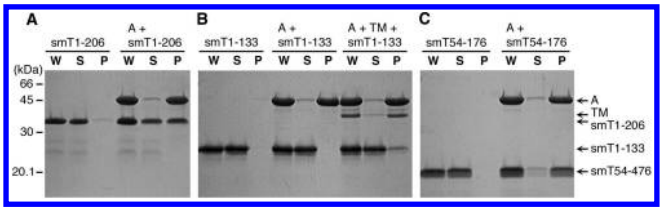

FIGURE 9: Binding of TnT fragments to actin. Actin and TnT fragments, smTnT1−206 (A), smTnT1−133 (B), or smTnT54−176 (C), were incubated in a solution containing 0.1 M KCl for 1 h. In some experiments in panel B, tropomyosin (TM) was included in the mixture. The mixtures were then centrifuged at 386000*g* for 20 min. The resultant supernatant (S), precipitates (P), and the mixtures before centrifugation (W) were subjected to SDS−PAGE: A, actin; TM, tropomyosin; smT1−206, smTnT1−206; smT1−133, smTnT1−133; smT54−176, smTnT54−176. The molecular mass markers are shown at the left.

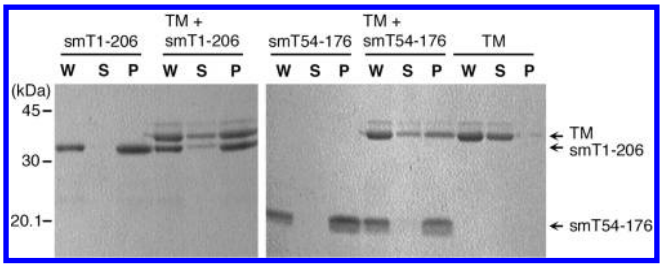

FIGURE 10: Binding of TnT fragments to tropomyosin. The mixture of the smTnT1−206 fragment and tropomyosin, the mixture of the smTnT54−176 fragment and tropomyosin, the smTnT1−206 fragment alone, the smTnT54−176 fragment alone, or tropomyosin alone was incubated for 1 h. Ni-charged resins were then added to the solution, followed by centrifugation. The resultant supernatant (S), precipitates (P), and the mixtures before centrifugation (W) were subjected to SDS−PAGE: TM, tropomyosin; smT1−206, smTnT1−206; smT54−176, smTnT54−176. The molecular mass markers are shown at the left.

For activation of actomyosin-ATPase, the actin-binding and tropomyosin-binding sequence (residues 54−176) is sufficient and the actin-binding sequence (residues 134−176) is necessary.

Evolutionary Relationship of TnT Structures among Chordates. Because functional variation in troponin, especially in TnT, was observed between ascidians and other chordates, we further examined the structural relevance of TnT among chordate species by phylogenetic analysis based on their amino acid sequences across Chordata (Figure 11). Consistent with the previously reported phylogenic relationship of TnI and TnC sequences among chordate species (*5*−*7*), our results indicate that cephalochordate (amphioxus, *Branchiostoma floridae* and *Branchiostoma belcheri*) TnT diverged from a chordate troponin ancestor, and vertebrate and ascidian (*H. roretzi* and *C. intestinalis*) TnTs constitute a monophyletic group with a common ancestor, even though they are functionally distinct from each other. The TnT of ascidian (*C. intestinalis* and *H. roretzi*) seems to have evolved to provide the troponin complex with the unique accelerator-type function.

DISCUSSION

Previous studies of troponin in protochordates have shown that ascidian (*H. roretzi*) smooth muscle troponin acts as a Ca^{2+}-dependent accelerator of actin−myosin interaction (*9*), whereas amphioxus striated muscle troponin functions as an inhibitor in the absence of Ca^{2+} just as vertebrate striated muscle troponin (*7*). On the other hand, evolutionary comparisons of the

Table 1: Functional Properties[a] of smTnT and Its Fragments

	tropomyosin binding	actin binding	activation of actomyosin ATPase
smTnT	+	+	+
smTnT1−206	+	+	+
smTnT1−133	+	−	−
smTnT54−176	+	+	+

[a]Positive activity (+) or negative activity (−).

