Characteristics of light chains of Chara myosin revealed by immunological investigation

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Abstract: Chara myosin is plant myosin responsible for cytoplasmic streaming and moves actin filaments at 60 µm/s, which is the fastest of all myosins examined. The neck of the myosin molecule has usually mechanical and regulatory roles. The neck of Chara myosin is supposed to bind six light chains, but, at present, we have no knowledge about them. We found Ca^{++}-calmodulin activated Chara myosin motility and its actin-activated ATPase, and actually bound with the Chara myosin heavy chain, indicating calmodulin might be one of candidates for Chara myosin light chains. Antibody against essential light chain from Physarum myosin, and antibodies against Chara calmodulin and chicken myosin light chain from lens membranes reacted with 20 kDa and 18 kDa polypeptides of Chara myosin preparation, respectively. Correspondingly, column purified Chara myosin had light chains of 20 kDa, and 18 kDa with the molar ratio of 0.7 and 2.5 to the heavy chain, respectively.

Keywords: Chara myosin, light chain, Chara calmodulin, calcium, motility, IQ motif

Introduction

In a long living cell of green algae, *Nitella* or *Chara*, the thin layer of the endoplasm is observable streaming very fast at a peed of ∼50 µm/s just beneath the cell wall by light microscopy. The motive force for this endoplasmic streaming is generated at the interface between cortical fibrils connecting chloroplast arrays and streaming endoplasm,[1)] and is postulated to be based on interaction between actin and myosin, in the similar way as muscle contraction.[2)] The streaming speed is, however, 10 times faster than the speed of muscle contraction. In the Characean cell, cortical fibrils were confirmed to be composed of actin filament bundles by observation of the arrowhead structure formed by binding with skeletal muscle heavy meromyosin.[3),4)] Myosin was thought to be contained in the streaming endoplasm, and putative Chara myosin was verified by observation under a dark field microscope that cortical fibrils moved actively on the glass surface when the cytoplasm was squeezed in an artificial medium.[3)] According to this finding, putative Chara myosin was purified from living cells by following the motile activity with the *in vitro* motility assay method using muscle actin filaments.[5),6)]

Investigation of the molecular structure has shown that Chara myosin is actually a plant myosin having the motor structure like animal myosin and belongs to class XI of the large myosin family and its structure is similar to class V myosin, that is, 41% identical.[7),8)] Chara myosin has the motor domain of the head with ATPase in the N-terminal region, followed by the neck consisting of six IQ motifs for binding of calmodulin or the calmodulin related protein as light chains, long α-helix to dimerize by forming the coiled-coil structure of the rod, and the globular tail in the C-terminal domain probably for binding with a cargo in order to transport it to its destination.

The neck of myosin molecule plays a vital role in cellular movement and acts as a lever arm which amplifies the small conformational change developed in the head by ATP hydrolysis and relatively

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Abbreviations: EDTA: ethylenediaminetetraacetic acid; DTT: dithiothreitol; TPCK: Tosyl phenylalanyl chloromethyl ketone; TAME: p-toluenesulfonyl-L-arginine methyl ester; Tris: tris(hydroxymethyl)aminomethane; BSA: bovine serum albumin; CBB: coomassie brilliant blue.

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advances the actin filament by one step.[9),10)] The neck structure of the heavy chain consists of long α-helix. Single and long α-helix itself would be too thin and too weak to produce large force for movement. Actually, the neck may be strengthened by binding with light chains.

The long α-helix of the neck has the particular amino acid sequence called the IQ motif which is the consensus sequence for calmodulin binding. Calmodulin was firstly found in brain,[11),12)] as a calcium binding activator of cyclic nucleotide phosphodiesterase, after discovery of troponin C as a key protein for Ca^{++}-regulation of muscle contraction.[13),14)] Calmodulin changes its conformation when it binds with the second messenger of Ca^{++} and forms the complex with the target protein having a particular Ca^{++}-dependent calmodulin binding motif in order to regulate numerous kinds of fundamental cellular function.[15)]

Calmodulin also binds to a particular amino acid sequence termed the IQ motif. The myosin molecule has the IQ motif sequence in the neck for binding the light chain,[16)] but the IQ motif is a calmodulin binding site in the Ca^{++}-independent manner.[17)] Therefore, myosin light chains usually do not bind Ca^{++}, when they bind with the heavy chain. It is interesting but unsolved question how and why the myosin head of the motor domain evolutionally acquired the neck by fusion with the IQ motif(s) of the calmodulin binding sequence.

Chara myosin has a long neck with six IQ motifs, like myosin V. Myosin V is thought to bind a vesicle at its tail and transport the vesicle by moving processively, step by step along an actin filament without dissociation.[18),19)] To walk along an actin filament in a long distance, the step size of walking myosin V should coincide with the length of a half pitch of the helical double stranded structure of the actin filament, 36 nm.[20),21)] This large step size requires the long neck of myosin consisting of six IQ motifs. Likewise, Chara myosin transports vesicles along cortical fibrils of actin filament bundles. For this reason, Chara myosin should be required to have the long neck consisting of six IQ motifs and move processively with the long step size.[22)]

Another important role of the myosin light chain is regulatory function. The activity of myosin is mostly regulated by Ca^{++} in various ways. Smooth muscle and non muscle myosin are activated, when their regulatory light chains are phosphorylated by myosin light chain kinase coupled with Ca^{++}-calmodulin.[23)] Although calmodulin binds Ca^{++} and the myosin light chain is calmodulin or the calmodulin related protein, the myosin light chain does not usually bind Ca^{++}. When the light chain binds with Ca^{++}, myosin I or myosin V loses its activity by dissociation of the light chain from the neck.[24),25)] Only exception is that the essential light chain of scallop myosin binds Ca^{++} and activates myosin ATPase.[26)]

In a Characean cell, the cytoplasm streams continuously, but suddenly ceases streaming by an external stimulus, such as touching, which evokes action potential at the cell membrane[27)] and gives rise to concomitant Ca^{++} entry into the cell.[28)] When the concentration of Ca^{++} in the endoplasm is increased from micromolar level to several ten micromolar, streaming of the endoplasm stops.[29)] On the other hand, with the conventional *in vitro* motility assay, fast sliding movement of F-actin on Chara myosin is insensitive to Ca^{++}, indicating that streaming cessation does not take place by direct binding of Ca^{++} with Chara myosin.[5)]

Protein phosphorylation triggered by Ca^{++} entry is suggested to cause streaming cessation by using the subcellular system.[30)] When the endoplasm is squeezed into the medium, vesicle movement is inhibited by adding the phosphatase inhibitor,[31)] and actually, Chara myosin treated with protein kinase C loses its motile activity.[31)] On the other hand, the ATPase and motility of smooth muscle and non-muscle myosin II are activated by phosphorylation through myosin light chain kinase activated by Ca^{++}-calmodulin binding. Myosins I and VI are also activated by phosphorylation of the heavy chain.[32),33)] Conversely, Chara myosin is inhibited by phosphorylation.[31)] It is unknown whether the phosphorylation site is the light chain or the heavy chain of Chara myosin.

