The fastest actin-based motor protein from the green algae, *Chara*, and its distinct mode of interaction with actin

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Abstract The endoplasmic streaming in Characean cells is an actin-dependent movement. The motor protein responsible for the streaming was partially purified and characterized. It was soluble at low ionic strength, an ATPase of a molecular mass of 225 kDa and activated more than 100 times by muscle F-actin. Surprisingly, in an in vitro motility assay, the motor protein moved muscle F-actin at 60 μm/s, which is similar to the velocity of streaming in a living cell and 10 times faster than muscle myosin. Proteolytic cleavage of actin impaired movement crucially on muscle myosin, but did not affect movement at all on the *Chara* motor protein, suggesting that the *Chara* motor protein would interact with actin via a set of sites different from those of muscle myosin.

Key words: Motility (in vitro); Actin-based movement; Cleaved actin; Motor protein; *Chara*

1. Introduction

In Characean cells, the endoplasm streams along the actin cables [1,2] at 30–100 μm/s. Direct observation of in vitro movement of endogenous actin cables revealed the presence of the motor protein responsible for this fast streaming in Characean cells [3]. If the streaming endoplasm contains myosin, the polarity of the actin cable explains the direction of streaming [4]. The molecular mechanism of actin-dependent nonmuscle cell motility is thought to be fundamentally the same as that in muscle contraction. Purification and characterization of the motor protein in a characean cell are important for the understanding of the molecular mechanism of endoplasmic streaming.

We partially purified and characterized the actin-dependent motor protein from Characean cells by following the motor activity with the in vitro motility assay [5,6]. Distinct characteristics of biochemical and motility aspects of the *Chara* motor protein will be described.

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HMM, heavy meromyosin.

2. Materials and methods

2.1. Purification of the Chara motor protein

An internodal cell of *Chara australis* was squeezed with fingers and about 0.1 ml of the squeezed cytoplasm was mixed with 0.1 ml of E-buffer containing 0.1 M Tris-HCl (pH 8.0), 20 mM EGTA, 2 mM $MgCl_2$, 5 mM ATP, 0.25 M sucrose, 1 mM DTT and protease inhibitors (1 μg/ml *p*-tosyl-L-arginene methyl ester, 10 μg/ml L-1-torylamide phenylethyl-chloromethyl ketone, 10 μg/ml *N*-benzoyl-L-arginine methyl ester, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation at 100,000 × *g* for 30 min, the motor activity located in the supernatant (the crude extract) was precipitated by 38% of saturated ammonium sulfate (pH 7.0). The precipitates were dissolved in B-buffer containing 30 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, protease inhibitors and 50% glycerol without ATP at 1/20 volume of the crude extract, and then muscle F-actin not containing ATP was added at the concentration of 0.4 mg/ml and the concentration of glycerol was adjusted to 40%. The motor activity bound to F-actin was precipitated by centrifugation at 250,000 × *g* for 90 min (Beckman TL-100 Ultracentrifuge USA), and separated from F-actin by centrifugation in B-buffer containing 10 mM ATP without glycerol at 250,000 × *g* for 30 min. The supernatant was applied to the ion exchange Mono Q, column (Pharmacia) after brief dialysis against the elution buffer (B-buffer containing 1 mM ATP). The proteins were dissociated from the column with KCl gradient.

2.2. In vitro motility assay

The sample solution was infused into a flow-chamber [7], and the motor protein was allowed to attach onto the glass surfaces on ice. Then F-actin labeled with rhodamine-phalloidin [8] was infused into the flow-chamber and its movement was observed in M-buffer (1 mM ATP, 2 mM $MgCl_2$, 20 mM DTT, 50 mM HEPES buffer at pH 7.6) containing the oxygen scavenger system [8] by fluorescence microscopy at room temperature (23°C) and recorded on a video tape [9]. When movements of various actin preparations on HMM were compared, the same collodion coated glass surface on which HMM attached was used, because the movement on HMM depended largely on the condition of collodion coated surface. All F-actin filaments were dissociated from HMM and washed away by perfusing M-buffer containing 0.2 M KCl [9], before the other actin preparation was examined. Actin and HMM were prepared from rabbit skeletal muscle by the methods described in [10] and [9], respectively.

