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Sea Lily Muscle Lacks a Troponin-Regulatory System, While It Contains Paramyosin

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Troponin, a Ca^{2+}-dependent regulator of striated muscle contraction, has been characterized in vertebrates, protochordates (amphioxus and ascidian), and many invertebrate animals that are categorized in protostomes, but it has not been detected in echinoderms, such as sea urchin and sea cucumber, members of subphylum Eleutherozoa. In this study, we examined the muscle of a species of isocrinid sea lilies, a member of subphylum Pelmatozoa, that constitute the most basal group of extant echinoderms to clarify whether troponin is lacking from the early evolution of echinoderms. Native thin filaments were released from the muscle homogenates in a relaxing buffer containing ATP and EGTA, a Ca^{2+}-chelator, and were collected by ultra-centrifugation. Actin and tropomyosin, but not a troponin-like protein, were detected in the filament preparation. The filaments increased Mg^{2+}-ATPase activity of rabbit skeletal muscle myosin irrespective of the presence or absence of Ca^{2+}. The results indicate that Ca^{2+}-sensitive factor, troponin, is lacking in the thin filaments of sea lily muscle as in those of the other echinoderms, sea urchin and sea cucumber. On the other hand, a paramyosin-like protein that is absent from chordates was detected in sea lily muscle as in the muscles of the other echinoderms and invertebrate animals of protostomes.

Key words: troponin, actin, paramyosin, muscle proteins, sea lily, *Metacrinus rotundus*

INTRODUCTION

Troponin is a mediator of Ca^{2+}-dependent regulation of striated muscle contraction in vertebrates (Ohtsuki et al., 1986). It is linked to actin filaments and functions in combination with tropomyosin. This troponin–tropomyosin regulatory system is also found in striated and obliquely striated muscles of a variety of invertebrate animals, although direct binding of Ca^{2+} to myosin regulatory light chain is also involved in actin-myosin interaction in most of the animals among protostomes (Lehman and Szent-Gyorgyi, 1975; Hooper et al., 2008). Among the animals in deuterostomes, troponin has been detected in vertebrate striated muscles, as well as in striated and smooth muscles of ascidians (Toyota et al., 1979; Endo and Obinata, 1981; Ohshiro et al., 2010) and in striated muscle of amphioxus (Dennisson et al., 2010). Interestingly, functional variation has been detected in troponin of chordates, namely vertebrates, amphioxus and ascidian. Troponin in vertebrates and amphioxus inhibits muscle contraction, or actin–myosin interaction, in the absence of Ca^{2+}, and Ca^{2+} removes the inhibitory action (Ohtsuki et al., 1986; Dennisson et al., 2010). However, troponin in both smooth and striated muscles of urochordate ascidian weakly inhibits the actin–myosin interaction in the absence of Ca^{2+}, but enhances it remarkably in the presence of Ca^{2+} (Endo and Obinata, 1981; Ohshiro et al., 2010). In other words, ascidian troponin functions as a Ca^{2+}-dependent accelerator of actin–myosin interaction, whereas in vertebrate and amphioxus striated muscle troponin functions as a Ca^{2+}-sensitive inhibitor. The ascidian muscle troponin may have acquired its unique property as a Ca^{2+}-dependent accelerator during evolution, while amphioxus and vertebrate striated muscle troponin may have succeeded the brake property from the ancestral troponin (Obinata and Sato, 2012). A comparative study of troponin in other animals may give insights into how the troponin-regulatory system evolved among the deuterostomes, and also explain how functional differences emerged between various muscles. Previous studies, however, have not detected troponin in actin filaments isolated from the muscles of echinoderms, such as sea urchin (Obinata et al., 1974) and sea cucumber (Takito and Konishi, 1987), subphylum Eleutherozoa. Deficiency of troponin in the echinoderm muscles seemed