Although light chains of myosin have important roles, but, at present, we have no knowledge about light chains of Chara myosin. Recombinant myosin Va can express its activity by binding calmodulin to all IQ motifs in the neck.[34)] The sequences of six IQ motifs in the Chara myosin heavy chain, however, are so unique that the individual light chains may be specific and most of them may not be replaced by cytoplasmic calmodulin.[35),36)]

Our present prime target is to obtain knowledge on Chara myosin light chains. We report here the following results. The motile activities and actin activated ATPase of Chara myosin were activated by Ca^{++}-calmodulin. Immunological examinations of partially purified Chara myosin showed that the

18 kDa polypeptide reacted with antibody against Chara calmodulin and monoclonal antibody against myosin light chain with molecular mass of 20 kDa from chicken lens membranes. The 20 kDa polypeptide in partially purified Chara myosin reacted with the antibody against the essential light chain of Physarum myosin II. Corresponding to these results, column-purified Chara myosin was revealed to consist of, at least, two kinds of light chains with molecular mass of 20 kDa and 18 kDa including the heavy chain of 240 kDa. Finally, we discuss some characteristics of Chara myosin light chains based on the IQ motifs to understand the characteristic properties of Chara myosin.

Materials and methods

Purification of Chara myosin. Muscle actin was purified from rabbit skeletal muscle as described.[37] Chara myosin was partially purified according to the method,[5] as described briefly in the followings. The cytoplasm of an internodal cell of *Chara corallina* (*Chara australis*) was squeezed into 100 µl of E-buffer (100 mM Trirs-HCl pH 8.0, 400 mM sucrose, 5 mM $MgCl_2$, 50 mM EGTA, 5 mM ATP, 1 mM DTT, 0.2 mg/ml leupeptin, 1 mM Pefabloc, 0.01 mg/ml pepstatin A, 1 mM NaN3, 0.02 mg/ml calpain inhibitorII, 150 µg/ml TAME, 150 µg/ml TPCK, 150 µg/ml N-a-bensoyl-L-arginine ethyl ester) and mixed at a volume ratio of 1 to 1. Aliquots of the mixture were collected and centrifuged at 100,000 g for 30 min. Proteins in the supernatant were precipitated by 38% saturated ammonium sulfate, and then resolved in Buffer B (50 mM HEPES pH 7.6, 2 mM $MgCl_2$, 2 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT, 0.2 mg/ml leupeptin, 1 mM Pefabloc, 2.5 µg/ml pepstatin A, 1 mM NaN_3, 5 µg/ml calpain inhibitorII, 150 µg/ml TAME, 150 µg/ml TPCK, 150 µg/ml N-a-bensoyl-L-arginine ethyl ester, 50% glycerol). In the absence of ATP, Chara myosin was prevented from dinaturation in the presence of 50% glycerol and could form the rigor complex with muscle F-actin. The rigor complex was precipitated by centrifugation at 200,000 g (Beckman ultracentrifuge, TLA 100.2 rotor, Beckman Fullerton, CA) and the precipitated Chara myosin complex was dissociated from F-actin in Buffer D (100 mM Trirs-HCl pH 8.0, 2 mM $MgCl_2$, 1 mM EGTA, 4 mM ATP, 1 mM DTT, 0.5 mM Pefabloc, 15 µg/ml TAME, 15 µg/ml TPCK, 15 µg/ml N-a-bensoyl-L-arginine ethyl ester) by adding 15 mM ATP. By immediate centrifugation at 200,000 g for 30 min Chara myosin was obtained in the supernatant (partially purified Chara myosin).

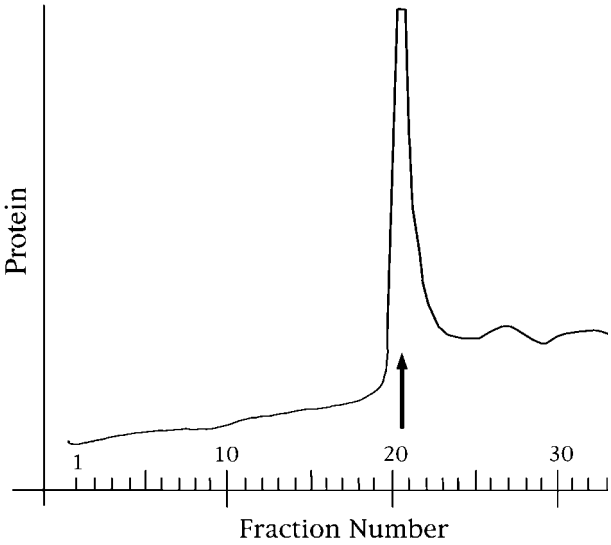

Fig. 1. Gel filtration of Chara myosin. Partially purified Chara myosin was concentrated and applied to superdex 200HR column. The fraction #20 was subjected to SDS-PAGE, the result is shown in Fig. 7. The fractions #21 and #22 were mixed and subjected to the measurement of the actin activated ATPase.

This was used for motility assay and other experiments in this work, unless otherwise described. All these processes were performed at 4 °C.

Purification of Chara myosin by gel filtration. In order to confirm the composition of Chara myosin, Chara myosin was extensively purified by gel filtration with superdex 200HR (Pharmacia, Uppsala Sweden). Partially purified Chara myosin was concentrated in a dialyzing bag by dehydration with solid sucrose. This concentrated Chara myosin solution was applied to the column equilibrated with the buffer containing 50 mM Tris-H-Cl (pH 8.0), 2 mM $MgCl_2$, 1 mM EGTA, 4 mM ATP, 1 mM DTT, 0.5 mM NaN_3 and 0.5 mM pefabloc and eluted with the same buffer under the condition commercially recommended. By using the peak fraction as shown in Fig. 1, the molecular composition (using the fraction No. 20) and the actin activated ATPase activity (using fractions No. 21 and 22) were examined.

Expression and purification of Chara calmodulin. Chara calmodulin clone (Accession number BAA94697) was the generous gift from A. Nakamura in Gunma University. It was ligated in the expression vector pET21d, and infected to BL21(DE3). In the cultivated solution of one liter, cells were collected by centrifugation and pellets were dissolved in 40 ml of 20 mM Tris-HCl (pH 8.0) and

10 mM EDTA. After disruption of cells by sonication, proteins containing in the supernatant of high speed centrifugation were collected by precipitation in 3% trichloro acetic acid. Precipitated specimen was dissolved in 40 ml of 20 mM Tris-HCl (pH 8.0) and 1mM EDTA, and fractionated with ammonium sulfate between 50% and 100% saturation.

Finally specimen was dissolved in 20 mM Tris-HCL (pH 8.0), 2 mM $MgCl_2$, 4 mM ATP and 1 mM DTT, and purified with gel-filtration using Superdex 200 HR equilibrated and eluted by the buffer with the same composition. The peak fraction was used for this course of the experiment. This sample had the typical characteristics of calmodulin of the calcium shift in SDS-PAGE.