2.3. Biochemical assays

Binding of ATP to proteins was assayed according to Maruta and Korn [11]. [α-^{32}P]ATP was from Amersham. The actin activated ATPase of the motor protein in the crude extract was assayed in M-buffer containing 1 mM DTT by using the flow chamber, since the motor protein specifically attached onto the glass surface. Aliquot of the sample solution was applied to the flow chamber (18 mm × 18 mm × 0.15 mm) and the motor protein was allowed to attach to the glass surface for more than 1 h on ice. Then the chamber was extensively rinsed with M-buffer, prior to infusing M-buffer containing 0.1 mg/ml of muscle F-actin. After incubationa at 23°C for an appropriate period, inorganic phosphate liberated in the chamber was measured by

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the Malachite green method [12]. ATPase activities of HMM activated by various actin preparations were assayed in M-buffer containing 0.15 mg/ml of F-actin and 0.1 mg/ml HMM, and the amount of inorganic phosphate liberated was measured according to LeBel et al. [13].

2.4. Enzymatic cleavage of actin

Muscle G-actin (2 mg/ml) was cleaved by subtilisin at 3 μg/ml for 60 min at 20°C or proteinase K at 0.01 mg/ml for 90 min at 25°C [14,15] and the reaction was terminated with 1 mM PMSF. Proteinase K-cleaved actin was polymerizable in the presence of phalloidin [15].

3. Results

3.1. In vitro motility of the Chara *motor protein*

We identified an actin-dependent motor protein in the cell extract from *Chara australis* using the in vitro motility assay (Fig. 1A). The *Chara* motor protein, even in the crude extract, specifically attached to a glass surface without any treatment, and moved muscle F-actin at a high speed. The movement was usually continuous at about 30 μm/s, but after extensive perfusion of the chamber with M-buffer, the sliding velocity increased up to 60 μm/s (Fig. 1B), though the movement became intermittent. This was the fastest movement of F-actin in vitro observed so far.

This in vitro movement was insensitive to the Ca^{2+} concentration (less than 5 mM), though endoplasmic streaming in vivo was regulated by Ca^{2+} [16] . The movement was very sensitive to KCl. The moving filaments were rarely observed at 30 mM or more, since the affinity of F-actin to the motor protein markedly decreased. Tropomyosin activated the movement of F-actin on muscle myosin [7], but it inhibited completely the binding and movement of F-actin on the *Chara* motor protein.

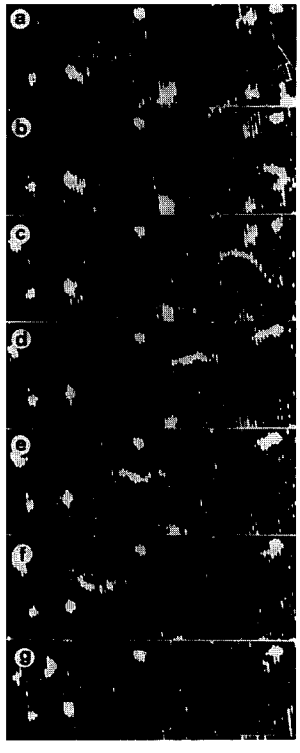
Incubation M-buffer without ATP eliminated the motility in a few minutes at room temparature. Reintroducing a fresh M-buffer into the chamber could not induce the movement again. The motility was, however, fully retained in the absence of ATP by adding glycerol 40–50%.