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strange, as troponin is detectable not only in the muscles of chordates but also in the muscles of many protostomes. Moreover, troponin has been detected in actin filament networks in non-striated muscle of the tardigrades (water bears) (Obinata et al., 2011) and in gonad myoepithelial cells of nematodes (Ono and Ono, 2004; Obinata et al., 2010), suggesting the possibility that troponin is involved in the regulation of a variety of motile cells. In this study, we focused on an isocrinid sea lily (stalked crinoid), a taxon that constitutes the most basal group of extant echinoderms (Rouse et al., 2013) and a member of subphylum Pelmatozoa, to clarify whether troponin is lacking from the beginning of the evolution of echinoderms. According to microscopic observations, crinoids, some feather stars, have obliquely striated muscle as many invertebrates of protostomes but an unusual Z-system exists in the crinoid muscle (Candia Carnevali and Saita, 1985; Candia Carnevali et al., 1986). However, morphological structures and proteins of the muscles of sea lily crinoids have not been examined, because the habitat of the animals is limited and the size of the muscle tissue is too small to obtain sufficient amounts for biochemical analysis. The body of the sea lily consists of a crown and a stalk (Fig. 1A). The crown is composed of a cup and arms. The cup is joined to the stalk at its topmost part, and many arms extend from the cup (Fig. 1B). In contrast to the stalk in which muscles have never been reported, the arms have flexor muscles. Each arm is supported by skeletal elements that are termed brachials (arm plates) located in longitudinal series along the arm. The arm plates are connected by a pair of muscle masses running from one plate to the next in adoral muscle fields situated near the oral surface of the plates (Hyman, 1955). In the absence of extensive molecular and genomic information, we were able to isolate native thin filaments from minute muscle tissue that connects the arm plates in the crown of a sea lily, *Metacrinus rotundus*, to characterize the protein constituents and the functional properties. We also examined whether sea lily muscle has paramyosin, which is distributed among a variety of animals of protostomes and sea urchin and sea cucumber (Szent-Gyorgyi et al., 1971; Obinata et al., 1975; Winkelman, 1976; Kantha et al., 1990), but not in urochordates and vertebrates.

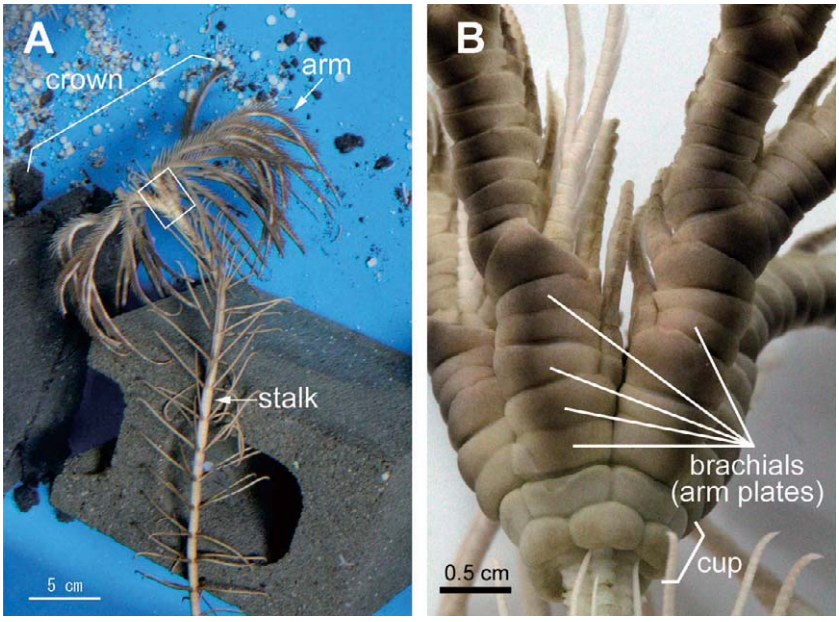

Fig. 1. A living adult of the isocrinid sea lily *Metacrinus rotundus* cultured in an aquarium. **(A)** The body of the sea lily consists of a crown and a stalk. The crown is formed by a cup, which is joined to the stalk at its topmost part. Many arms extend from the cup. **(B)** Enlargement of the rectangle in **(A)**, showing the cup and the basal parts of the arms. The arms spring pentamerously from the cup, and fork repeatedly.

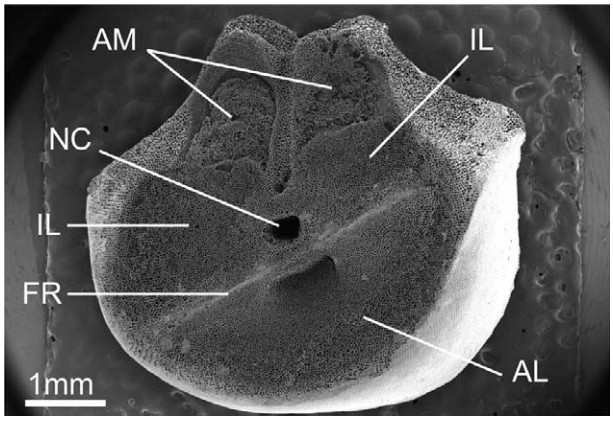

Fig. 2. SEM of a proximal articular facet of a skeletal ossicle isolated from an arm plate (brachial). The arm plate was treated with bleach to remove soft tissues. A pair of adoral muscle fields becomes visible on the facet of the ossicle after removal of the muscle masses. AL, aboral ligament field; AM, adoral muscle field; FR, fulcral ridge; IL, interarticular ligament field; NC, nerve canal.

