Inhibitory effect of tropomyosin on Chara myosin motility

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(Communicated by Setsuro Ebashi, M.J.A., Oct. 12, 2000)

Abstract: Chara myosin is a plant myosin involved in cytoplasmic streaming of Characean cells and the fastest of all myosins observed so far with in vitro motility assay. Movement of F-actin on Chara myosin was completely inhibited by muscle tropomyosin. In concert with this, the actin activated Chara myosin ATPase was also completely inhibited by tropomyosin. The movement of the thin filament of F-actin with native tropomyosin, the complex of tropomyosin and troponin, was inhibited on Chara myosin and the actin activated ATPase of Chara myosin was also inhibited by native tropomyosin. With careful observations, Chara myosin occasionally moved actin filaments in the presence of native tropomyosin and Ca²⁺. This indicates that the effect of tropomyosin of the thin filament in the “ON” state is not exactly the same as that of tropomyosin alone on F-actin.

Key words: Chara myosin; tropomyosin; actin activated ATPase.

Introduction. Tropomyosin was first found by Bailey in skeletal muscle. It has a long rod shape composed of α-helical coiled coil and its function in muscle was not understood until native tropomyosin was discovered by Ebashi. Native tropomyosin, the complex of tropomyosin and troponin, binds to the groove of F-actin (thin filament) and inhibits muscle contraction in the absence of Ca²⁺. Binding of Ca²⁺ to troponin C causes relocation of tropomyosin on the F-actin surface, and thin filaments assume the “ON” state to interact with myosin.

In nonmuscle cells, there are several isoforms of tropomyosin, but no troponin. Function of tropomyosins in nonmuscle cells has been thought to strengthen the structure of microfilaments and regulates the mode of interaction of F-actin with various actin binding proteins, by which dynamic changes of cytoskeleton are induced in the process of cell signaling.

With the in vitro motility assay method, tropomyosin was found to augment myosin motility in vitro. Motility of smooth muscle myosin II is activated almost twice by tropomyosin and motility of skeletal muscle myosin II is also activated by 20 to 30% by tropomyosin or native tropomyosin.

Many myosin isoforms have been found and myosin family is now a large family consisting of 15 classes. Recently, we isolated plant myosin named Chara myosin from green algae, Chara australis (corallina). Chara myosin has unique properties. Firstly, Chara myosin moves F-actin at the strikingly high speed of 60 μm/s, that is ten times faster than the sliding velocity with muscle myosin II. Secondly, F-actin which is composed of actin cleaved by proteases at the position of 40-50 loop in subdomain 2, can slide on Chara myosin at the same velocity with intact F-actin, whereas movement of F-actin on skeletal myosin II is strongly inhibited by such cleavage.

Here, we report the third unique property of Chara myosin that tropomyosin and native tropomyosin can completely inhibit the movement of F-actin on Chara myosin in vitro.

Materials and methods. Protein preparations. Actin was prepared from muscle by the method as described in. Native tropomyosin was prepared from rabbit skeletal muscle according to Ebashi, and from this preparation of native tropomyosin, tropomyosin was purified by ion exchange chromatography in the presence of 6 M urea. Chara myosin was partially purified from internodal cells of Chara corallina. Briefly, the whole cytoplasm was squeezed out of an internodal...
cell with fingers into the extraction buffer containing Mg-ATP and protease inhibitors. Hundred micro liters of squeezed cytoplasm were immediately mixed with equal volume of the extraction buffer. After clarification by centrifugation at 100,000 × g for 30 min, Chara myosin in the supernatant was precipitated with 38% saturated ammonium sulfate. Collected precipitates were dissolved in the buffer containing protease inhibitors and 50% glycerol, and stored at –80°C before use. This preparation was used within a week.

For ATPase assay in a solution, Chara myosin was further purified by binding with and dissociated from F-actin. Prior to ATPase assay, inorganic phosphate in the preparation was removed with a centrifuge column of microcon 30 (Amicon, Beverly, MA, U.S.A.).