For observation of the calcium shift, Chara calmodulin or Chara myosin was incubated in either 2 mM $CaCl_2$ or 2 mM EGTA for 10 min on ice before the sample buffer was added, and then SDS-PAGE was performed with 15% acrylamide gel. Chara calmodulin showed Ca^{++} shift very similar to that of bovine calmodulin.

Polyclonal antibody against Chara calmodulin. Chara calmodulin purified by gelfiltration was concentrated in a dialyzing bag with polyethylene glycol (MW 3000, Nakarai, Kyoto) from 1 mg/ml to about 10 mg/ml. The specimen was mixed with Titer Max gold (TiterMax, Inc, Norcross, Georgia U.S.A.) and 100 µl (about 0.5 mg of Chara calmodulin) of this mixture was injected to a mouse. After four weeks of injection blood was collected. The obtained serum reacted specifically with Chara calmodulin, but it did not react with bovine calmodulin and regulatory light chain of smooth muscle myosin from chicken gizzard.

Overlay assay. Partially purified Chara myosin was subjected to SDS-PAGE and transferred onto membrane, Immobilon-P (Millipore, Billerica, MA U.S.A.). Membrane was blocked with 10% BSA for more than 2 hours and then it was divided into five pieces. One piece was incubated with 1 mg/ml Chara calmodulin in a buffer containing Tris-HCl (pH 8.0) and 2 mM Ca^{++} at 4 °C overnight and the second one was in the presence of 2 mM EGTA instead of Ca^{++}. After washing membrane with the buffer without calmodulin, remaining proteins were fixed with 0.2% glutaraldehyde in 25 mM phosphate buffer at pH 7.0.

After washing extensively, membrane was treated with antibody against Chara calmodulin for 30 min by 1/1000 dilution of serum containing antibody against Chara calmodulin and reaction was detected by using Vectastain ABC-kit (Vector Laboratories, Inc. Burlingame, CA U.S.A.).

Immunological assay. Chara myosin purified by F-actin binding was concentrated by microcon 30 (Millipore, Billerica, MA U.S.A.) to about 1/8 volumes and subjected to SDS-PAGE with 18% acrylamide gel. Polypeptides separated in the gel were transferred to PVDF membrane under the condition of 1.5 mA/cm^2 for 30 min. The membrane was treated with 10% BSA overnight to block the surface. Polyclonal antibodies against essential light chain of Physarum polysephalum myosin II (generous gift from A. Nakamura in Gunma University, Japan) and against Chara calmodulin and monoclonal antibody against myosin light chain (20 kDa) from chicken lens membrans (Sigma-Aldrich, St.Louis, MD U.S.A., product No. M4401), were used for investigation of Chara myosin light chains. Monoclonal andibody (M4401) was reported to react best with regulatory light chain of chicken gizzard myosin (Sigma-Aldrich). The primary antibody was reacted with the membrane at 1/2000 dilution for 30 min. The reaction was detected by using the ECL kit (Phermacia, Uppsala Sweden) for polyclonal antibodies, and by using ABC-kit for antibody against regulatory light chain according to the commercially recommended methods.

***In vitro* motility assay.** The sliding velocity of F-actin on Chara myosin was measured with the *in vitro* motility assay method as described.[5] Movement was observed in the standard assay buffer (STD buffer) containing 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.2 mM EGTA, and the oxygen scavenger system (0.22 mg/ml glucose oxidase, 4.5 mg/ml glucose, and 0.36 mg/ml catalase). After the Chara myosin solution was infused into the flow chamber, myosin was allowed to attach onto the glass surface for 30 min on ice. Chara myosin can bind to the glass surfaces of coverslip and slideglass without any treatment. Actin filaments labeled with rhodamine-phalloidin in STD buffer was introduced into the flow chamber. For observation of the effect of Ca^{++} and/or calmodulin on motility, 0.1 mM Ca^{++} and/or 0.1 mg/ml bovine calmodulin (Sigma-Aldrich, St.Louis, MD U.S.A.) was added in STD buffer. Observations and records were performed by using a video microscope equipped with epifluorescence microscope (Olympus, Tokyo Japan) and a SIT camera (C2400, Hamamatsu photonics, Hamamatsu Japan).

ATPase assay. The actin activated Chara myosin ATPase was assayed in STD buffer without

the oxygen scavenger system in the presence of 0.4 µg/ml Chara myosin and 0.5 mg/ml skeletal muscle F-actin, by measuring inorganic phosphate liberated with the malachite green method.[38] Purified Chara myosin used was the mixture of peak fractions No. 21 and 22 of gel filtration.

Results

Effect of calmodulin on motility and actin activated ATPase of Chara myosin. In spite of the inhibitory effect of Ca^{++} on cytoplasmic streaming in a living cell, in the *in vitro* motility assay, Ca^{++} seemed to have no effect on Chara myosin motility, as shown in Fig. 2A. Motility was firstly assayed in the standard assay medium (STD) for every assay chamber (the gray column), and then examined by perfusion of the medium with the different condition indicated for testing (the black column). In the presence of Ca^{++}, the sliding velocity (13.3 µm/s on average) was the same as that in STD without Ca^{++} (12.3 µm/s), within an experimental error. By adding calmodulin (CaM), however, the sliding velocity increased by 24%, and when both of Ca^{++} and calmodulin were added, the sliding velocity increased by 35% (16.3 µm/s). This Ca^{++}-calmodulin effect was reversible since the velocity slowed down to the initial level when the standard buffer was infused to the assay chamber in order to wash out Ca^{++} and calmodulin (the shaded column in Fig. 2A). These results indicated that calmodulin could bind to the Chara myosin heavy chain and augment its motility in the presence of Ca^{++}.

Movement of actin filaments is produced through transduction of energy liberated by ATP hydrolysis in the interaction between actin and myosin. So that, to certify the correlation between motility and the actin activated ATPase activity of myosin, the ATPase activities of Chara myosin were measured in the same medium conditions as those that motilities were observed. To remove contaminated highly active ATPase(s), partially purified Chara myosin was extensively purified with gel filtration. The actin activated ATPase activities in the presence of Ca^{++}, and calmodulin, slightly increased by 42% and 24%, respectively. By adding both Ca^{++} and calmodulin, the actin activated ATPase activity was significantly increased by 91% (Fig. 2B). These results about Ca^{++} and calmodulin effects on actin activated Chara myosin ATPase were in good agreement with those on Chara myosin motility as described above.