MATERIALS AND METHODS

Animals

Adults of the isocrinid sea lily *Metacrinus rotundus* were collected using a gill net from a depth of about 130 m in the southeastern area of the Uraga Channel in Sagami Sea, Japan. After transport to the Marine and Coastal Research Center of Ochanomizu University, the sea lilies were maintained in sea water in the dark at 14°C as described in the previous paper (Amemiya et al., 2013). Muscles were isolated from tissues containing a pair of muscle masses running from one arm plate to the next in adoral muscle fields situated near the oral surface of the plates (Fig. 2). Arms were removed from the adult crowns, and arm plates were isolated from the arms by cutting with a blade. Tissues containing the muscle masses on the proximal and distal facets of each arm plate were shaved off by tweezers under a dissection microscope. The isolated tissues were stored in a solution containing 50% glycerol, 50 mM KCl, 1 mM $MgCl_2$, 10 mM phosphate buffer (pH 7.0), 0.1% NaN_3, 1 mM 2-mercaptoethanol and 1 mM PMSF (phenylmethylsulfonyl fluoride) at –20°C until used.

Preparation of a skeletal ossicle in an arm plate for scanning electron microscopy (SEM)

An arm plate was isolated from an arm removed from an adult

crown by cutting with a blade. A skeletal ossicle in the arm plate was isolated by treating the plate with bleach containing 5% sodium hypochlorite for 24 hours. The ossicle was rinsed with distilled water, dehydrated with a graded ethanol series and air-dried. The dried ossicle was attached to stubs via double-sided adhesive tape, coated with gold in an E-101 ion sputter coater (Hitachi, Tokyo, Japan), and observed with a JSM-6510LV SEM (JEOL, Tokyo, Japan).

Antibodies

Primary antibodies to muscle proteins used were (1) SkA-06, mouse monoclonal antibody to sarcomeric α-actin (Hayakawa et al., 1996), (2) SUPM-1, mouse monoclonal antibody to sea urchin paramyosin (Tsuchiya et al., 1992), (3) rabbit polyclonal antibody raised against purified sea urchin paramyosin (Obinata et al., 1975). Alkaline phosphatase (AP)-labeled goat anti-mouse IgG and AP-labeled goat anti-rabbit IgG were purchased from Bio-Rad (Richmond, CA).

Gel electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% or 12.5% polyacrylamide gel and a discontinuous Tris-glycine buffer system (Laemmli, 1970). The proteins for SDS-PAGE were dissolved with an SDS-extracting solution containing 2% SDS, 2% 2-mercaptoethanol, 63 mM Tris-HCl (pH 6.7), 25% glycerol and 0.2 mM PMSF. Urea-shift SDS-PAGE was carried out with 10% polyacrylamide separating gel containing 0.1% SDS or 0.1% SDS plus 5 M urea (Ishimoda-Takagi et al., 1997). Two-dimensional PAGE was performed by a combination of isoelectric focusing (IEF) (O'Farrell, 1975) in the first dimension and SDS-PAGE in the second dimension using 10% polyacrylamide gel. For western blotting, the proteins were transferred electrophoretically from the polyacrylamide gel to a nitrocellulose membrane in a solution containing 25% ethanol, 20 mM Tris-HCl, 150 mM glycine, pH 8.3 and 0.05% SDS, for 1 h (Towbin et al., 1992). The nitrocellulose membrane was treated with 5% skim milk in Tris-buffered saline (TBS) and then incubated with primary antibodies for 1 h at room temperature, followed by treatment with AP-labeled second antibody for 1 h at room temperature. The AP-labeled antibodies bound to the membrane were detected by incubation with a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT).