3.2. Purification of the motor protein from Chara *australis*

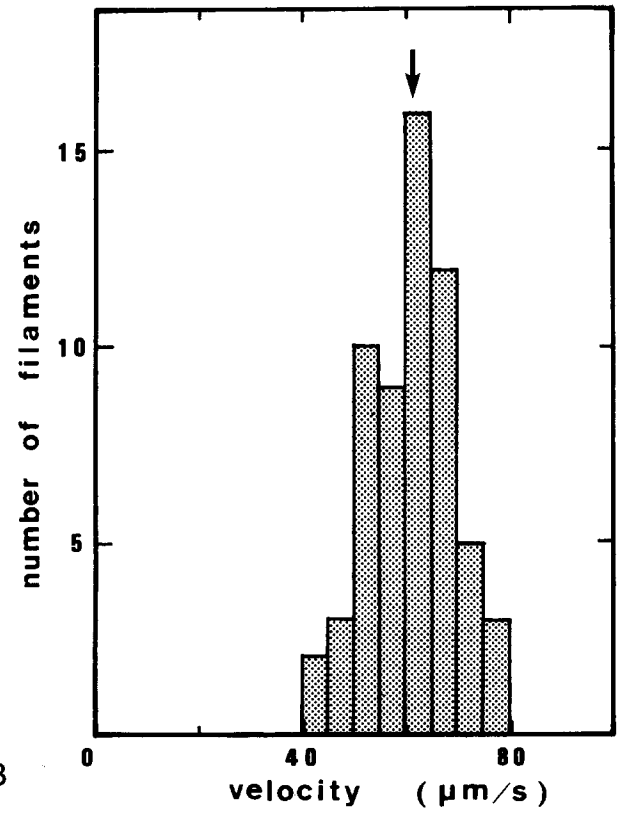
Following this in vitro motility, we attempted to purify the motor protein. The motile activity contained in the crude extract (Fig. 2b) was precipitated by 38% saturated ammonium sulfate (Fig. 2c). The activity was bound to and cosedimented with muscle F-actin in B-buffer containing 40% glycerol without ATP. Only the 225 kDa polypeptide was concentrated (Fig. 2d). The motile activity was separated from F-actin by centrifugation in the presence of 10 mM ATP (Fig. 2, e: supernatant and f: precipitate) and eluted at about 0.35 M KCl from the ion exhange column. The peak of the motile activity coincided with the peak of 225 kDa polypeptide (Fig. 2h). The protein of this 225 kDa polypeptide was an ATP binding protein, as it was crosslinked with ATP under UV irradiation (Fig. 2j). Taken together with these results, we concluded that the *Chara* motor protein was composed of the 225 kDa polypeptide.

3.3. In vitro motility of cleaved actin on the Chara *motor protein*

On the surface coated with muscle HMM, F-actin composed of muscle actin which were cleaved by subtilisin and proteinase K moved very slowly at 2.4 and 0.4 μm/s, respectively, while



A



B

Fig. 1. Sliding movement of actin filaments on the *Chara* motor protein. (A) Serial photographs taken at every 4th frame (4/30 s). An arrow in (a) and arrowheads in each photograph point to the direction of movement and the moving actin filament, respectively. The filament looks longer than the real one because of the after image in (c) to (f). Bar: 20 μm. (B) Velocity distribution. The arrow points to an average velocity of 60.1 ± 8.4 μm/s (n = 60).

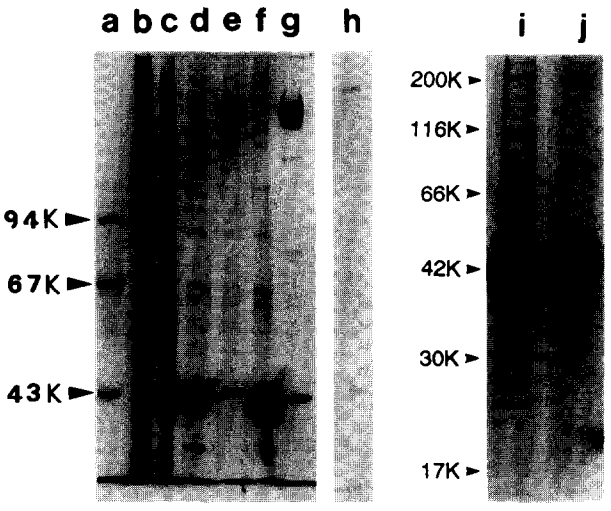


Fig. 2. Purification and ATP binding assay of the *Chara* motor protein: SDS-PAGE patterns. lane a: molecular markers, lane b to f, h and j are described in the text. lane g: muscle myosin. lane i: the same sample as lane j stained with Coomassie brilliant blue. lanes a to h: 7.5% polyacrylamide gel, lanes i and j: 12.5% gel.

