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The Molecular Structure of the Fastest Myosin from Green Algae, *Chara*

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***Chara* myosin in green algae, *Chara corallina*, is the fastest myosin of all those observed so far. To shed light on the molecular mechanism of this fast sliding, we determined the primary structure of *Chara* myosin heavy chain (hc). It has a motor domain, six IQ motifs for calmodulin binding, a coiled-coil structure to dimerize, and a globular tail. *Chara* myosin hc is very similar to some plant myosins and has been predicted to belong to the class XI. Short loop 1 and loop 2 may account for the characteristics of mechanochemical properties of *Chara* myosin.** © 2000 Academic Press

Characean cells show very fast endoplasmic streaming which has been known to be the actin-based movement. The endoplasm streams along cortical fibrils composed of bundles of F-actin filaments (1). When the bundle was decorated with muscle HMM, arrowheads of F-actin filaments pointed to the direction opposite to the streaming (2). The direction of streaming was understood by the idea that myosin would reside in the endoplasm, in analogy to muscle contraction. The presence of myosin in the endoplasm was suggested by observation of active movements of F-actin bundles squeezed out of the cell in an artificial medium (3). Under an optical microscope, the bundles continued moving, even in a vigorous flow of the medium, so that motor proteins were supposed to be attached to the glass surface. When the motor protein isolated from *Chara corallina* (which is also called *Chara australis*) or *Nitella* was attached to a glass surface, muscle F-actin filaments were found to slide on the attached motor protein at a strikingly fast speed of 60 μm/s, ten times faster than that on skeletal muscle myosin (4, 5). This motor protein was recently purified from *Chara corallina* and named *Chara* myosin (5). It had a molecular mass of ~225 kD judged from SDS-PAGE and its ATPase was activated by muscle F-actin more than 100 times (5, 6).

The molecular mechanism of muscle contraction has been currently explained by the lever arm model (7). It is a serious question if the lever arm model can be applied to the fast sliding of *Chara* myosin because its neck is not ten times longer than that of muscle myosin (8) and its actin activated ATPase is not ten times higher than that of muscle myosin (data reported in Sumiyoshi, H., and Higashi-Fujime, S. (1998) *J. Biophys.* [in Japanese] **38** Suppl. 2), s70; and referred to in Sugi, H., and Kobayashi, T. (1999) *J. Muscle Res. Cell Motil.* **20,** 325–327. To gain insights into the mechanism of fast sliding of *Chara* myosin, we determined the primary structure of *Chara* myosin. *Chara* myosin heavy chain (hc) is very similar to those of some plant myosins of class XI, and has a coiled coil structure suggesting a two-headed myosin. Characteristics of the molecular structure of *Chara* myosin and its significance in endoplasmic streaming are described below.

MATERIALS AND METHODS

Purification of mRNA from Chara corallina. Total RNA was extracted according to the AGPC method (9) with some modifications. Ten grams of *Chara corallina* were powdered in liquid nitrogen and suspended in 100 ml of solution D (4.2 M guanidinium thiocyanate, 11.4 mM Na citrate (pH 7.0), 0.5% N-Lauroyl sarcosine Na salt, 0.72% β-mercaptoethanol), 100 ml of water-saturated phenol and 10 ml of 2 M Na acetate (pH 5.2). The sample was homogenized by Polytron and 20 ml of chloroform was added. After the sample was shaken vigorously, it was centrifuged at 10,000× g for 20 min at 4°C in order to separate the aqueous phase from phenol. According to the method of (10), total RNA in the aqueous phase was selectively precipitated by adding a solution of 1.2 M NaCl and 0.8 M Na citrate and then isopropanol, both up to 42% volume of the aqueous phase. The precipitated total RNA was solved in the extraction buffer of QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). By using this kit, about 10 μg of mRNA was purified.