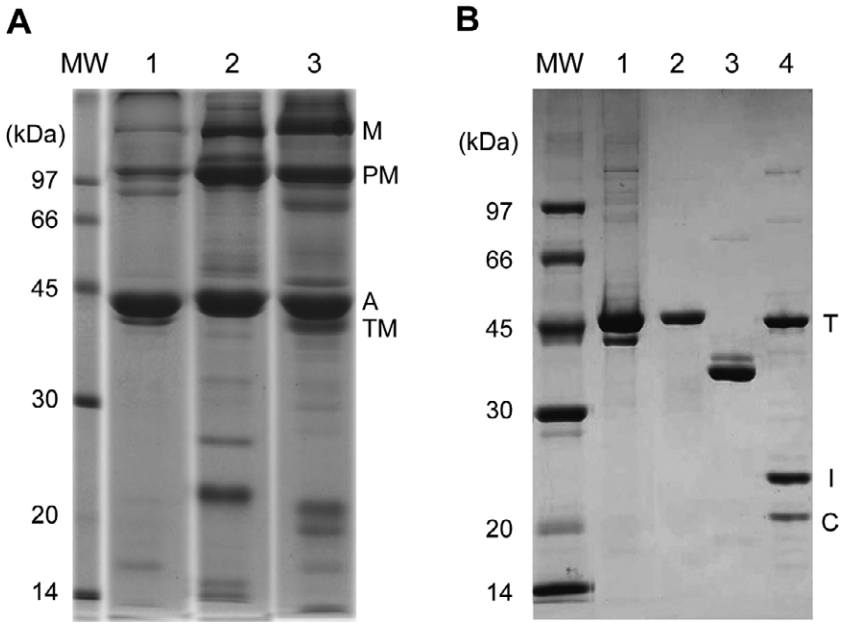

Fig. 3. SDS-PAGE patterns of whole lysates **(A)** of sea lily, sea urchin and sea cucumber muscle and thin filaments **(B)** isolated from sea lily muscle. **(A)** Equivalent amounts of proteins from sea lily (lane 1), sea urchin (lane 2) and sea cucumber (lane 3) were displayed on 10% polyacrylamide gel by SDS-PAGE. M, myosin heavy chain; PM, paramyosin; A, actin; TM, tropomyosin. The molecular weight markers (MW) are shown at the left side of lane 1. **(B)** Native thin filaments isolated from sea lily muscle were examined by SDS-PAGE. Lane 1: native thin filaments of sea lily, lane 2–4: marker proteins as references. Rabbit skeletal muscle actin (lane 2), chicken skeletal muscle tropomyosin (lane 3) and chicken skeletal (pectoralis) muscle troponin composed of three troponin components (T, I, C) (lane 4). The size of troponin T of this muscle is similar to that of actin, as previously described (Matsuda et al., 1981). The molecular weight markers (MW) are shown at the left side of lane 1.

Isolation of native thin filaments

The fractionation procedure is schematically shown in Fig. 4. Roughly 100 mg of muscle tissue were homogenized in 2 ml of a KMI solution (0.05 M KCl, 2 mM $MgCl_2$, 10 mM imidazole, pH 6.5) containing 0.1% Triton-X100, 0.5 mM PMSF, 40 μg/ml trypsin inhibitor, 1 mM 2-mercaptoethanol (Me), 0.05% sodium azide with a dounce tissue grinder and then by sonication for a short period (5 sec). The homogenate was centrifuged at 10,000 g for 20 min to obtain supernatants (S1) and precipitates (P1). The precipitates (P1) were incubated in the relaxing buffer, a KMI solution containing 5 mM ATP, 1 mM EGTA, 0.5 mM PMSF, 40 μg/ml trypsin inhibitor, 1 mM 2-mercaptoethanol, 0.05% sodium azide for 10 min on ice and re-homogenized by sonication for 3 sec, followed by centrifugation at 10,000 g for 30 min to obtain supernatants (S2) and precipitates. The supernatant (S2) was subjected to ultracentrifugation, at 74,000 g for 40 min, to collect released thin filaments.

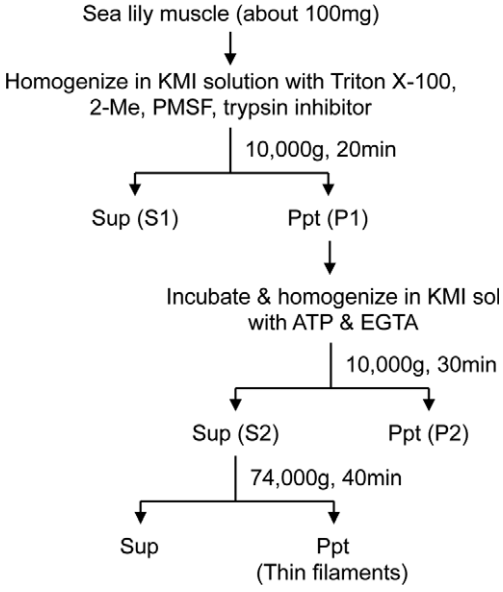

Fig. 4. Schematic representation of the procedure for isolation of native thin filaments, as described in Material and Methods.

Electron microscopic observation of isolated thin filaments

Negative staining was performed with 1.5% uranyl acetate as described by Hanson and Lowey (1963). Electron microscopic observation was carried out with a Hitachi H-7500.

Preparation of other proteins

Myosin was prepared from rabbit back muscle according to the method of Perry (1955). Actin and tropomyosin were prepared as previously described (Dennisson et al., 2010).

ATPase measurements

The ATPase assays were carried out according to our previously reported method (Obinata, 1969). The assay conditions are indicated in the legend for Fig. 8.