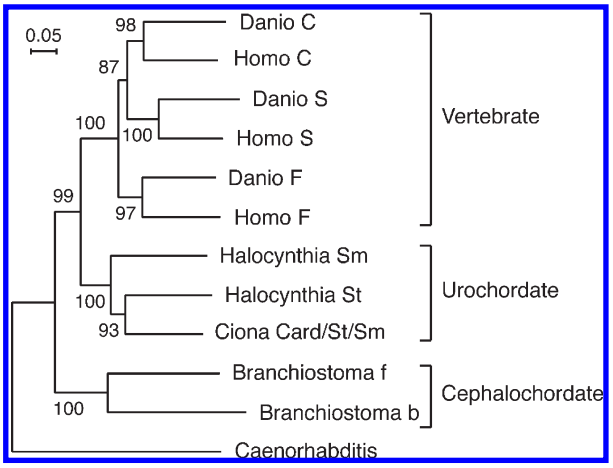

FIGURE 11: Phylogenetic relationships among cephalochordate (amphioxus), urochordate (ascidian), and vertebrate TnT amino acid sequences. Numbers at the forks indicate the percentage of 1000 bootstrap resamplings that support these topological elements. The scale bar indicates an evolutionary distance of 0.05 amino acid substitution per position in the sequence. The GenBank accession numbers for TnT of *Danio rerio* fast (Danio F), slow (Danio S), and cardiac (Danio C) muscle and TnT of *Homo sapiens* fast (Homo F), slow (Homo S), and cardiac (Homo C) muscle are NP_857636, AA162595, NP_690853, P45378, P13805, and P45379, respectively. The GenBank accession numbers for TnT of *H. roretzi* smooth muscle (Halo Sm), larval striated muscle (Halo St), *B. floridae* muscle (Branchiostoma f), and *B. belcheri* muscle (Branchiostoma b) are BAA09463, BAA12720, EEA60346, and BAI67730, respectively. The accession numbers for TnT of *C. intestinalis* cardiac and smooth and larval striated muscle (Ciona Card/St/Sm) is KH.C4.57.v1.A.SL1-1 in the Ghost database. The *Caenorhabditis elegans* (Caenorhabditis) sequence, used as an outgroup, is from GenBank entry Q27371.

troponin molecular structure have shown that ascidian (*H. roretzi*) smooth muscle troponin and vertebrate striated muscle troponin diverged from a common ancestral troponin after the divergence of amphioxus striated muscle troponin from a chordate ancestral protein (*6*, *7*). On the basis of these functional and structural data, it was suggested that vertebrate striated muscle troponin inherited the properties of the protein ancestor of amphioxus troponin, while ascidian troponin evolved differently to acquire its unique functional characteristics (*7*). It was, thus, a matter of great interest whether troponin in ascidian striated muscle is the accelerator-type troponin as in ascidian smooth muscle or the brake-type troponin as in amphioxus striated muscle. The results of this study show definitively that troponin in both striated and smooth muscles of ascidian (*C. intestinalis*) functions similarly as a Ca^{2+}-dependent activator of the actin−myosin interaction, a characteristic caused by the properties of TnT and TnI. Moreover, the troponin genes that encode striated and smooth muscle troponin components, especially TnT (this study) and TnI (*7*), appear to have evolved differently between cephalochordates (amphioxus) and urochordates (ascidian). The evolution of

TnT in ascidian (*C. intestinalis* and *H. roretzi*) seems to be particularly important for providing the troponin complex with the unique accelerator-type function.

In this study, we examined the functional characteristics of troponin of *C. intestinalis* with recombinant proteins. TnC and TnI generated in an *E. coli* expression system were soluble in a physiological salt solution, but recombinant TnT was dissolved in a solution containing 6 M urea. Urea at this concentration (6 M) has been used to separate each component of troponin without losing the intrinsic nature and their activity (*34*). In our previous investigation, ascidian (*H. roretzi*) TnT purified from body-wall muscles using 6 M urea exhibited an accelerating effect on actin−myosin interaction (*9*), while amphioxus TnT purified in the presence of urea did not exhibit any accelerating effect on the interaction (*7*). Therefore, it is not likely that the functional property of the urea-treated recombinant TnT of *C. intestinalis* represents artifacts caused by misfolding of the protein molecules.

We previously demonstrated that regulatory proteins of ascidian (*H. roretzi*) smooth muscle function with not only the ascidian actomyosin system (*13*) but also the vertebrate striated muscle actomyosin system (*9*) as a Ca^{2+}-dependent accelerator of actin−myosin interaction. In this study, we used only vertebrate skeletal muscle myosin and actin for testing functional properties of troponin and its components of ascidian (*C. intestinalis*), because purification of myosin from muscle tissue of *C. intestinalis* was quite difficult. We assume that troponin of *C. intestinalis* should also function with the ascidian actomyosin system in a manner similar to that of the vertebrate actomyosin system as in the case of *H. roretzi*.

