Detection of a troponin I-like protein in non-striated muscle of the tardigrades (water bears)

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Abbreviations: TnI, troponin I; TnC, troponin C

Tardigrades, also known as water bears, have somatic muscle fibers that are responsible for movement of their body and legs. These muscle fibers contain thin and thick filaments in a non-striated pattern. However, the regulatory mechanism of muscle contraction in tardigrades is unknown. In the absence of extensive molecular and genomic information, we detected a protein of 31 kDa in whole lysates of tardigrades that cross-reacted with the antibody raised against nematode troponin I (TnI). TnI is a component of the troponin complex that regulates actin-myosin interaction in a Ca^{2+} -dependent and actin-linked manner. This TnI-like protein was co-extracted with actin in a buffer containing ATP and EGTA, which is known to induce relaxation of a troponin-regulated contractile system. The TnI-like protein was specifically expressed in the somatic muscle fibers in adult animals and partially co-localized with actin filaments in a non-striated manner. Interestingly, the pharyngeal muscle did not express this protein. These observations suggest that the non-striated somatic muscle of tardigrades has an actin-linked and troponin-regulated system for muscle contraction.

Introduction

Muscle is classified into striated and non-striated muscles on the basis of the organization of actin and myosin filaments. In striated muscles, actin and myosin filaments are organized into cross-striated sarcomeric structures, while in non-striated muscles, actin and myosin filaments are arranged in parallel without striations or assembled into a lattice-like network. Distinct differences exist in the regulatory systems for actin-myosin interaction between striated and non-striated muscles. In vertebrate striated muscles including skeletal and cardiac muscle, the actin-myosin interaction is regulated in a Ca^{2+} -dependent manner by troponin that is linked to actin filaments.^{1,2} Troponin is a complex of three components: troponin T (TnT), a tropomyosin-binding component, troponin I (TnI), an inhibitory component of the actin-myosin interaction and troponin C (TnC), a Ca^{2+} -binding component.² Troponin functions as an inhibitor of the actin-myosin interaction at low Ca^{2+} concentrations, but the inhibition is released by binding of Ca^{2+} to troponin. In vertebrate smooth muscle, however, phosphorylation/dephosphorylation of myosin regulatory light chain is the major mechanism to regulate actin-myosin interaction.^{3,4} In many invertebrates, direct binding of Ca^{2+} to myosin regulatory light chain is essential for actin-myosin interaction, although troponin is also involved in the regulation of striated muscles in several invertebrate species.^{5,6}

Although troponin is predominantly expressed in striated muscles in both vertebrate and invertebrate animals, there are several instances for the presence of troponin in non-striated muscles. Troponin has been detected in multinucleated smooth muscle cells of ascidian body wall^{7,8} and adductor smooth muscle of Akazara scallop.⁹ In these muscles, troponin functions as a Ca^{2+} -dependent activator of actin-myosin interaction. Recently, expression of troponin was detected in mammalian vascular smooth muscle, but its physiological roles in contractile regulation are not demonstrated.¹⁰ More recently, troponin was detected in myoepithelial cells, primitive non-striated muscle-like cells, which are present in the somatic gonad of the nematode *Caenorhabditis elegans*. In these cells, actin and myosin are organized in non-striated filamentous networks, and troponin C and troponin I are associated with actin filaments.^{11,12} Their contraction is tightly coupled with oocyte maturation and troponin plays an essential role for the contractile regulation.¹¹⁻¹³ Furthermore, TnI is involved in the regulation of chromosomal stability and cell polarity in early *Drosophila* embryos.¹⁴ These observations suggest that troponin may play important roles not only in striated muscles but also in non-striated muscle or even in non-muscle cells. Therefore, it will be interesting to investigate how the actin-linked troponin regulatory system is distributed among non-striated muscles of a variety of animals.