RESULTS

Protein components of native thin filaments isolated from sea lily muscle tissue

Before isolating thin filaments from muscle tissue, protein constituents of sea lily muscle were analyzed by SDS-PAGE. Whole proteins extracted from the muscle with an SDS-extracting solution were displayed on SDS-polyacrylamide gel and compared with the muscle proteins of other echinoderms, sea urchin and sea cucumber (Fig. 3A). The overall profile of major protein constituents does not appear to differ significantly among these echinoderms, although difference was detected in several minor protein bands. A major protein band of near 45 kDa in sea lily (lane 1) showed almost the same mobility as actin (A) in sea urchin (lane 2) and sea cucumber (lane 3). This protein of sea lily was identified with actin by using anti-actin antibody as described below (Figs. 6 and 9). Notably, the relative proportion of actin within the whole set of protein extracts was much higher in sea lily than in sea urchin and sea cucumber. Higher molecular weight proteins that correspond to paramyosin (PM) and myosin heavy chain (M), which were characterized in sea urchin (Obinata et al., 1975) and sea cucumber (Takito and Konishi, 1987), were also detected in sea lily, but the relative proportion of these proteins among the whole muscle proteins were much lower in sea lily than in sea urchin and sea cucumber. The size of tropomyosin (TM) differed slightly in these animals; since the size of sea urchin tropomyosin is close to that of actin (Obinata et al., 1974), they were scarcely separated under this electrophoresis condition (Fig. 3A, lane 2).

Native thin filaments were isolated from the sea lily muscle homogenates by applying slight modification of previously described methods (Obinata et al., 1974; Obinata et al., 2011). To minimize proteolysis of muscle proteins during the isolation procedure, PMSF and trypsin inhibitor were included in the homogenization buffer. Thin filaments were released from the tissue homogenate by a relaxing solution containing ATP and EGTA and precipitated by ultracentrifugation (Fig. 4). An electron micrograph of negatively stained thin filament preparation is shown in Fig. 5. Although globular materials were present in the preparation, many thin filaments were detected. The structure of the filaments appears similar to those of native thin filaments isolated from other animals such as sea urchin (Obinata et al., 1974) and ascidian (Toyota et al., 1979) muscles. The globular materials may be aggregates of disorganized actin, because SDS-PAGE analysis showed that the thin filament preparation was enriched with actin and tropomyosin and the other protein components were scarcely detected. The SDS-PAGE pattern of thin filaments from sea lily is shown in Fig. 3B (lane 1). The main band, which has almost the same mobility as that of rabbit skeletal muscle actin (lane 2), was des-

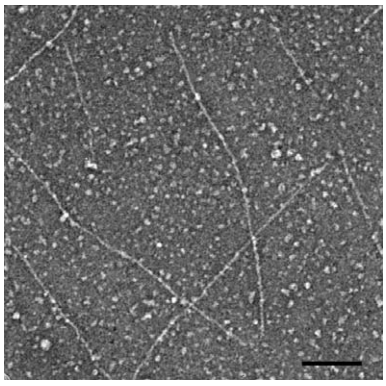

Fig. 5. Electron micrograph of native thin filaments that were isolated from sea lily muscle. Bar: 200 nm.

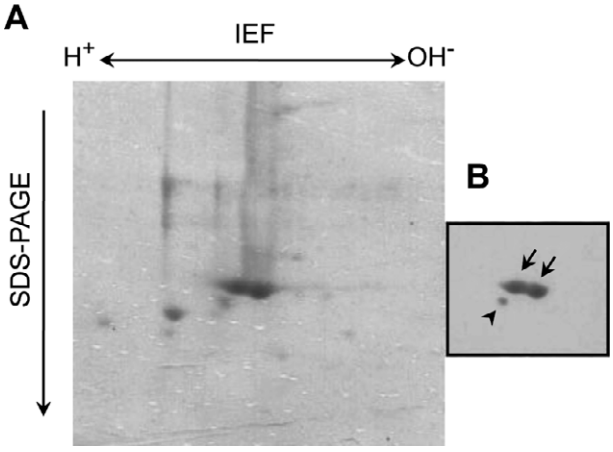

Fig. 6. Two-dimensional gel electrophoresis pattern of sea lily muscle actin. Whole lysates of sea lily muscle were subjected to two-dimensional gel electrophoresis, a combination of isoelectric focusing and SDS-PAGE, and subsequent Coomassie staining **(A)** or western blotting with anti-sarcomeric α-actin antibody **(B)**. Arrows indicate major actin spots and arrowhead indicates minor actin spot.