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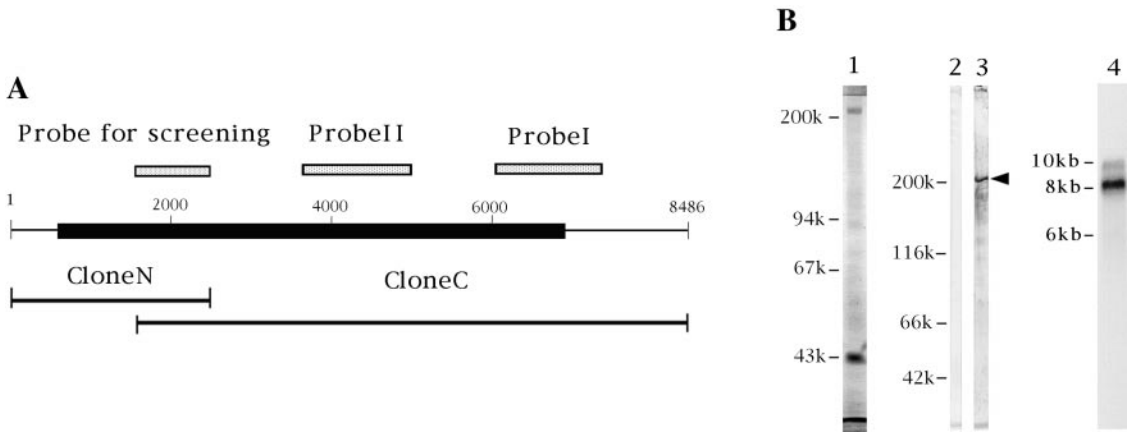

FIG. 1. The structure of *Chara* myosin cDNA. (A) The entire structure of *Chara* myosin cDNA. Clone C and Clone N are shown. The open reading frame is indicated by a bold line. Probes I and II were used for Northern hybridization. (B) Western blot and Northern hybridization. SDS-PAGE (7.5% gel) of *Chara* myosin dissociated from F-actin (lane 1). Western blots of proteins separated in 6% gel with nonimmunized serum (lane 2) and antibody against *Chara* myosin (lanes 3). An arrowhead indicates the *Chara* myosin hc reacted. Northern hybridization with probe II (lane 4). The results obtained by using probe I and II were the same. Molecular weight markers are shown in the left of lanes 1 and 2.

Construction of cDNA library, its cloning and sequencing. Messenger RNA was reversely transcripted with the oligo(dT) primer by Superscript II (Life Technologies, Rockville, MD). Double-stranded cDNA was synthesized according to the method of (11). The cDNAs longer than 1 kbp selected by passing through Sephacryl S-500 HR (Amersham Pharmacia Biotech, Uppsala, Sweden) were ligated into expression vectors of λZAP (Stratagene, La Jolla, CA) at the *Eco*R I site and packaged with Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA). The cDNA expression library was screened by using polyclonal antibody against *Chara* myosin hc with a detection method of ECL (Amersham Pharmacia Biotech, Uppsala, Sweden) and a phage vector with a 6.7 kbp cDNA insert was isolated. pBK-CMV carrying this cDNA was prepared by *in vivo* excision from the λZAP vector (named Clone C, Fig. 1). Clone C was sequenced with the restriction enzyme digestion method or the primer walking strategy. Since the *Pst* I fragment of 2 kbp had multiple repeats, the fragment was sequenced with GPS-1 Genome Priming System (New England Biolabs, Beverly, MA). Both strands of Clone C were sequenced using BigDye terminator cycle sequencing kit (PE Biosystems, Foster City, CA) and ABI prism 377/310 genetic analyzer (PE Biosystems, Foster City, CA).