Binding of calmodulin to the Chara myosin heavy chain. As Chara myosin motility and ATPase were activated by Ca^{++} and calmodulin, it was suggested that Chara myosin could bind calmodulin. To test this possibility, we performed the overlay assay as shown in Fig. 3. After SDS-PAGE of Chara myosin, polypeptides were transferred to PVDF membrane. Membrane was divided into 5

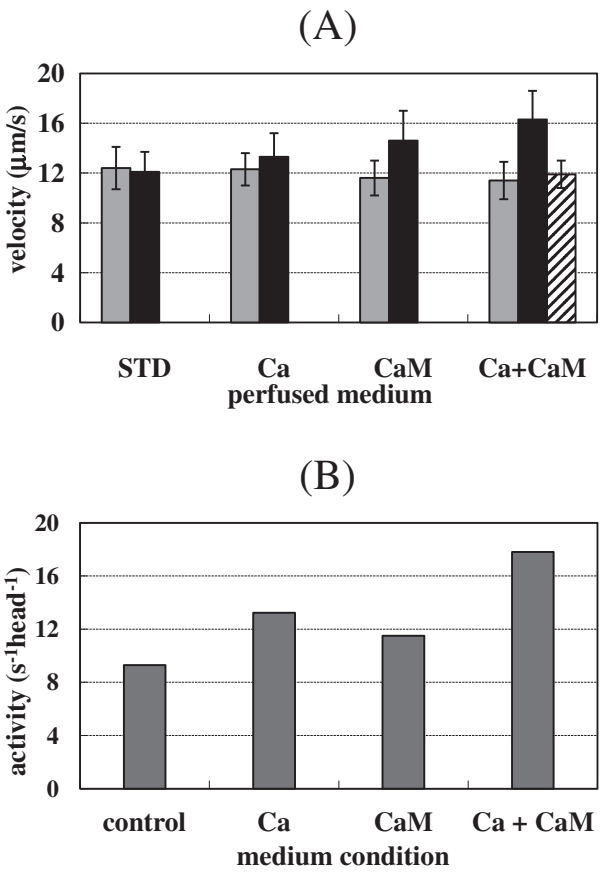

Fig. 2. Activation of Chara myosin motility by Ca^{++}-calmodulin. (A) Activation of motility. Sliding velocities were measured by the *in vitro* motility assay. Firstly, the standard buffer (STD) containing F-actin was infused (gray columns), and then to the same flow cell, the buffer STD containing Ca^{++} (Ca) and/or calmodulin (Ca+CaM, or CaM) was infused (black columns). Calcium did not seem to activate motility, because the change was within an experimental error. Addition of calmodulin, and both calmodulin and Ca^{++} activated motility by 24% and 35%, respectively. This effect of Ca^{++}-calmodulin was reversible as shown by recovery to the initial level by perfusion of the buffer STD (the shaded column). (B) Actin activated ATPase of Chara myosin. The actin activated Chara myosin ATPase activities were measured in the same buffer condition used for the motility assay, but the oxygen scavenger system was removed. The Ca^{++} and calmodulin on ATPase showed the effects very similar to that on motility. The activations of Ca^{++}, calmodulin and Ca^{++}-calmodulin were 42, 24, and 91%, respectively.

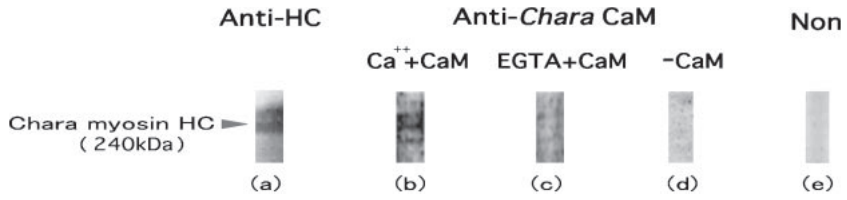

Fig. 3. Binding of Chara calmodulin with the Chara myosin heavy chain. Partially purified Chara myosin was transferred to membrane after SDS-PAGE. The membrane blocked with BSA was divided into 5 pieces. The one was treated with (a) antibody against Chara myosin heavy chain, the other two, (b) and (c), were treated with antibody against Chara calmodulin after treated with Chara calmodulin at 1 mg/ml in the presence of 2 mM $CaCl_2$ and 2 mM EGTA, respectively. The fourth one was treated with (d) antibody against Chara calmodulin without treatment with Chara calmodulin, and the fifth (e) with non-immunized serum.

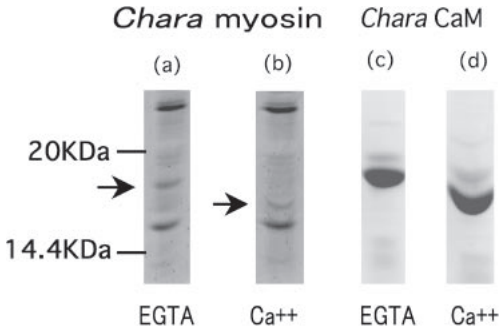

Fig. 4. The mobility shift of the 18 kDa polypeptide by Ca^{++}. Chara myosin was found to have 18 kDa polypeptide which showed mobility shift in the presence of 1 mM EGTA (a) and 1 mM $CaCl_2$ (b). This shift was very similar to the Ca^{++}-shift of Chara calmodulin (c) and (d). Proteins were stained with CBB.

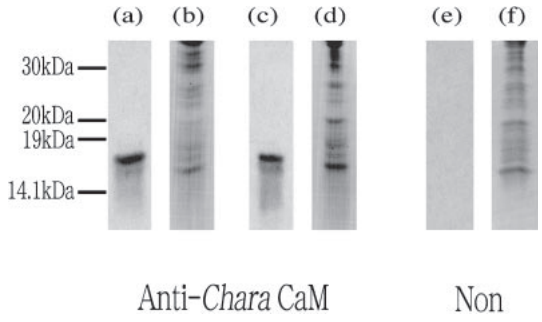

Fig. 5. Antibody against Chara calmodulin reacted with the 18 kDa polypeptide bound to Chara myosin. Chara myosin dissociated from F-actin contained the 18 kDa band reacted with antibody against Chara calmodulin (a, b). To avoid contamination of cytoplasmic calmodulin, partially purified Chara myosin solution was treated with microcon 50 for removing free calmodulin. Chara myosin still contained the 18 kDa band reacted with antibody against Chara calmodulin (c, d). The lanes b, d and f: CBB stained, the lane a and c: reacted with antibody against Chara calmodulin, the lane e: treated with non-immunized serum.

pieces. The position of the Chara myosin heavy chain was confirmed by the antibody against the Chara myosin heavy chain (lane a). One piece was treated with Chara calmodulin in the presence of Ca^{++} (b), the other was treated with Chara calmodulin but in the absence of Ca^{++} (c), or without calmodulin (d). Binding of Chara calmodulin to the Chara myosin heavy chain was confirmed immunologically by using antibody against Chara calmodulin after fixiation. Only in the presence of Ca^{++}, calmodulin could bind with the Chara myosin heavy chain. This result agreed well with the results on the activation of motility and ATPase of Chara myosin in the presence of Ca^{++} and calmodulin.