Although two ascidian species, *C. intestinalis* and *H. roretzi*, belong to Urochordata and show a similar body organization, they are phylogenetically categorized in different orders. In addition, between the two species, distinct differences are present in the number of genes encoding troponin components, the troponin isoforms generated and the isoforms used in adult smooth muscle and larval striated muscle (*5*, *14*−*18*). Regardless of such differences in genes and protein isoforms, the results of this study show that the troponin complex and its components, TnT and TnI, of both species showed very similar functional properties. Therefore, it is very likely that ascidian troponin achieved a unique evolution to acquire the unique property of accelerator-type troponin. The activating effect of ascidian troponin on the actomyosin system in the presence of Ca^{2+} is suitable for ascidian muscle, because the ATPase activity of ascidian actomyosin is much lower compared to that of vertebrate skeletal muscle actomyosin, roughly one-third to one-fifth of that of vertebrates (*13*).

TnT of vertebrate striated muscle can enhance actomyosin ATPase activity by 30−40%, although the activation level is much lower than that of ascidian TnT (*11*). The domain of TnT responsible for the activation of actomyosin ATPase has been mapped (*33*): the activation is mediated by interaction of TnT with actin and tropomyosin, and the regions responsible for actin binding and tropomyosin binding are located in the central region of the TnT sequence. We have specified the sequences that are homologous with actin-binding and tropomyosin-binding domains of vertebrate striated muscle TnT in the ascidian TnT sequence. Functional analysis of the ascidian TnT fragments showed that actin-binding and tropomyosin-binding domains are located in the central regions of the primary sequence and both actin-binding and tropomyosin-binding domains are required for the activation of actomyosin ATPase as in the case of vertebrate TnT. Therefore, overall domain structures seem to be similar between ascidian and vertebrates. Nevertheless, the activation level in ascidian is much higher than in vertebrates. The specific region of ascidian TnT that is responsible for the high level of activation must be identified in the future.