Since Clone C did not extend to the 5′-end of the coding region, another cDNA library was constructed. The construction method was the same as described above, except that *Chara* mRNA was reversely transcripted by Thermoscript RT (Life Technologies, Inc., Rockville, MD) with each of nine primers designed according to the first 882 bp of Clone C, and then first-strand cDNAs were mixed. The library was screened with hybridization method using the *Sca* I fragment of Clone C (1,800–2,679 bp in the total cDNA sequence) as a probe, which was labeled with DNA labeling kit (Nippon Gene, Toyama, Japan). The cDNA of 2.6 kbp was cloned (Clone N, Fig. 1) by the method described above. This cDNA was found to be transcripted with the primer of 5′-AACCCTGCACAGCTGATTCGG-3′ (2598–2618 bp in the total cDNA sequence). Clone N was sequenced by the primer walking method or by generating deletion mutants with exonuclease III (TaKaRa Shuzo, Kyoto, Japan). The nucleotide sequence data reported in this paper appeared in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession No. AB034154.

Northern blot analysis. Messenger RNA was separated electrophoretically on 1.0% agarose gel containing formaldehyde and transferred onto Nytran SuPerCharge Nylon membrane (Schleicher & Schuell, Dassel, Germany). Two probes were prepared, probe I and II: digestion fragments of Clone C with *Pst* I and *Bam*H I (6,132–7,191), and with *Eco*R V (3,732–4,992), respectively. DNA probes were labeled by the same method as described above. Probes were hybridized for 16 h at 42°C in the buffer containing 10% dextran sulfate, 1% SDS, 50% deionized formamide, 2× SSC (0.3 M NaCl and 0.03 M Na citrate at pH 7.0) and 5× Denhardt's solution. After the membrane was washed at 42°C in the washing buffer (0.1× SSC, 0.1% SDS) for 30 min twice, the signal was visualized by autoradiography at −80°C for 2 h.

Production of polyclonal antibody against Chara myosin. *Chara* myosin was partially purified from *Chara corallina* according to the method as described (5). After SDS-PAGE (Fig. 1), the band of *Chara* myosin hc of ~225 kD was excised from the gel and the protein was eluted electrophoretically. The eluent was dialysed against PBS. The mixture of the dialysate and incomplete freund's adjuvant was injected into mice. The immunological activity was detected with Western blot by using ABC kit (Vector Laboratories, Burlingame, CA). The antibody strongly reacted with the 225 kD polypeptide (Fig. 1).

Microsequencing of polypeptide fragments of Chara myosin. The band of *Chara* myosin hc was excised from the gel and the protein was digested in the gel with Achromobacter lysyl endoprotease according to the method described (12). Peptide fragments isolated by micro-bore liquid chromatography on a Vydac C18 column (1.0 × 250 mm) were subjected to Edman sequencing (ABI protein sequencer 494, PE Biosystems, Foster City, CA).

In vitro motility assay. *In vitro* motility was assayed with muscle F-actin as described (13). To examine the effect of antibody on *Chara* myosin motility, the minimum concentration of *Chara* myosin required for motility assay was first examined by dilution with TBS containing 5 mM ATP, 5 mM $MgCl_2$ and 1 mM DTT. At a concentration just above the minimum concentration (usually about 0.01 mg/ml), *Chara* myosin was reacted with the antibody for 60 min at 22°C, and then allowed to attach to the surface for 30 min on ice. The antibody was purified with Protein G PLUS/Protein A Agarose beads (Oncogene Research Products, Cambridge, MA). Since the antibody specifically reacted with *Chara* myosin hc, it was not further purified.

Indirect immunofluorescence microscopy. An internodal cell was cut at both ends. The cell was fixed by perfusion of the vacuole with Buffer A (5 mM EGTA, 6 mM $MgCl_2$, 200 mM sorbitol) containing 1%

paraformaldehyde, 2% glutaraldehyde and 100 mM K-phosphate buffer (pH 7.0). After 30 min fixation, the cell was reacted with antiserum against *Chara* myosin hc at 1/2,000 dilution with Buffer B (Buffer A containing 30 mM PIPES (pH 7.0)) for 60 min at room temperature, and then reacted with FITC conjugated anti-mouse IgG at 1/200 dilution (Calbiochem, La Jolla, CA). The stained cell was longitudinally cut and washed with Buffer B, and then observed under a epifluorescence microscope BH2 (Olympus, Tokyo, Japan) with the filter G 520.