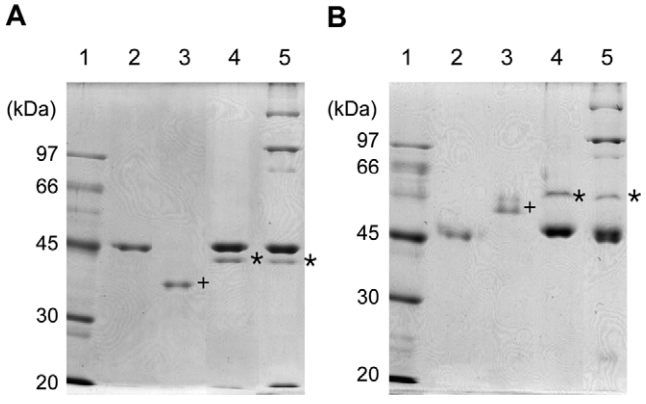

Fig. 7. Urea-shift SDS-PAGE analysis of tropomyosin in sea lily muscle. Isolated thin filaments and whole lysates of sea lily were subjected to SDS-PAGE in the absence **(A)** or presence **(B)** of 5 M urea. Lane 1: molecular weight marker, lane 2: rabbit actin, lane 3: chick muscle tropomyosin, lane 4: sea lily thin filaments, lane 5: whole lysates of sea lily muscle. Chick tropomyosin band was marked with cross (+). Sea lily protein bands that showed mobility shift in the presence of urea are marked with asterisks (⋆).

ignated as actin based on the size and positive interaction with anti-actin antibody (see below). A minor component with a slightly lower molecular weight than actin, probably tropomyosin, was also present in the thin filament preparation, but other protein components, such as troponin subunits, were not detected. When actin was examined by a combination of two dimensional electrophoresis and western blotting, three actin spots, two major (arrows) and one minor (arrowhead), were distinguished (Fig. 6B). They differ slightly in size and pI. The antibody raised against chicken skeletal muscle actin recognized all of them.

To confirm whether tropomyosin is associated with sea lily actin filaments, we carried out SDS-PAGE analysis of the isolated thin filaments in the absence (Fig. 7A) or presence (Fig. 7B) of urea, as it is known that mobility of tropomyosin on the gel is specifically altered in the presence of urea (Sender, 1971). The mobility of the protein band (marked by asterisk) that is located just below actin band in isolated thin filaments (lane 4) and whole protein extracts (lane 5) markedly altered in the presence of urea; in fact, the particular bands marked by asterisk moved faster than actin in the absence of urea (Fig. 7A), but did more slowly than actin in the presence of urea (Fig. 7B). As reported, similar mobility shift was clearly observed in purified chicken muscle tropomyosin (lane 3, marked by cross) under the same condition. Therefore, it is very likely that the sea lily protein marked by asterisk is tropomyosin, and that the size of sea lily tropomyosin is larger than that of chicken skeletal muscle tropomyosin.

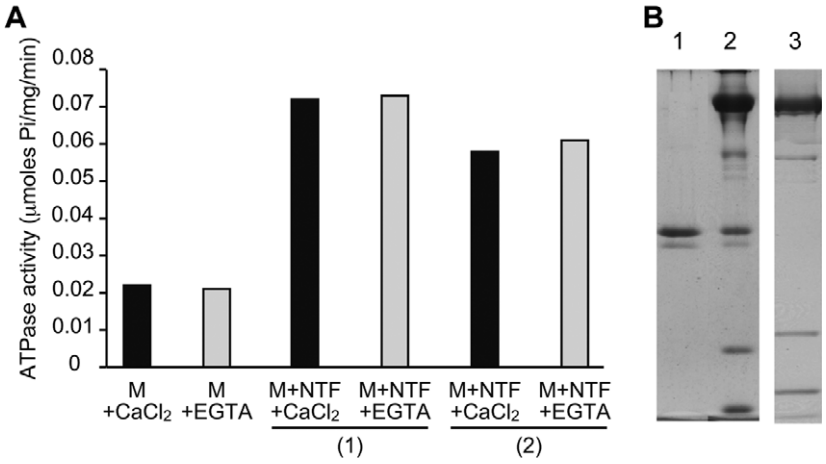

Fig. 8. Effects of native thin filaments isolated from sea lily muscle on Mg^{2+}-ATPase activity of myosin. **(A)** Mg^{2+}-ATPase activity of rabbit myosin with sea lily thin filaments (M+NTF) or without the thin filaments (M) was measured in the presence of Ca^{2+} or EGTA. Two representative assay results, (1) and (2), are demonstrated. The reaction mixture included 53 mM KCl, 6.6 mM $MgCl_2$, 1 mM ATP, 20 mM Tris-HCl buffer, pH 7.4, either 0.1 mM $CaCl_2$ (black bars, +$CaCl_2$), or 1 mM EGTA (gray bars, +EGTA), 0.18 μM rabbit myosin, and sea lily thin filaments. Reaction time: 10 min at 25°C. **(B)** SDS-PAGE patterns of the specimen used for ATPase assay. Lane 1: sea lily thin filaments before adding to myosin, lane 2: mixture of myosin and thin filaments (M+NTF), lane 3: myosin without thin filaments (M).