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Figure 1. Detection of TnI and actin in total lysates of tardigrades by western blotting. (A) Whole lysates of tardigrades (~100 animals/lane) were subjected to SDS-PAGE and subsequent Coomassie staining (lane 1) or western blotting with anti-TnI (lane 2) or anti-actin antibody (lane 3). Positions of molecular weight markers are indicated on the left. (B) Comparison of the sizes of TnI (or TnI-like protein) and actin in nematodes and tardigrades. Whole lysates of nematodes (N) (*C. elegans*: 10 worms/lane) and tardigrades (T) (~100 animals/lane) were subjected to western blotting with anti-actin or anti-TnI antibody. Positions of *C. elegans* TnI (N-TnI) and tardigrade TnI-like protein (T-TnI) are indicated on the right. Positions of molecular weight markers are indicated on the left.

In this study, we focused on tardigrades, also known as water bears, as a model animal to examine troponin in non-striated muscles, because non-striated muscle cells are present throughout the entire body.^{15,16} Most of them show smooth muscle-like morphology; namely, actin filaments and myosin filaments are mostly aligned in parallel without striations as in vertebrate smooth muscle cells.^{15,17,18} Phylogenetic studies revealed that Tardigrada is categorized in the Ecdysozoa together with Nematoda, Arthropoda and others.^{19,20} Based on such phylogenetic relationship between Tardigrada and Nematoda, it was expected that immunological probes that are raised against nematode proteins might be useful for the analysis of tardigrade motile and regulatory systems. In this study, we were able to detect a troponin I-like protein that is co-localized with actin filaments in tardigrade non-striated muscle, suggesting that troponin may be involved in the contractile regulation of this muscle.

Results

Detection of a Troponin I-like protein in whole lysates of tardigrades by western blotting. Since recent phylogenetic studies of the animal kingdom revealed that tardigrades and nematodes are related in a phylogenetic tree,²⁰ we tested whether the antibody against the *Ascaris* TnI can be used for detecting tardigrade TnI. The rabbit

Figure 2. Co-extraction of tardigrade TnI-like protein with native thin filaments is enhanced by ATP and EGTA. (A) Schematic representation of the procedure for extraction of native thin filaments, as described in Materials and Methods. (B and C) SDS-PAGE and western blotting of isolated actin filaments from P1 (actin filaments extracted in the absence of ATP and EGTA) (B) and P2 (actin filaments extracted in the presence of ATP and EGTA) (C). These fractions were subjected to SDS-PAGE and subsequent Coomassie staining (lane 1) or western blotting with anti-actin (lane 2) or anti-TnI antibody (lane 3).

Figure 3. Localization patterns of TnI-like protein and F-actin in the freeze-fractured tardigrade bodies. Lateral (a–c) and dorsal views (d–f) of freeze-fractured whole animals are demonstrated. Tardigrades were stained with anti-*Ascaris* TnI antibody (a and d) and rhodamine-phalloidin (b and e). Merged images are shown in c and f (anti-TnI antibody in green and rhodamine-phalloidin in red). Asterisks indicate the pharynx that was stained with phalloidin but not with anti-TnI antibody. Bars, 100 μ m.

polyclonal antibody that was raised against *Ascaris* troponin I²¹ specifically recognizes nematode TnI in *Ascaris*²¹ and *C. elegans*.^{12,22} As shown in Figure 1A, the antibody reacted specifically with a single band of roughly 31 kDa in the tardigrade extracts (Fig. 1A, lane 2). Actin was also clearly detected in the tardigrade extracts with the monoclonal anti-actin antibody (C4) (Fig. 1A, lane 3), which is known to react with actin in a variety of organisms. The sizes of actin and the protein (TnI-like protein) detected by anti-*Ascaris* TnI antibody in the tardigrade extracts were compared with those of nematode proteins (Fig. 1B). The size of tardigrade actin was almost the same as that of nematode actin (Fig. 1B), whereas the size of tardigrade TnI-like protein is slightly smaller than that of nematode TnI (Fig. 1B). This is within the range of the various sizes of TnI proteins from vertebrate and invertebrate animals. Based on the size and the specific binding to the antibody, the 31 kDa protein is most likely a tardigrade TnI protein and designated as TnI-like protein in this work.