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REFERENCES

1. Ohtsuki, I., Maruyama, K., and Ebashi, S. (1986) Regulatory and cytoskeletal proteins of vertebrate skeletal muscle. *Adv. Protein Chem. 38*, 1–68.
2. Takeda, S., Yamashita, A., Maeda, K., and Maeda, Y. (2003) Structure of the core domain of human cardiac troponin in the Ca^{2+}-saturated form. *Nature 424*, 35–41.
3. Hooper, S. L., Hobbs, K. H., and Thuma, J. B. (2008) Invertebrate muscles: Thin and thick filament structure; molecular basis of contraction and its regulation, catch and asynchronous muscle. *Prog. Neurobiol. 86* (2), 72–127.
4. Putnam, N. H., Butts, T., Ferrier, D. E., Furlong, R. F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J. K., Benito-Gutiérrez, E. L., Dubchak, I., Garcia-Fernàndez, J., Gibson-Brown, J. J., Grigoriev, I. V., Horton, A. C., de Jong, P. J., Jurka, J., Kapitonov, V. V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L. A., Salamov, A. A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin-I, T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L. Z., Holland, P. W., Satoh, N., and Rokhsar, D. S. (2008) The amphioxus genome and the evolution of chordate karyotype. *Nature 453*, 1064–1072.
5. Cleto, C. L., Vandenberghe, A. E., MacLean, D. W., Pannunzio, P., Tortorelli, C., Meedel, T. H., Satou, Y., Satoh, N., and Hastings, K. E. (2003) Ascidian larva reveals ancient origin of vertebrate-skeletal-muscle troponin I characteristics in chordate locomotory muscle. *Mol. Biol. Evol. 20*, 2113–2122.
6. Yuasa, H. J., Cox, J. A., and Takagi, T. (1998) Diversity of the troponin C genes during chordate evolution. *J. Biochem. 123*, 80–1190.
7. Dennisson, J. G., Tando, Y., Ogasawara, M., Kubokawa, K., Sato, N., and Obinata, T. (2010) Functional characteristics of amphioxus troponin in regulation of muscle contraction. *Zool. Sci. 27*, 461–469.
8. Terakado, K., and Obinata, T. (1987) Structure of multinucleated smooth muscle cells of the ascidian *Halocynthia roretzi*. *Cell Tissue Res. 247*, 85–94.
9. Endo, T., and Obinata, T. (1981) Troponin and its components from ascidian smooth muscle. *J. Biochem. 89*, 1599–1608.
10. Potter, J. D., Sheng, Z., Pan, B. S., and Zhao, J. (1995) A direct regulatory role for troponin T and a dual role for troponin C in the regulation of muscle contraction. *J. Biol. Chem. 270*, 2557–2562.
11. Malnic, B., Farah, C. S., and Reinach, F. C. (1998) Regulatory properties of the NH_2- and COOH-terminal domains of troponin T. *J. Biol. Chem. 273*, 10594–10601.
12. Ohshiro, K., Dennisson, J., Tandoh, Y., Sato, N., and Obinata, T. (2008) Comparative study of protochordate troponin in the regulation of muscle contraction. In Proceedings of the 9th International Congress on Cell Biology, pp 79−83, ICCN2008, Medimond, Italy.
13. Obinata, T., Ooi, A., and Takano-Ohmuro, H. (1983) Myosin and Actin from Ascidian Smooth Muscle and Their Interaction. *Comp. Biochem. Physiol. 76B*, 437–442.
14. Endo, T., Matsumoto, K., Hama, T., Ohtsuka, Y., Katsura, G., and Obinata, T. (1996) Distinct troponin T genes are expressed in embryonic/larval tail striated muscle and adult body wall smooth muscle in ascidian. *J. Biol. Chem. 271*, 27855–27862.
15. Yuasa, H. J., Kawamura, K., Yamamoto, H., and Takagi, T. (2002) The structural organization of Ascidian *Halocynthia roretzi* of troponin I genes. *J. Biochem. 132*, 135–141.
16. Yuasa, H. J., Sato, S., Yamamoto, H., and Takagi, T. (1997) Structure of the ascidian, *Halocynthia roretzi*, troponin C gene. *J. Biochem. 121*, 671–676.
17. Chiba, S., Awazu, S., Itoh, M., Chin-Bow, S. T., Satoh, N., Satou, Y., and Hastings, K. E. M. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis* IX. Genes for muscle structural proteins. *Dev. Genes Evol. 213*, 291–302.

18. MacLean, D. W., Meedle, T. H., and Hastings, K. E. M. (1997) Tissue-specific alternative splicing of Ascidian troponin I isoforms. *J. Biol. Chem. 272*, 32115–32120.
19. Perry, S. V. (1955) Myosin adenosinephosphatase. *Methods Enzymol. 2*, 582–588.
20. Spudich, J. A., and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem. 246*, 4866–4871.
21. Bailey, K. (1948) Tropomyosin: A new asymmetric protein component of the muscle fibril. *Biochem. J. 43*, 271–279.
22. Itzhaki, R. F., and Gill, D. M. (1964) A micro-biuret method for estimating proteins. *Anal. Biochem. 9*, 401–410.
23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680–685.
24. Tanokura, M., Imaizumi, M., Yamada, K., Shiraishi, F., and Ohtsuki, I. (1992) Preparation and characterization of troponin C from bullfrog skeletal muscle. *J. Biochem. 112*, 800–803.
25. Head, J. F., and Perry, S. V. (1974) The interaction of the calcium-binding protein (troponin C) with bivalent cations and the inhibitory protein (troponin I). *Biochem. J. 137*, 145–154.
26. Obinata, T. (1969) The myosin of developing chick embryo. *Arch. Biochem. Biophys. 132*, 184–197.
27. Taussky, H. H., and Shorr, E. (1953) A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem. 202*, 675–685.
28. Ryan, T. A. (1959) Multiple comparisons in psychological research. *Psychol. Bull. 56*, 26–47.
29. Ryan, T. A. (1960) Significance tests for multiple comparison of portions, variances, and other statistics. *Psychol. Bull. 57*, 318–328.
30. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics 23*, 2947–2948.
31. Saitou, N., and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol. 4*, 406–425.
32. Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution 39*, 783–791.
33. Oliveira, D. M., Nakaie, C. R., Sousa, A. D., Farah, C. S., and Reinach, F. C. (2000) Mapping the domain of troponin T responsible for the activation of actomyosin ATPase activity. *J. Biol. Chem. 275*, 27513–27519.
34. Potter, J. D. (1982) Preparation of troponin and its components. *Methods Enzymol. 85*, 241–263.