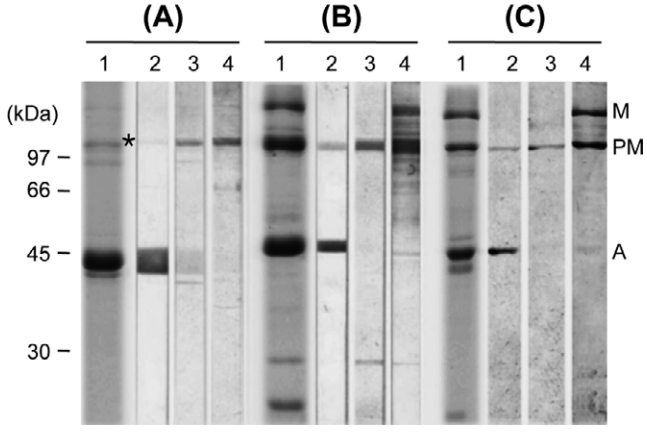

Fig. 9. Detection of paramyosin in total lysates of sea lily muscle by western blotting. Whole lysates of sea lily **(A)**, sea urchin **(B)** and sea cucumber **(C)** muscles were subjected to SDS-PAGE and subsequent Coomassie staining (lane 1 in each case) or western blotting with anti-sarcomeric α-actin (lane 2), a monoclonal anti-sea urchin paramyosin antibody (lane 3) or polyclonal anti-sea urchin paramyosin antibody (lane 4). The positions of molecular weight markers are shown on the left side. An asterisk marks the sea lily paramyosin band. A, actin; M, myosin heavy chain; PM, paramyosin.

Interaction of thin filaments with myosin

Effects of thin filaments on myosin ATPase activity were examined within a day after isolation. When sea lily thin filaments were added to rabbit myosin, the Mg^{2+}-ATPase activity of the myosin was remarkably increased at low ioninc strength as shown in Fig. 8A. The increased ATPase activity was scarcely inhibited by removing free Ca^{2+} with EGTA, a Ca^{2+}-chelating agent. The thin filament fractions used for ATPase assay did not have ATPase activity at all (data not shown). The ATPase assay was carried out repeatedly with different thin filament preparations and basically the same results were obtained. Two representative results are shown in Fig. 8A, (1) and (2). SDS-PAGE patterns of thin filaments, myosin, and the mixture of thin filaments and myosin used for ATPase assay are shown in Fig. 8B. The results of ATPase assay indicate that Ca^{2+}-regulatory proteins on the isolated sea lily thin filaments are negligible, as suggested by SDS-PAGE analysis of the thin filament proteins as shown in Fig. 3B, lane 1 and Fig. 8B, lane 1.

Detection of paramyosin in sea lily muscle lysates

Since a protein band that has roughly the same mobility as paramyosin of sea urchin and sea cucumber was detected in sea lily muscle extract (Fig. 3A), interaction of the sea lily protein with the antibody raised against sea urchin paramyosin was examined. Whole lysates of sea lily muscle were displayed on SDS-polyacrylamide gel and blotted on nitrocellulose membrane, and then reacted with anti-actin antibody, monoclonal or polyclonal antibody raised against sea urchin paramyosin. The lysates of sea urchin and sea cucumber muscles were similarly treated with the antibodies as references. As shown in Fig. 9A (lane 2) as

well as Fig. 6B, sea lily actin band showed positive reaction with anti- sarcomeric α-actin antibody. Sea urchin actin as well as sea cucumber actin was also recognized by the anti-actin antibody (Fig. 9B and C). A sea lily protein with a higher molecular weight (marked by an asterisk)—about 100 kDa as judged by the mobility in SDS-PAGE—interacted with both monoclonal and polyclonal anti-sea urchin paramyosin antibodies (Fig. 9A, lane 3 and 4). The size of this protein is quite similar to that of sea urchin and sea cucumber paramyosin, which also interacted with anti-paramyosin antibodies (Fig. 9B and C). The polyclonal anti-paramyosin antibody cross-reacted obviously with myosin heavy chain band of sea urchin and sea cucumber but this antibody did not give positive staining in the region of myosin heavy chain of sea lily. This difference between sea lily and the other echinoderms may be due to the antigenic difference in myosin heavy chain or that relative proportion of myosin in sea lily muscle proteins is much lower than that in sea urchin and sea cucumber. The results of western blotting strongly suggest that paramyosin exist in sea lily muscle, as in the muscle of the other echinoderms.