Ca^{++}-shift of Chara calmodulin and 18 kDa protein of Chara myosin. Calmodulin exhibits the mobility shift in SDS-PAGE by Ca^{++}, so called the Ca^{++}-shift. If Chara myosin had calmodulin as its light chain(s), there would reside the band showing the Ca^{++}-shift in SDS-PAGE of Chara myosin. As shown in Fig. 4, Chara myosin had the 18 kDa polypeptide, which showed the Ca^{++}-shift as shown in (a) and (b). Molecular mass and the extent of the mobility shift were very similar to those of Chara calmodulin as shown in (c) and (d). This suggests that calmodulin must be one of the candidates of the Chara myosin light chain.

Preparation of Chara myosin used here, however, was partially purified by F-actin binding and then dissociation in the presence of ATP. Therefore, the result obtained could not rule out the possibility if the specimen was contaminated by cytoplasmic calmodulin. Consequently, free calmodulin was removed by passing the specimen through microcon 50 (Pharmacia, Uppsala Sweden). Chara myosin preparation still contained 18 kDa polypeptide reacting with antibody against Chara calmodulin at the similar extent (Fig. 5(c) and (d)), when compared with the preparation before treatment with microcon (Fig. 5(a) and (b)). This suggested that calmodulin could be the light chain of Chara myosin.

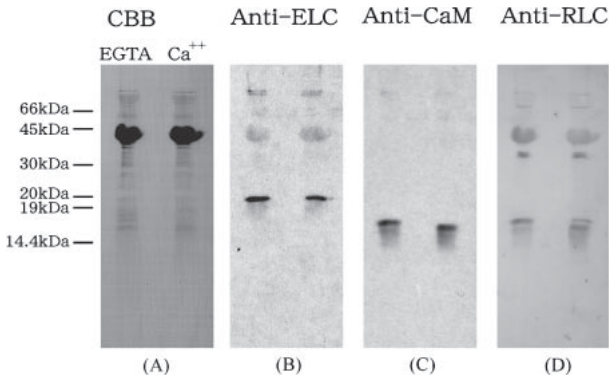

Fig. 6. Searching the light chain of Chara myosin by the immunological method. In Chara myosin preparation the polypeptide with 20 kDa molecular mass was strongly reacted with antibody against the essential light chain of myosin from Physarum polysephlum (Anti-ELC), and the 18 kDa polypeptide was reacted with monoclonal antibody against myosin light chain of 20 kDa from chicken lens membranes (Anti-RLC), and also antibody against Chara calmodulin (Anti-CaM). In each panel, the left and right lanes show the specimen eluted in the presence of 1 mM EGTA and 1 mM $CaCl_2$, respectively. The 18 kDa band reacted with anti-CaM (C) and also the band reacted with anti-RLC(D) clearly exhibited the calcium shift. Thus, the 20 kDa and 18 kDa polypeptides were strongly suggested to be the light chains of Chara myosin. The membrane was stained with CBB (A).

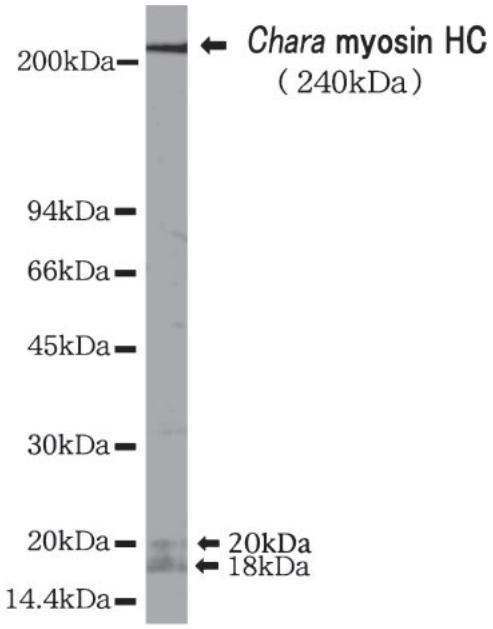

Fig. 7. Column purified Chara myosin. Partially purified Chara myosin by F-actin association and dissociation was further purified with gel filtration after the concentration, as shown in Fig. 1. The peak fraction (No. 20) was concentrated with microcon 30 to 1/13 fold of the initial volume and subjected to SDS-PAGE. The gel was stained with coomassie brilliant blue. The molecular weights of three bands were 240 kDa, 20 kDa and 18 kDa, and the latter two might be those reacted with antibodies against the light chain of Physarum myosin and Chara calmodulin. The stoichiometry was roughly estimated to be, 240 kDa of the heavy chain: 20 kDa : 18 kDa = 1 : 0.7 : 2.5. The 18 kDa band seemed to have the faint upper band, suggesting that there would be another light chain with the molecular mass very close to 18 kDa.

Light chains of Chara myosin. The light chain binds the specific sequence of the IQ motif in the neck region of the myosin heavy chain. Chara myosin has six IQ motifs, most of which are very unique if compared with the typical IQ motif for calmodulin binding.[36] We further investigated the possibility whether Chara myosin would have the light chain other than calmodulin by using antibodies against light chains of the other type of myosin. Chara myosin contained the 18 kDa polypeptide reacting with monoclonal antibody against myosin light chain from chicken lens membranes (Fig. 6D). This polypeptide showed calcium shift in SDS-PAGE and had a very similar molecular mass to that reacted with antibody against Chara calmodulin (Fig. 6C). In addition to this band, Chara myosin was suggested to have another light chain, because the polypeptide with 20 kDa molecular mass strongly reacted with antibody against the essential light chain of Physarum myosin II (Fig. 6B).

Purification of Chara myosin. As described above, we had the evidence that Chara myosin preparation had light chains of 18 kDa, and 20 kDa polypeptides reacting with antibodies against Chara calmodulin and chicken myosin light chain, and antibody against the essential light chain of Physarum myosin II, respectively. We tried to identify that Chara myosin really bound the light chains of 20 kDa and 18 kDa polypeptides. After F-actin binding and then dissociation, partially purified Chara myosin was further purified through gel filtration, as shown in Fig. 1. Purified Chara myosin was revealed to consist of the 240 kDa heavy chin, 18 kDa and 20 kDa light chains, by SDS-PAGE (Fig. 7). Molar ratios of light chains 18 kD and 20 kD to the heavy chain were roughly estimated to be 2.5 and 0.7, respectively. The band of 18 kDa contained a faint upper band and seemed to be composed of two polypeptides with molecular mass of 18.2 kDa and 17.8 kDa.

Discussion

Cytoplasmic streaming in the Characean cell ceases suddenly by external stimulus concomitant with Ca^{++} entry to the cytoplasm. The *in vitro* motility assay of Chara myosin, however, showed

that actin filaments moved continuously without changing the sliding velocity when Ca^{++} was added.[5] However, we have reported here that *in vitro* motility and the actin activated ATPase of Chara myosin are activated by adding calmodulin and Ca^{++} (Fig. 2).