Association of the tardigrade TnI-like protein with thin filaments isolated from tardigrades. In order to examine

whether the TnI-like protein is associated with actin filaments in tissues, native actin filaments were extracted and isolated from tissue homogenates. Since the amount of animals was very small (only a few milligrams), whole animals without tissue dissection were homogenized by sonication in a small volume of a low-salt buffer containing Triton X-100 (Fig. 2A). To minimize depolymerization of actin filaments, phalloidin was included in the homogenization buffer. Under these conditions, actin was released in the soluble fraction (Fig. 2A, S1) and filamentous (F-) actin was precipitated by ultracentrifugation (Fig. 2A, P1; B, lanes 1 and 2). However, the TnI-like protein was not detected by western blot (Fig. 2B, lane 3), indicating that TnI (or the TnI-like protein)-free actin filaments possibly from non-muscle tissues were released under these conditions. Next, a second extraction was performed from the insoluble fraction (P0) with the same low-salt buffer except that it contained ATP and EGTA (Ca^{2+} -chelater), which are known to cause relaxation of troponin-regulated actomyosin structures. Under these relaxing conditions, a considerable amount of actin filaments were released and precipitated (Fig. 2A, P2; C, lanes 1 and 2) together with the TnI-like protein (Fig. 2C, lane 3). These observations indicate that the TnI-like protein is associated with actin filaments that are integrated into ATP- and Ca^{2+} -sensitive intracellular structures. These properties resemble those of troponin-regulated thin filaments in vertebrate striated muscle and strongly suggest that the tardigrade TnI-like protein is a regulatory component of thin filaments in the somatic muscle.

Specific expression of the TnI-like protein in the somatic muscle in tardigrades. We determined tissue expression and localization of the TnI-like protein in adult tardigrades by immunofluorescence microscopy. Animals were permeabilized by freeze-fracturing and stained with the anti-TnI antibody. Rhodamine-phalloidin has been reported to stain strongly F-actin in the somatic muscle of tardigrades^{16,20} and was used in double-staining experiments. The TnI-like protein was specifically detected in a filamentous network pattern throughout the entire body and legs (Fig. 3a and d). This pattern was similar to that of F-actin in the somatic muscle as visualized by phalloidin-staining (Fig. 3b and e). The TnI-like protein was clearly detected in nearly all somatic muscle cells including longitudinal musculature in both dorsal and ventral sides, dorsoventral musculature, lateral musculature and leg musculature (Fig. 3a and d). Remarkably, however, the TnI-like protein was not detected in the pharyngeal muscle (Fig. 3a and d, asterisks), although it was strongly stained by rhodamine-phalloidin (Fig.

3b and e, asterisks). These observations suggest that the TnI-like protein is a component of the contractile regulatory system in the somatic muscle and that the pharyngeal muscle uses a distinct regulatory system.

Intracellular localization of the TnI-like protein was analyzed at higher magnifications. The somatic muscle cells are mono-nucleated and elongated to roughly 40–50 μm in length. The TnI-like protein was detected in the entire cytoplasm without any striated patterns (Fig. 4a). F-actin was similarly detected in the cytoplasm without any striations (Fig. 4b). High-magnification images showed that both TnI-like protein and F-actin localized to faint filamentous structures, suggesting that they co-localized in the thin filaments (Fig. 4a and b, insets). Perhaps, the thin filaments are densely packed in the myofibrils that individual filaments (or bundles) are not distinguishable.

Besides co-localization of the TnI-like protein and F-actin, we also found distinct localization patterns of the TnI-like protein in the somatic muscle cells. First, the TnI-like protein was present in the diffuse cytoplasm around the nuclei (Fig. 5a, asterisk) where F-actin was not enriched (Fig. 5c, asterisk). The single nucleus of a somatic muscle cell is present at one side of the cell (Fig. 5a and b), and a small amount of cytoplasm with no contractile apparatuses was visible around the nucleus. The TnI-like protein was detected in this cytoplasmic region but not in the nucleus (Fig. 5a, asterisk). This diffuse localization of TnI-like protein might make its filamentous localization less clear (Fig. 4a).

Second, the TnI-like protein was only weakly accumulated or absent from the cell-cell or cell-substrate attachments (Fig. 6). Ends of the somatic muscle cells are attached to the epidermis where discrete cell-substrate attachment structures are formed (Shaw 1974).¹⁵ At the cell-substrate attachment sites, F-actin was accumulated to dense structures (Fig. 6b, arrows). However, the TnI-like protein was absent from these F-actin-rich structures (Fig. 6a and c, arrows). The left attachment site in the micrograph coincided with the cell-cell contact region of the longitudinal muscle cells where F-actin, but not TnI-like protein, was enriched.