RESULTS

Primary Structure of Chara Myosin Heavy Chain (hc)

As shown in Fig. 2A, we determined the primary structure of *Chara* myosin hc composed of 2167 amino acid (aa) residues from the cDNA with 8486 bp. Two cDNA clones, Clone C with 6687 bp and clone N with 2617 bp, overlapped 798 bp and were enough to obtain the full length of the coding region. In the flanking 5′ untranslated region (UTR) there were 609 bp and in the 3′UTR there were 1373 bp containing the specific sequence of the polyadenylation signal. The deduced amino acid sequence exactly matched the results of microsequencing of *Chara* myosin hc, the amino acid sequences of seven polypeptide fragments (Fig. 2A, sequences enclosed by rectangles). The calculated molecular mass was 246,334 D which was slightly larger than that of 225 kD estimated from the mobility in SDS-PAGE. According to Northern hybridization, the probes hybridized to mRNA of ~8.5 kb which was the exact length expected (Fig. 1B).

The structural analysis revealed that *Chara* myosin hc had a motor domain in the N-terminal region, six IQ motifs, a long coiled coil structure to dimerize and a globular tail. *Chara* myosin was strikingly similar to some unconventional myosins from plants belonging to myosin class XI in the head and the tail domains. The intermediate rod or stalk domain of *Chara* myosin was not similar to any other myosins.

The head and the neck domains of Chara myosin hc. The head domain of *Chara* myosin (aa residues 1–739 just before the IQ domain) was 65, 64, 63 and 62% identical to those of plant myosins from *Helianthus annuus* (sunflower), *Zea mays* (maize), *Arabidopsis thaliana* MYA1 and MYA2, respectively. It was also similar to chicken class V myosin *dilute* (41% identical) or *Dictyostelium* myosin IJ (43% identical). From search on the nonroot phylogenetic tree (Felsenstein, J. 1993 PHYLIP ver. 3.5c) *Chara* myosin was suggested to belong to the class XI in myosin family, since myosin family was classified by sequence similarity in the head domain.

In the N-terminal region, skeletal myosin had ~80 aa extension in comparison with the sequence of myosin I. This extension of *Chara* myosin was ~50 aa, and similar to those of myosin classes XI and V (Fig. 2B). From X-ray crystallographic analysis of skeletal myosin, this region resides near the ATP binding pocket (14), but its functional significance is unknown. The sequence specific to the ATP binding site "GESGAGKT" was found at aa 160–167 of *Chara* myosin. The 25/50-kD junction of loop 1 near the ATP binding site is one of variable regions. The corresponding region of *Chara* myosin was shorter than that of skeletal myosin and did not show close similarity to other myosins. Two charged residues to form salt bridges to the phosphate in switch 1 and switch 2 were also found in *Chara* myosin, 218R and 443E, respectively.

The consensus sequences for the actin binding site and the interface between actin and myosin (15) were also highly conserved in *Chara* myosin hc. Just upstream of the actin binding site (AB in Fig. 2A), the 50/20-kD junction of loop 2 varies from myosin to myosin in both its length and alignment of residues (Fig. 2B). This region of *Chara* myosin hc was very short and did not contain so many Lys residues as myosin II. The highly reactive cysteins SH1 (667C) and SH2 (656C) were both found in the position corresponding to those of chicken skeletal myosin, though the position of SH1 displaced by one aa. After this region, there was a long α-helix of a neck domain.

The neck domain (740-884) was characterized by six IQ motifs specific for calmodulin binding. Although the residues "VQ" or "FQ" substituted for 'IQ' and the motif was slightly modified in comparison with those of chicken myosin V (16), *Chara* myosin would probably have six calmodulin or calmodulin-related molecules as light chains for a heavy chain.