Chara myosin is highly homologous to Myosin V, 41% identical to myosin V Dilute.[7),8)] Myosin V lost its motility even in the absence of Ca^{++} after assayed in the buffer containing Ca^{++} at pCa = 4, whereas full recovery of motility required adding calmodulin in the absence of Ca^{++}.[24)] Plant myosin having 170 kDa molecular mass purified from lily pollen tube moved F-actin at 6 µm/s in the absence of Ca^{++}. This myosin also lost its motility in the presence of Ca^{++}, and its motility recovered by adding calmodulin,[39)] like myosin V. Myosin from Tobacco BY-2 cell was also inhibited its motility by Ca^{++}.[40)] Thus, myosin V and some plant myosins lost their motility in the presence of Ca^{++}. This phenomenon is understood by the idea that in the presence of Ca^{++}, calmodulin light chain dissociates from the myosin heavy chain.[24),41)] Unlike myosin V, Chara myosin motility is not affected by Ca^{++}, and the heavy chain of Chara myosin is expected to have the site for binding of Ca^{++}-calmodulin as shown in Fig. 3, in good agreement with activation of motility and actin-activated ATPase by Ca^{++}-calmodulin (Fig. 2). How can we consistently understand these properties of Chara myosin different from myosin V?

Calmodulin plays an important role in signal transduction. Calmodulin bound with Ca^{++} associates with the target protein and regulates various kinds of cell function. Calmodulin binding region of the target protein is characterized by a basic amphipathic α-helix consisting of about 20 amino acid residues.[42)] To transmit the Ca^{++} signal to the target protein, Ca^{++}-calmodulin binds the site with the specific sequence termed 1-(5)-8-14, or 1-5-10 in this α-helix of the target.[43)] The number indicates the position of the hydrophobic amino acid residue in the calmodulin binding site. In contrast, the IQ motif, for example found in the myosin neck region, is the sequence specific to Ca^{++}-independent binding of calmodulin. Among myosins belonging to various classes, however, the sequence type 1-(5)-8-14 for Ca^{++}-dependent calmodulin binding is found in a few myosins, such as rat myr4, fruit fly NinaC, and myosin from *A. thaliana*[43)] as shown in Table 1.

Myr4 is class I myosin from rat brains, and the Ca^{++}-dependent calmodulin binding IQ sequence is located in the tail region, but not in the neck region of this myosin.[44)] Nina C is myosin found in the photoreceptor cell of a fruit fly *Drosophila melanogaster*, and has a role in phototransduction and calmodulin distribution. In IQ motifs of NinaC, the Ca^{++}-dependent calmodulin binding sequence 1-(5)-8-14 resides as shown in Table 1. Mutation at the site of, either long or short NinaC myosin, resulted in defective photo response. Binding of Ca^{++}-calmodulin to these sites of Nina C is required *in vivo* for termination of phototransduction.[45)]

Table 1. Ca^{++}-dependent calmodulin binding sequence in myosin

Myosin		hydrophobic residue ↓ ↓ ↓ ↓
Chara (IQ4)	817	AIR IQSAIRSLAARRVL CVL
A.thaliana	827	VIH LQSAVRGWLARKHF NSM
Drosophila NinaC*	1040	VIK VQSMMRALLARKKV KGG
Drosophila NinaC**	1076	ASK IQKAFRGFRDRVRL PPL
Rat myr4	856	NVL FSCHVRKVNRFSKV EDR

The Ca^{++}-dependent calmodulin binding sequence, the type 1-5-8-14, found in myosin, particularly in IQ motif in the neck except myr4. The sequence of myr4 in the table is located in the tail. The hydrophobic residues at the position 1, 5, 8 and 14 are pointed by arrows and indicated by blue letters.
* The amino acid residue at the 5 position can be sometimes replaced by the residue other than the hydrophobic one.
* NinaC long protein [*Drosophila melanogaster*]
** NinaC short protein [*Drosophila melanogaster*]

Plant myosin from *A. thaliana* also has the Ca^{++}-dependent calmodulin binding sequence in the IQ motif of the neck (Table 1). This fact indicates that *A. thaliana* myosin may contain the Ca^{++} binding light chain, but the biological role of this light chain is unknown. Chara myosin belongs to class XI, the same class as *A. thaliana* myosin. Actually forth IQ sequence, IQ4, of Chara myosin has such characteristics of the Ca^{++}-dependent calmodulin binding sequence, that is, ^{820} **IQSAIRSLAARRVL** (Table 1). The characteristic hydrophobic residues in the sequence are expressed in bold.

The forth IQ sequence, IQ4, of Chara myosin is actually the Ca^{++}-independent binding sequence for calmodulin, but at the same time, it has the property of the sequence for Ca^{++}-dependent calmodulin binding. The Ca^{++}-sensitive light chain bound with IQ4 might probably dissociate from the heavy chain during extraction and purification in the extraction buffer containing a high concentration of EGTA. Then, the neck of Chara myosin might become

defective. With this idea, we can understand the phenomenon in part that the sliding velocity with the *in vitro* motility assay, 15–30 μm/s, is much slower than the velocity of the endoplasmic streaming, 30–50 μm/s. By adding calmodulin and Ca^{++}, motility and the ATPase activity of Chara myosin were recovered, but partially (Fig. 2). This suggested that added calmodulin in the presence of Ca^{++} could bind to IQ4 but it would be insufficient substitution for the Chara myosin light chain dissociated, provably because of requirement of the light chain specific for IQ4.

Myosin V has six IQ motifs in the neck and its light chains are calmodulin. Unlike IQ4 of Chara myosin, these motifs of myosin V do not have the Ca^{++}-dependent calmodulin binding motif. In the presence of Ca^{++} but absence of calmodulin in the medium, calmodulin bound with the neck would dissociate from the heavy chain when it binds Ca^{++}. Therefore, calmodulin should be added to recover the myosin activity. The light chains of Chara myosin would be unique for the respective particular IQ sequences in the neck. They could not be able to bind Ca^{++} except the light chain bound with IQ4. If the affinity of the light chain to the heavy chain is very high and dissociation constants of the light chains are nanomolar level, they can keep binding to the neck during preparation. Therefore, Chara myosin purified from the cell moves actin filament and does not change the sliding velocity by adding Ca^{++} in the assay medium.

Myosin V mutated by adding two alanine residues in between IQ3 and IQ4 exhibited slow sliding movement and the short step size, roughly corresponding to myosin having three IQ motifs.[46] This result will support our understanding that the siding velocity of F-actin on extracted or purified Chara myosin, which might have no light chain bound with IQ4, is about a half of the velocity of intact myosin or of the speed of endoplasmic streaming.