Discussion

In this study, we have detected a protein of about 31 kDa that is recognized by anti-*Ascaris* TnI antibody. This protein was isolated together with actin filaments from tardigrade tissue homogenates under the conditions that relax muscle by dissociating actin filaments from myosin. These characteristics strongly suggest that this protein is a TnI of tardigrade. This is the first demonstration of the presence of a troponin (or troponin-like) component in tardigrades. Currently, the whole genome sequence of tardigrades is not available. The TnI-like protein is a strong candidate of a regulator of Ca^{2+} -dependent interaction of actin filaments with myosin, because this TnI-like protein was co-fractionated with native actin filaments in

Figure 4. Filamentous localization of TnI-like protein and F-actin in muscle cells. Somatic muscle cells were co-stained with anti-TnI antibody (a) and rhodamine-phalloidin (b). Both TnI-like protein and F-actin were present in muscle cells in non-striated manners. High-magnification images (insets) show faintly filamentous staining patterns for TnI-like protein and F-actin. Bars, 10 μm .

a relaxation buffer containing ATP and EGTA, as expected for a component of the troponin complex. We attempted to detect other troponin component, TnC, with anti-nematode TnC antibody,²¹ but no specific reactivity in the tardigrade lysates was found (our unpublished data). Nonetheless, the presence of TnC in tardigrades has been suggested by two database entries of partial cDNA sequences from *Hypsibius dujardini* (GenBank accession numbers: CO742021.1 and CO741776.1), which are homologous to troponin C, but their functions are unknown.

It has been reported that the pharynx is composed of non-striated muscle cells with the morphology similar to muscle cells in body and leg regions,^{17,23} but we did not observe positive staining of the pharynx by the anti-TnI antibody. In *C. elegans*, four TnI isoforms are present and differentially expressed in the pharynx and body wall muscle.^{12,24,25} Likewise, tardigrades may have multiple muscle-type-specific TnI isoforms, and a pharynx-specific isoform might not be recognized by the anti-*Ascaris* TnI antibody. It is also possible that the tardigrade pharynx uses a regulatory system for contraction that is independent of troponin.

For many years, troponin has been regarded as a Ca^{2+} -dependent regulator of actin-myosin interaction that is characteristic of striated muscles.^{1,2} However, several studies have demonstrated the instances for expression of troponin in non-striated muscles and involvement of troponin in contractile regulation of smooth muscles and myoepithelial cells.^{7,9-12} Here, we have demonstrated another example for the expression of

Figure 5. F-actin-independent localization of TnI-like protein in the cytoplasm around the nuclei. Somatic muscle cells were co-stained with anti-TnI antibody (a), DAPI (b) and rhodamine-phalloidin (c). Merged image is shown in (d) (anti-TnI antibody in green, DAPI in blue and rhodamine-phalloidin in red). Asterisks indicate a nucleus in a muscle cell. TnI-like protein was present in the cytoplasm around the nucleus (a), but F-actin was absent (c). Bar, 10 µm.

troponin, or troponin-like protein, in non-striated muscle of tardigrade. Further biochemical and molecular genetic studies on tardigrade troponin are likely to provide new insights into the evolution of regulatory systems for muscle contraction and diverse functions of troponin-related proteins in various cell types and organisms.

Materials and Methods

Animals. Tardigrades, freshwater species (Eutardigrada: Hypsibius sp), were purchased from Connecticut Valley Biological Supply (Southampton, MA). Wild-type strain N2 of the nematode *C. elegans* was obtained from the Caenorhabditis Genetic Center (Minneapolis, MN).