*In vitro motility assay.* In *in vitro* motility was assayed by using rabbit skeletal muscle actin labeled with rhodamine-phalloidin. Chara myosin in 50% glycerol solution of the crude preparation was diluted 6 to 10 times with Diss buffer (50 mM Tris-HCl pH 7.6, 1 mM Mg-ATP, 1 mM DTT, 0.5 mM NaNO₃, 0.5 mM peflabloc, 0.05 mg/ml leupeptin, 1 µg/ml of TPCK and TAME (Tos-Arg-OMe-HCl)) and then myosin was allowed to attach to the glass surface without any coating for about 30 min on ice. Motility was assayed in the buffer containing 50 mM Hepes (pH 7.6), 1 mM Mg-ATP, 20 mM DTT, 3 nM rhodamine-phalloidin labeled F-actin and the oxygen scavenger system, at room temperature. Tropomyosin or native tropomyosin was mixed with F-actin in the assay buffer prior to observation of effects of these proteins. Movements of F-actin were observed with an epifluorescence microscope (Olympus BH2, Olympus, Tokyo, Japan) equipped with a video camera (Hamamatsu photonics c-2400, Hamamatsu, Japan) and recorded on a video tape.

**ATPase assay.** Actin activated Chara myosin ATPase activity was assayed in Diss Buffer containing F-actin (0.34 mg/ml), tropomyosin (0.15 mg/ml) and Chara myosin (∼5 µg/ml) by measuring the concentration of inorganic phosphate liberated by ATP hydrolysis with malachite green method as described. The ATPase activity was also analyzed by using a motility assay chamber, in which Chara myosin was applied to the glass surface, under the same condition for observation of *in vitro* motility except that a large amount of actin (0.4 mg/ml) and native tropomyosin (0.4 mg/ml) were added. In this experiment, the surface was coated with nitrocellulose to make sure that all Chara myosin molecules bound to the surface more tightly than to the uncoated glass surface. The amount of Chara myosin applied to a chamber was about 0.5 µg. Assay chambers were prepared as many as required to follow the time course of reaction. At appropriate time intervals after reaction was started, the cover slip of the chamber was stripped and the inside glass surfaces were washed with 100 µl of 2% solution of trichloroacetic acid. The whole washing solution was collected and phosphate in the solution was assayed.

**Results.** (1) *In vitro motility inhibited by tropomyosin.* After observation of moving F-actin filaments on Chara myosin, the motility assay buffer containing tropomyosin at a concentration of 20 µg/ml was perfused into the assay chamber. When the assay buffer containing F-actin-tropomyosin reached to the field of microscope, sliding filaments began to dissociate from the surface. F-actin with tropomyosin in the assay buffer infused was not attached onto the surface and finally the sliding movements were not observable.

When F-actin with tropomyosin was perfused into a freshly prepared assay chamber, the number of moving filaments decreased with increasing the concentration of tropomyosin, as shown in Fig. 1. In spite of inhibitory effect of tropomyosin on sliding movement, the velocity of moving filaments did not change (data not shown). At 2 µg/ml of tropomyosin, there existed 10 times molar excess of tropomyosin to actin molecules in the assay buffer.
buffer and the fraction of moving filaments was 80%. Requirement of a large excess tropomyosin for effective inhibition of movement might be due to the extremely low concentration of F-actin (about 3 nM).

(2) Inhibitory effects of native tropomyosin on Chara myosin motility. F-actin filament slid on Chara myosin at 20 to 25 µm/s. To this assay chamber, perfusion of F-actin containing native tropomyosin (40 pg/ml) caused dissociation of sliding F-actin from the surface in the absence of Ca²⁺ and perfused thin filaments (F-actin-native tropomyosin) were not attached to the surface at all. No filament sliding was observed in the absence of Ca²⁺ (Fig. 2).