We previously reported that all IQ motif sequences of Chara myosin have very low probability for calmodulin binding, indicating that each IQ motif of Chara myosin would not bind calmodulin but bind the light chain specific to the individual IQ motif with calmodulin related sequence.[36] To examine some properties of Chara myosin light chains, we performed immunological investigation. We have reported here that Chara myosin has light chains of the 20 kDa polypeptide reacting with the antibody against the essential light chain of *Physarum polycephalum* myosin II, and the 18 kDa polypeptide reacting with the antibody against Chara calmodulin and also the 18 kDa polypeptide reacting with the antibody against chicken myosin light chain of 20 kDa (Fig. 6). In accordance with these results, it was really revealed that column purified Chara myosin consisted of the heavy chain with 240 kDa molecular mass and light chains with 20 kDa and 18 kDa molecular mass (Fig. 7), and molar ratios of these light chains to the heavy chain were roughly 0.7 and 2.5. The 18 kDa band seems to have a faint upper band, suggesting that Chara myosin has at least three kinds of the light chain consisting of 20 kDa, 18.2 kDa and 17.8 kDa polypeptides. The antibodies against calmodulin and against chicken myosin light chain of 20 kDa reacted with the 18kDa polypeptide in Chara myosin preparation, but they might react with the different polypeptide with similar molecular mass.

Why does Chara myosin need such a variety of the light chain? One of the reasons for this may be regulation. The light chain bound with IQ4 may receive Ca^{++} signal, but its physiological meaning is unknown, at present. As Chara myosin transports various kinds of the cargo, some light chain(s) will take the specific regulatory role for a particular vesicle. Phosphorylation of Chara myosin is implicated in sudden cessation of cytoplasmic streaming.[31] This phosphorylation site has not been investigated yet. In spite of sudden cessation of endoplasmic streaming upon mechanical stimulation, its recovery usually takes several minutes. This long term recovery process may include some processes, not only dephosphorylation of myosin, but also other process including large conformational change.

Myosin V takes the unique conformation of the folded form in the inhibited state, where the tail and the long rod bend to form the triangular shape of the molecule by interacting with light chains and the head.[47] In analogy to the inhibitory state of myosin V, if Chara myosin takes a folded form in an inhibitory state, Chara myosin may dissociate from the actin cable and also the cargo. To take the folded form, specific interaction between the light chains and the rod and also the tail may be important to stabilize the structure, and in case of Chara myosin several kinds of light chains may be required to stabilize this form. But the folded form of Chara myosin has not been confirmed yet.

As Chara myosin has a long neck and the high duty ratio, it probably move processively with a long step size of about a half pitch of the actin filament

along the actin cable.[22] If Chara myosin is not a processive motor, it dissociates from the cable in a second step and can not transport the cargo. If Chara myosin moves processively with a step size shorter than the half pitch of an actin filament, myosin rotates around the actin filament. As the result, moving myosin disrupts the actin cable, or myosin has to dissociate from the cable and can not transport the cargo. Thus, Chara myosin must be a processive myosin with a long step size, and moves at the speed of cytoplasmic streaming of 30–50 μm/s. To perform this high speed by a processive movement, particular coordination between neck flexibility and actin-myosin binding states will be important.

Loop 2 of myosin usually has many positively charged residues and is thought to interact with N-terminal negatively charged residues of actin. Loop 2 of Chara myosin is very short and has no net charge.[36] This interaction is closely related to weak binding of myosin to actin and coordinate with each other for long run of processive movement.[48] The interface between actin and loop 2 of Chara myosin may differ from those of other class of myosins. Unique sequences of IQ motifs and specific light chains will produce the particular flexibility of the neck, and this, together with the short loop 2, will secure processive movement of the fastest Chara myosin.

At present, plant myosin was purified only from Characean cells or tobacco BY-2 cells and has extensively been examined. Further investigations with these cell-purified myosins provide us with useful evidences to understand physiology including regulatory mechanism on cytoplasmic streaming in the living plant cell.

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References

1) Kamiya, N. and Kuroda, K. (1956) Velocity distribution of the protoplasmic streaming in *Nitella* cells. Bot. Mag. (Tokyo) **69**, 544–554.
2) Higashi-Fujime, S. (1991) Reconstitution of active movement *in vitro* based on the actin-myosin interaction. Int. Rev. Cytol. **125**, 95–138.
3) Higashi-Fujime, S. (1980) Active movement in vitro of bundle of microfilaments isolated from *Nitella* cell. J. Cell Biol. **87**, 569–578.
4) Palevitz, B.A., Ash, J.F. and Hepler, P.K. (1974) Actin in the green alga, *Nitella*. Proc. Natl. Acad. Sci. U.S.A. **71**, 363–366.
5) Higashi-Fujime, S., Ishikawa, R., Iwasawa, H., Kagami, O., Kurimoto, E., Kohama, K. and Hozumi, T. (1995) The fastest actin-based motor protein from the green algae, *Chara*, and its distinct mode of interaction with actin. FEBS Lett. **375**, 151–154.
6) Yamamoto, K., Kikuyama, M., Sutoh-Yamamoto, N. and Kamitubo, E. (1994) Purification of actin based motor protein from *Chara corallina*. Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci. **70**, 175–180.
7) Morimatsu, M., Nakamura, A., Sumiyoshi, H., Sakaba, N., Taniguchi, H., Kohama, K. and Higashi-Fujime, S. (2000) The molecular structure of the fastest myosin from green algae, *Chara*. Biochem. Biophys. Res. Commun. **270**, 147–152.
8) Kashiyama, T., Kimura, N., Mimura, T. and Yamamoto, K. (2000) Cloning and characterization of a myosin from characean alga, the fastest motor protein in the world. J. Biochem. **127**, 1065–1070.
9) Uyeda, T.Q., Kron, S.J. and Spudich, J.A. (1990) Myosin step size. Estimation from slow sliding movement of actin over low densities of heavy meromyosin. J. Mol. Biol. **214**, 699–710.
10) Howard, J. (1997) Molecular motors: structural adaptations to cellular functions. Nature **389**, 561–567.
11) Kakiuchi, S. and Yamazaki, R. (1970) Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain studies on cyclic 3′,5′-nucleotide phosphodiesterase (3). Biochem. Biophys. Res. Commun. **41**, 1104–1110.
12) Cheung, W.Y. (1971) Cyclic 3′,5′-nucleotide phosphodiesterase. Effect of divalent cations. Biochim. Biophys. Acta **242**, 395–409.
13) Ebashi, S. (1963) Third component participating in the superprecipitation of 'natural actomyosin'. Nature **200**, 1010.
14) Ebashi, S., Ebashi, F. and Kodama, A. (1967) Troponin as the Ca^{++}-receptive protein in the contractile system. J. Biochem. **62**, 137–138.
15) Schaub, M.C. and Heizmann, C.W. (2008) Calcium, troponin, calmodulin, S100 proteins: from myocardial basics to new therapeutic strategies. Biochem. Biophys. Res. Commun. **369**, 247–264.
16) Cheney, R.E. and Mooseker, M.S. (1992) Unconventional myosins. Curr. Opin. Cell Biol. **4**, 27–35.
17) Rhoads, A.R. and Friedberg, F. (1997) Sequence motifs for calmodulin recognition. FASEB J. **11**, 331–340.
18) Mehta, A.D., Rock, R.S., Rief, M., Spudich, J.A., Mooseker, M.S. and Cheney, R.E. (1999) Myosin-V is a processive actin-based motor. Nature **400**, 590–593.
19) Walker, M.L., Burgess, S.A., Sellers, J.R., Wang, F., Hammer, J.A. III., Trinick, J. and Knight, P.J. (2000) Two-headed binding of a processive myosin to F-actin. Nature **405**, 804–807.
20) Rief, M., Rock, R.S., Mehta, A.D., Mooseker, M.S., Cheney, R.E. and Spudich, J.A. (2000) Myosin-V stepping kinetics: A molecular model for proces-