intact F-actin slid at 6.0 μm/s (Fig. 3A). On the *Chara* motor protein, however, subtilisin-cleaved actin could slide at 53.9 μm/s and even proteinase K-cleaved actin slid at 61.0 μm/s, while intact F-actin slid at 60.1 μm/s (Fig. 3A and B). Proteinase K-cleaved actin slid on the *Chara* motor protein 150 times faster than on muscle HMM. The movements of cleaved actins were not impaired at all on the *Chara* motor protein.

3.4. *Actin activated ATPase of the* Chara *motor protein*

Actin activated ATPase activity of the *Chara* motor protein was measured by using the flow chamber as described in section 2. The phosphate release increased linearly with time (≤20 min), indicating reliability of this method (data not shown). As shown in Table 1, muscle F-actin activated the motor protein ATPase 125 times. Proteinase K-cleaved and Subtilisin-cleaved actin also activated the motor protein ATPase to a similar extent. On the contrary, activation of muscle HMM ATPase by subtilisin-cleaved and Proteinase K-cleaved actin decreased to 39.0% and 17.6% of that by intact F-actin, respectively. These results showed good correlation between the ATPase activity and the sliding velocity.

4. Discussion

Actin-dependent motor protein from *Chara* was the fastest motor observed so far: F-actin slid at 60 μm/s at 23°C. The motor protein had an apparent molecular mass of 225 kDa and was soluble at low ionic strength. When the endoplasm was selectively squeezed into E-buffer, the motility was often found in both the precipitate and the supernatant of low speed centrifugation, indicating that the motor protein was associated with a large structure. The protein would possibly be membrane-associated as suggested by vesicle movement along actin cables [17,18] and by in vitro elongation of tubular membrane upon