In the presence of Ca²⁺, however, a few filaments were occasionally found to be attached to the surface and slide in a short distance of several micrometers long. After observation of no movements of thin filaments in the absence of Ca²⁺, thin filament were found moving occasionally when Ca²⁺ was added, as shown in Fig. 2 (black bars). The total distance of tracks of moving filaments in a restricted area in a second was about 5% of that of pure F-actin. The average sliding velocity of this occasional movement of thin filament in the presence of Ca²⁺ was the same as that of pure F-actin within an experimental error (gray bars in Fig. 2). By repeated exchange of the assay solution by successive perfusion of pure F-actin and thin filament in the presence or absence of Ca²⁺, filaments started moving or stopped moving and dissociated from the surface, repeatedly.

(3) Inhibition of Actin activated Chara myosin ATPase by tropomyosin and native tropomyosin. As in vitro movements of F-actin filaments on Chara myosin were inhibited by tropomyosin and by native tropomyosin, we examined if actin activated ATPase activity of Chara myosin was also inhibited by tropomyosin and by native tropomyosin.

First, actin activated ATPase of Chara myosin was measured in a solution by using Chara myosin preparation purified with F-actin binding. This preparation consisted mainly of Chara myosin and a small amount of actin remaining in the supernatant during centrifugation, but its own ATPase activity was too low to measure with the malachite green method. By adding F-actin in the assay buffer, the amount of phosphate liberated by Chara myosin ATPase increased linearly with time (Fig. 3A). When tropomyosin was added, this actin activated Chara myosin ATPase was completely inhibited (Fig. 3A).

Next, we measured the ATPase activity by using the motility assay chamber. The amount of phosphate liberated increased linearly with time despite usage of a series of different chambers for measurements of the time course of reaction. Therefore, obtained linearity of phosphate liberation indicated that the ATPase assay using the surface was reliable. As shown in Fig. 3B, F-actin greatly activated Chara myosin ATPase attached to the surface, but did not activate it when native tropomyosin was added, irrespective of Ca²⁺ concentration.

Thus, sliding movement on Chara myosin and its actin activated ATPase activity were strongly inhibited by tropomyosin and also native tropomyosin, in contrast to the activation in case of skeletal and smooth muscle myosin II.

Discussion. Native tropomyosin plays a key role in excitation and contraction coupling in muscle. The binding of Ca²⁺ to troponin C of native tropomyosin leads thin filaments to interact with myosin by relocation of tropomyosin on the groove of an F-actin filament. In vitro movement of F-actin on skeletal muscle myosin II is also activated by Ca²⁺ in the presence of native tropomyosin.

On the other hand, Chara myosin does not move F-actin combined with tropomyosin at all. Chara myosin is two headed plant myosin belonging to class XI. The amino acid sequence of its head portion is 41% identical to that of myosin V. However, Myosin V moves F-actin in the presence of tropomyosin at a similar velocity to that of pure F-actin. Interestingly, motility of monomeric brush border myosin I was completely inhibited by...
Tropomyosin inhibits Chara myosin

It is unknown how tropomyosin inhibited Chara myosin motility completely. Tropomyosin interacts with subdomains 3 and 4 of actin subunit in the filament. Muscle myosin II interacts with subdomain 1 and has additional contact with subdomain 3. Therefore, our results suggest that Chara myosin may interact with subdomain 3 and/or 4 of actin in the filament, in competing for part of the binding site with tropomyosin.

Native tropomyosin leads the thin filament to the “ON” state in the presence of Ca$^{2+}$ due to relocation of tropomyosin on F-actin. This position of tropomyosin is thought to be the same as that of tropomyosin without troponin. But our careful observations of Chara myosin motility reported here indicate that native tropomyosin in the presence of Ca$^{2+}$ is not exactly in the same position as that of tropomyosin alone. Native tropomyosin is suggested to have a significant role in regulation of motility in addition to simple switching between “ON” and “OFF” states.

Acknowledgement. We thank Prof. F. Oosawa, M.J.A., in Aichi Institute of Technology for critical reading of this manuscript. We also thank Prof. S. Ebashi, M.J.A., for his support of this work. This work was supported by the Grant-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Science, Sports and Culture of Japan to S. H-F (No. 12030213-00).

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