sivity. Proc. Natl. Acad. Sci. U.S.A. **97**, 9482–9486.
21) Veigel, C., Wang, F., Bartoo, M.L., Sellers, J.R. and Molloy, J.E. (2002) The gated gait of the processive molecular motor, myosin V. Nat. Cell Biol. **4**, 59–65.
22) Sumiyoshi, H., Ooguchi, M., Ooi, A., Okagaki, T. and Higashi-Fujime, S. (2007) Insight into the mechanism of fast movement of myosin from *Chara corallina*. Cell Motil. Cytoskeleton **64**, 131–142.
23) Sellers, J.R. (1985) Mechanism of the phosphorylation-dependent regulation of smooth muscle heavy meromyosin. J. Biol. Chem. **260**, 15815–15819.
24) Cheney, R.E., O'Shea, M.K., Heuser, J.E., Coelho, M.V., Wolenski, J.S., Espreafico, E.M., Forscher, P., Larson, R.E. and Mooseker, M.S. (1993) Brain myosin-V is a two-headed unconventional myosin with motor activity. Cell **75**, 13–23.
25) Williams, R. and Coluccio, L.M. (1994) Novel 130-kDa rat liver myosin-1 will translocate actin filaments. Cell Motil. Cytoskeleton **27**, 41–48.
26) Szent-Györgyi, A.G., Szentkiralyi, E.M. and Kendrick-Jonas, J. (1973) The light chains of scallop myosin as regulatory subunits. J. Mol. Biol. **74**, 179–203.
27) Hayama, T., Shimmen, T. and Tazawa, M. (1979) Participation of Ca^{2+} in cessation of cytoplasmic streaming induced by membrane excitation in *Characeae* internodal cells. Protoplasma **99**, 305–321.
28) Tominaga, Y., Shimmen, T. and Tazawa, M. (1983) Control of cytoplasmic streaming by extracellular Ca^{2+} in permeabilized *Nitella* cells. Protoplasma **116**, 75–77.
29) Williamson, R.E. and Ashley, C.C. (1982) Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature **296**, 647–650.
30) Tominaga, Y., Wayne, R., Tung, H.Y.L. and Tazawa, M. (1987) Phosphorylation-dephosphorylation is involved in Ca^{2+}-controlled cytoplasmic streaming of Characean cells. Protoplasma **136**, 161–169.
31) Morimatsu, M., Hasegawa, S. and Higashi-Fujime, S. (2002) Protein phosphorylation regulates actomyosin-driven vesicle movement in cell extracts isolated from the green algae, *Chara corallina*. Cell Motil. Cytoskeleton **53**, 66–76.
32) Maruta, H. and Korn, E.D. (1977) Acanthamoeba cofactor protein is a heavy chain kinase required for actin activation of the Mg^{2+}-ATPase activity of Acanthamoeba myosin I. J. Biol. Chem. **252**, 8329–8332.
33) Buss, F., Kendrick-Jones, J., Lionne, C., Knight, A.E., Côté, G.P. and Paul Luzio, J. (1998) The localization of myosin VI at the golgi complex and leading edge of fibroblasts and its phosphorylation and recruitment into membrane ruffles of A431 cells after growth factor stimulation. J. Cell Biol. **143**, 1535–1545.
34) Baker, J.E., Krementsova, E.B., Kennedy, G.G., Armstrong, A., Trybus, K.M. and Warshaw, D.M. (2004) Myosin V processivity: Multiple kinetic pathways for head-to-head coordination. Proc. Natl. Acad. Sci. U.S.A. **101**, 5542–5546.
35) Yap, K.L., Kim, J., Truong, K., Sherman, M., Yuan, T. and Ikura, M. (2000) Calmodulin target database. J. Struct. Funct. Genomics **1**, 8–14.
36) Higashi-Fujime, S. and Nakamura, A. (2009) Cell and molecular biology of the fastest myosins. Int. Rev. Cell Mol. Biol. **276**, 301–347.
37) Higashi-Fujime, S. (1983) Phosphorylation of myosin light chain modulates the *in vitro* movement of fibrils composed of actin and myosin filaments from skeletal muscle. J. Biochem. **94**, 1539–1545.
38) Kodama, T., Fukui, K. and Kometani, K. (1986) The initial phosphate burst in ATP hydrolysis by myosin and subfragment-1 as studied by a modified malachite green method for determination of inorganic phosphate. J. Biochem. **99**, 1465–1472.
39) Yokota, E., Muto, S. and Shimmen, T. (1999) Inhibitory regulation of higher-plant myosin by Ca^{2+} ions. Plant Physiol. **119**, 231–240.
40) Yokota, E., Yukawa, C., Muto, S., Sonobe, S. and Shimmen, T. (1999) Biochemical and immunocytochemical characterization of two types of myosins in cultured tobacco bright yellow-2 cells. Plant Physiol. **121**, 525–534.
41) Krementsov, D.N., Krementsova, E.B. and Trybus, K.M. (2004) Myosin V: regulation by calcium, calmodulin, and the tail domain. J. Cell Biol. **164**, 877–886.
42) O'Neil, K.T. and DeGrado, W.F. (1990) How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices. Trends Biochem. Sci. **15**, 59–64.
43) Rhoads, A.R. and Friedberg, F. (1997) Sequence motifs for calmodulin recognition. FASEB J. **11**, 331–340.
44) Bähler, M., Kroschewski, R., Stöffler, H.E. and Behrmann, T. (1994) Rat myr 4 defines a novel subclass of myosin I: identification, distribution, localization, and mapping of calmodulin-binding sites with differential calcium sensitivity. J. Cell Biol. **126**, 375–389.
45) Porter, J.A., Minke, B. and Montell, C. (1995) Calmodulin binding to *Drosophila* NinaC required for termination of phototransduction. EMBO J. **14**, 4450–4459.
46) Sakamoto, T., Wang, F., Schmitz, S., Xu, Y., Xu, Q., Molloy, J.E., Veigel, C. and Sellers, J.R. (2003) Neck length and processivity of myosin V. J. Biol. Chem. **278**, 29201–29207.
47) Wang, F., Thirumurugan, K., Stafford, W.F., Hammer, J.A. 3rd., Knight, P.J. and Sellers, J.R. (2004) Regulated conformation of myosin V. J. Biol. Chem. **279**, 2333–2336.
48) Hodges, A.R., Krementsova, E.B. and Trybus, K.M. (2007) Engineering the processive run length of myosin V. J. Biol. Chem. **282**, 27192–27197.

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