The stalk and the tail domains of Chara myosin hc. The highly hydrophobic region from 875K to 1,635V was expected to form a coiled coil structure with a high score (Fig. 2C), indicating that the *Chara* myosin hc would dimerize through this coiled coil to make a two-headed structure. The domain from 1,076Q to 1,630S was specifically characterized by tandem repeats of a 33 aa repeating unit (Fig. 2A), but in some of the repeats, one or two residues were replaced by others. The tandem repeat was interrupted three times by another repeat sequence consisting of 53 aa residues. There, the coiled coil structure was broken by the predicted random coil consisting of ~18 aa residues (wavy lines in Fig. 2A). This type of tandem repeat was not found in any other myosins. MYA3 from *Arabidopsis thaliana* has a 33 aa repeating unit. But the repeating unit of *Chara* myosin had no homology to that of MYA3.

After the tandem repeat, the remaining C-terminal region did not show particular structural characteristics and would form a globular tail. The later half of the globular tail domain showed again great similarity to class XI myosins.

Inhibition of the Fastest Movement by the Antibody Used for Screening

The identity of immunologically screened cDNA was confirmed as *Chara* myosin hc by the results of micro-

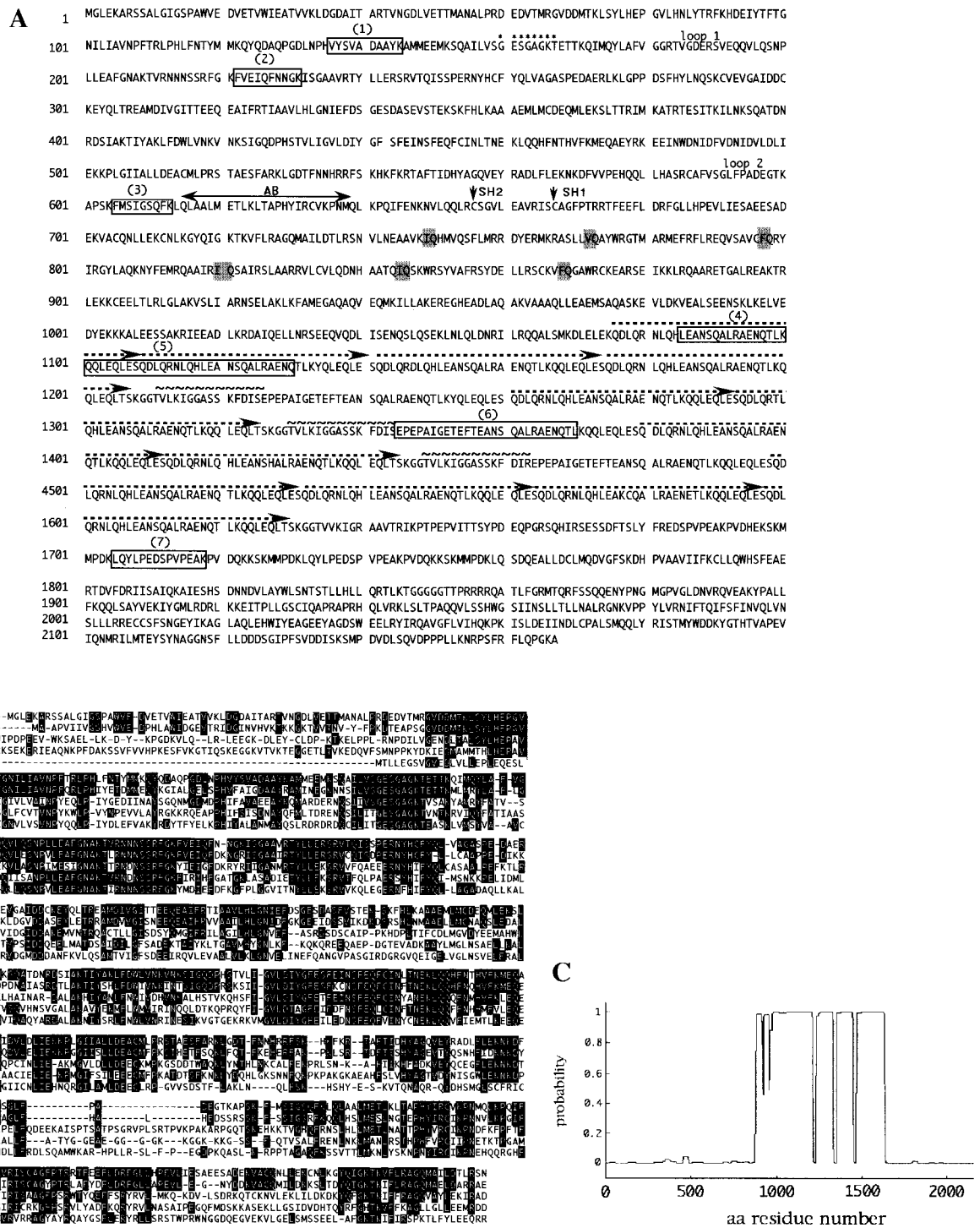

FIG. 2. The deduced amino acid sequence of *Chara* myosin hc and its structural analyses (A) The deduced aa sequence. asterisks, ATP binding sequence; AB, actin binding sequence; small arrows, SH1 and SH2; shaded letters, IQ or its related aa in the IQ motif; the dotted line with an arrowhead, the repeating unit; wavy lines, the predicted peptide microsequencing. (B) Comparison of the primary structure of the head domain of myosin. Chara, *Chara* myosin; MYA1, myosin from *Arabidopsis thaliana* MYA1; ChicMV, chicken brain myosin V; Chicsk, chicken skeletal myosin; BovMI, bovine brush border myosin I. The aa identical to the sequence of *Chara* myosin is shown with a white letter in black. (C) The probability of the coiled-coil formation searched with COILS ver. 2.2.