DISCUSSION

In this study, we examined muscle proteins of sea lily by a methodology of comparative biochemistry under the situation without molecular and genomic information. An advantage using biochemical methods is that functional properties of proteins can be clarified. We were able to isolate native thin filaments directly from the muscle tissue of sea lily, although the amount of the muscle located between arm plates was quite small. In the isolated filaments, actin and tropomyosin were detected, as major components of the filaments, but troponin-like proteins were not detectable. Isolated filaments were used for functional assay as soon as possible after the isolation, because the isolated filaments might be unstable in vitro although filament constituents were not degraded during storage for several days at least as judged by the SDS-PAGE pattern. The sea lily thin filaments remarkably enhanced ATPase activity of rabbit myosin, but the activity was not affected by the presence or absence of Ca^{2+}. It has been reported that in the case of molluscan thin filaments, tropomyosin and troponin easily dissociate out of actin filaments at a low Mg^{2+} concentration, and therefore a higher Mg^{2+} concentration is to be included in the solution through the preparation procedure of thin filaments and also in the assay of Ca^{2+}-dependent regulation of actomyosin ATPase (Lehman, 1983). In our present study, different Mg^{2+} concentrations, 1 to 10 mM, were tested in the preparation procedure and also in the ATPase assay, but the constituents of thin filaments and Ca^{2+}-sensitivity of the ATPase activity of reconstituted actomyosin, a mixture of myosin and thin filaments, were not altered by changing Mg^{2+} concentration. Therefore, it is reasonable to conclude that the Ca^{2+}-sensitive factor, troponin or its homolog, is lacking in the thin filaments of sea lily as observed in sea urchin (Obinata et al., 1974) and sea cucumber muscles (Takito and Konishi, 1987). Notably, in the case of sea urchin, a sequence showing weak homology with troponin T has been reported (GenBank accession: XP_791139), but the recombinant protein made with this message in our laboratory did not show any troponin T-like activity (data not shown). In addition, there is a report describing troponin C-like protein in sea urchin, but it has not been clarified whether this protein is actually troponin C, a Ca^{2+}-binding component of troponin, or calmodulin, another Ca^{2+}-binding protein that is distributed in a variety of animals and plants as well (Carpenter et al., 1984; Muesing et al., 1984). Currently, there is no reliable information showing that troponin exists in sea urchin. Ca^{2+} sensitive factor(s) for regulation of sea lily muscle probably should be sought in thick filaments or myosin filaments as has been found for many invertebrate animals of protostomes (Lehman and Szent-Gyorgyi, 1975; Hooper et al., 2008).

Considering that troponin is localized in the thin actin filaments of the muscles of many invertebrates of protostomes and chordates, it is assumed that ancestor of deuterostomes must have had troponin in thin filaments and the troponin gene has been inherited to chordates. Troponin gene might have lost or suppressed during evolution from deuterostomes ancestor to echinoderms. A matter of particular interest is that whether troponin is present in the thin filaments of the muscle of hemichordates that are classed as members of Ambulacraria together with echinoderms (Freeman et al., 2012), but currently, no information is available as to muscle proteins of hemichordates.

Paramyosin is known as a protein that constitutes the core of thick myosin filaments in striated, obliquely striated, and smooth muscles of many invertebrate animals that are categorized in protostomes (Winkelman, 1976; Kantha et al., 1990). Among deuterostomes, paramyosin has been observed in the smooth muscles of echinoderms, sea urchin (Obinata et al., 1975) and sea cucumber (Winkelman, 1976). Chordate muscle, both smooth and striated, lacks paramyosin, although it was detected in the notochord of amphioxus, which shows muscle-like structures (Guthrie and Banks, 1969; Castellani-Ceresa and Lanzavecchia, 1982). Here, we demonstrated that the muscle of sea lily, which constitutes the most basal group of extant echinoderms, contains paramyosin, suggesting that paramyosin gene expression was initiated at the initial stage of the evolution to echinoderms or inherited from the ancestor of deuterostomes. Presence of paramyosin and the absence of troponin seem to be a common feature of echinoderm muscles. Considering these characteristics, echinoderm muscle is quite different from the muscles of chordates, but seems more like the muscles of protostomes, although both echinoderms and chordates are categorized in deuterostomes. In this respect, hemichordate muscle is again of interest, namely whether paramyosin exists in this muscle.

In this study, our analysis of sea lily muscle proteins remained limited, as the small size of the muscle limits the amount of muscle proteins that can be obtained. When genomic information in crinoids and related animals becomes available in future, expression and characteristics of crinoid muscle proteins may be more clearly elucidated by using a combination of the methods of molecular biology and biochemistry.

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