Gel electrophoresis and western blotting. SDS-PAGE was carried out using a 12% polyacrylamide gel and a discontinuous Tris-glycine buffer system.²⁶ Western blot analysis was performed essentially as described previously in reference 27, with slight modification. Briefly, whole animals, tardigrades and nematodes (*C. elegans*), or the fraction including isolated thin filaments were lysed in an SDS-lysis buffer (2% SDS, 80 mM Tris-HCl, 5% β-mecaptoethanol, 15% glycerol and 0.5% bromophenol blue, pH 6.8) and subjected to SDS-PAGE (12% acrylamide gel) and blotting. Rabbit anti-*Ascaris* troponin I (TnI) antibody^{21,22} was diluted at 1:2,000 to 1:8,000 in Signal Enhancer HIKARI (Nacalai USA, Inc., San Diego, CA USA) and used

to detect troponin I. Mouse anti-actin monoclonal antibody (C4, MP Biochemicals, Irvine, CA) was used to detect actin. Horseradish peroxidase-labeled secondary antibodies were used to detect primary antibodies. The reactivity was detected with a SuperSignal West Pico chemiluminescence reagent (Pierce Chemical, Rockford, IL).

Isolation of native thin filaments. The fractionation procedure is schematically shown in Figure 2A. Roughly 5 mg of tardigrades (about 200 animals) were incubated in 50 µl of KMT solution [0.05 M KCl, 2 mM MgCl₂, 0.2% Triton-X100, 20 mM HEPES (pH 7.5), 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.05% sodium azide and 20 µg/ml phalloidin] for 10 min on ice and then homogenized by sonication for a short period (3 sec). The homogenate was centrifuged at 10,000 g for 10 min to obtain supernatants (S1) and precipitates (P0). The precipitates (P0) were incubated in the KMT solution containing 5 mM ATP and 1 mM ethyleneglycolbis (aminoethyl ether)-N-N' tetra acetic acid (EGTA) (a relaxing buffer) for 10 min on ice and re-homogenized by sonication for 3 sec, followed by centrifugation at 10,000 g for 10 min to obtain supernatants (S2) and precipitates. Both supernatants, S1 and S2, were subjected to ultracentrifugation (436,000 g for 15 min) to collect released actin filaments. The precipitates (P1) derived from S1 and the precipitates (P2) derived from S2 were examined by SDS-PAGE and western blotting with anti-TnI antibody and anti-actin antibody.

Fluorescence microscopy. Tardigrades were mildly compressed between a poly-lysine coated slide glass and a coverslip and frozen by dry ice, and then freeze-fractured by rapidly lifting off the coverslip with a razor blade as described by Epstein et al.²⁸ for *C. elegans* embryos. The slides were immediately immersed in 4% paraformaldehyde in cytoskeletal buffer (138 mM KCl, 3 mM MgCl₂, 2 mM EGTA and 10 mM MES-KOH, pH 6.1) containing 0.32 M sucrose and incubated for 10 min at room temperature, followed by treatment with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 30 mM glycine for 10 min. They were then incubated with rabbit anti-TnI antibody, 0.2 µg/ml tetramethylrhodamine-phalloidin and 0.2 µg/ml 4'6-diamidino-2-phenylindole (DAPI) in PBS containing 0.5% Triton X-100 and 30 mM glycine for 1 h and washed with PBS containing 0.5% Triton X-100 and 30 mM glycine. Binding of the primary antibody was visualized by Alexa488-conjugated goat anti-rabbit IgG (Invitrogen, San Diego, CA USA). Samples of the tissues stained with antibodies were mounted with ProLong Gold (Invitrogen, Carlsbad, CA USA) and observed by epifluorescence using a Nikon Eclipse TE2000 inverted microscope with a CFI Plan Fluor ELWD 40X (dry, NA 0.60) or Plan Apo 60X (oil, NA 1.40) objective. Images were captured by a SPOT RT monochrome CCD camera (Diagnostic Instruments) and processed by IPLab imaging software (BD Bioscience, San Jose, CA USA) and Adobe Photoshop CS3.

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Figure 6. Absence of TnI-like protein from the cell-cell and cell-substrate attachments. Somatic muscle cells were co-stained with anti-TnI antibody (a) and rhodamine-phalloidin (b). Merged image is shown in (c) (anti-TnI antibody in green and rhodamine-phalloidin in red). Arrows indicate the sites of attachments of dorsoventral muscle cells to the epidermis. The left site coincides with the cell-cell contact of longitudinal muscle cells. F-actin, but not TnI-like protein, was enriched in these attachment sites. Bar, 10 µm.

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