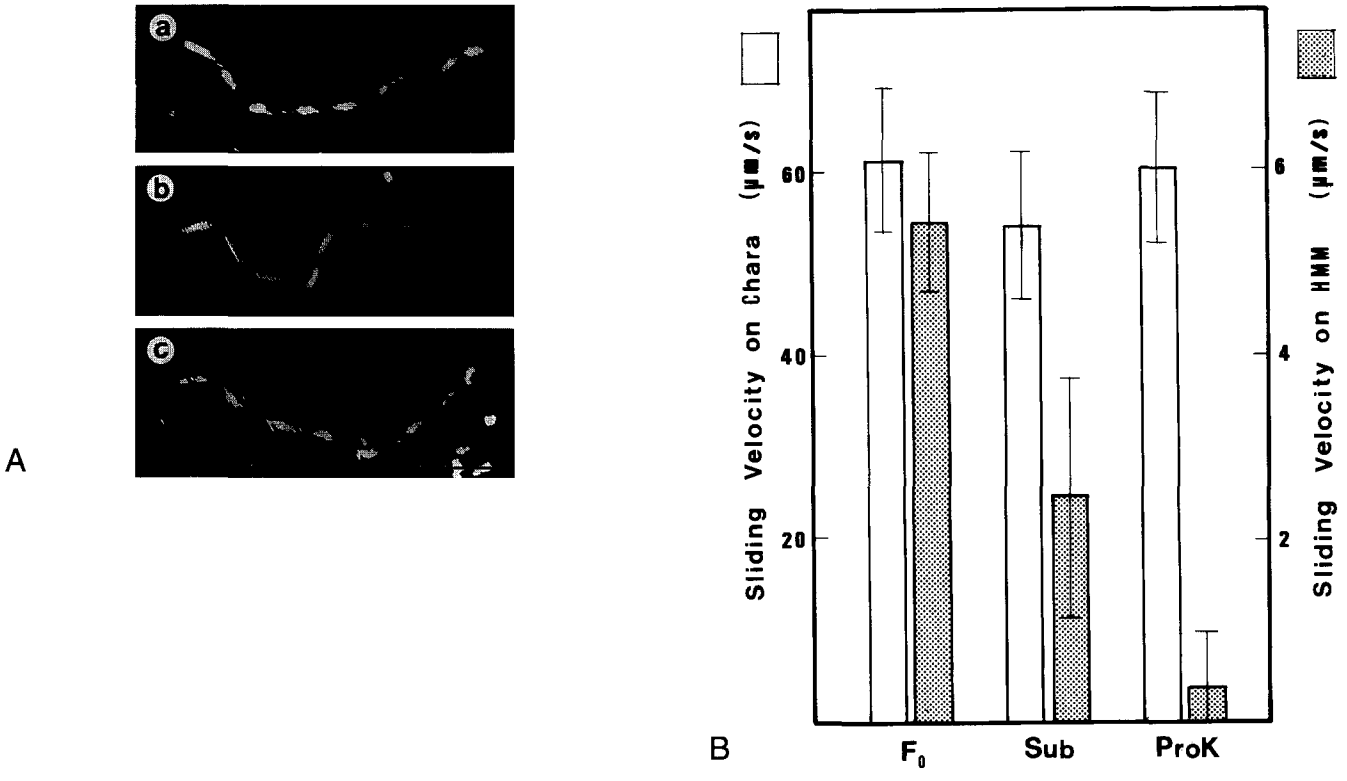


Fig. 3. Motility of cleaved actin on the *Chara* motor protein. (A) The movement was traced for 1 s by using the image processor of DVS 3000 (Hamamatsu Photonics, Hamamatsu Japan). (a) intact actin, (b) subtilisin-cleaved and (c) proteinase K-cleaved actin. (B) Sliding velocities on the *Chara* motor protein (open column) and muscle HMM (shaded column). Bars indicate standard daviations. F_o: intact actin (n = 60), Sub: subtilisin-cleaved actin (n = 81), ProK: proteinase K-cleaved actin (n = 60), on the *Chara* motor protein. (n = 25 for measurements on HMM.)

Table 1
Chara motor ATPase activated by muscle F-actin

Cleavage of actin	Expt. 1	Expt. 2	Expt. 3
F-actin	36.4 (100)	14.2 (100)	27.3 (100)
Proteinase K cleaved actin	30.3 (83.2)	10.0 (70.4)	–
Subtilisin cleaved actin	–	13.3 (93.7)	21.3 (78.0)
Without actin	0.3 (0.8)	–	–

The activity is expressed in μM/min. The percentage of the activity in comparison with uncleaved actin is shown in parentheses.

addition of F-actin [19]. The membrane-associated myosin, brush border myosin I moved F-actin two orders slower than the *Chara* motor protein [20]. However, the fact that tropomyosin inhibited in vitro motility of brush border myosin I [20] and antibody to Acanthamoeba myosin I reacted weakly to the motor protein from a Characean cell of *Nitella* [21], suggested the close evolutional relationship between myosin I and the *Chara* motor protein. Some unconventional myosins were reported to have calmodulin as light chains [22]. The *Chara* motor protein did not react to monoclonal antibody to calmodulin in the immunoblotting experiment (data not shown). It is unknown whether the *Chara* motor protein has light chain(s) or not. Since the motility of the *Chara* motor protein in vitro was insensitive to Ca^{2+}, the endoplasmic streaming in a cell could possibly be regulated by Ca^{2+} indirectly through the other pathway, such as phosphorylation [23].

Cleavage of an actin molecule at the site between Met^{47} and Gly^{48} [15] caused crucial damage of motility on muscle HMM. The *Chara* motor protein, however, moved the same cleaved actin at 60 μm/s, that is, the same rate as the intact actin. The effect of tropomyosin on the motility was opposite: activation on muscle myosin [7] but complete inhibition on the *Chara* motor protein. These results indicated that the *Chara* motor protein does not share the same set of binding sites on the actin molecule with muscle myosin, and may undergo serial conformational changes different from those of muscle myosin during a mechanochemical cycle.

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