sequencing of its peptide fragments. To demonstrate that the antibody used for screening was really the one against the fastest motor protein of *Chara* myosin, we investigated the inhibitory effect of the antibody on the motor activity by the *in vitro* motility assay. In fact, the motor activity was greatly inhibited by the antibody

TABLE 1

Inhibitory Effect of the Antibody Against *Chara* Myosin hc on *Chara* Myosin Motility

	Number of sliding filaments				
		Ab (IgG + IgM) treated		Nonimmunized (IgG + IgM) treated	
Sample	Nontreated	0.01 mg/ml	0.03 mg/ml	0.01 mg/ml	0.03 mg/ml
Surface 1 A	28	11	0	37	19
B	30	11	0	40	24
Surface 2 A	35	9	0	19	25
B	37	12	0	37	23
Average	32.5	10.8	0	33.3	20.3
Velocity (μm/s, n = 10)	20.5 ± 3.3	19.9 ± 3.3	—	20.8 ± 2.4	19.6 ± 2.1

Note. The number of sliding filaments was counted on the surface restricted in 50×50 μm^2. Measurement was done in two areas (A and B) for each of two preparations (surfaces 1 and 2). For this experiment, partially purified *Chara* myosin after ammonium sulfate precipitation was used. The concentration of *Chara* myosin reacted with antibody was roughly estimated to be 0.01 mg/ml as judged from CBB staining of SDS-PAGE gel. When the preparation of *Chara* myosin dissociated from F-actin was used, the results were the same.

(Table 1). With increasing the concentration of the antibody, the number of sliding filaments diminished and sliding filaments were often detached from the surface. The sliding distance of a filament from attachment to detachment became short, but the sliding velocity did not change as far as the movement could be observed. On *Chara* myosin treated with nonimmunized immunoglobulins and also on untreated *Chara* myosin, filaments moved continuously and the number of sliding filaments did not diminish.

Localization of Chara Myosin in a Living Cell

Chara myosin is expected to be responsible for force generation in the endoplasmic streaming and vesicular transport along actin cables. Actually, immunofluorescence microscopy showed, as in Fig. 3, that *Chara* myosin bound to cargoes and actin cables, and also existed diffusely in the endoplasm. In an isolated endoplasmic droplet, there are a lot of vesicles and rotating polygons composed of actin filaments which repeat formation and destruction. This indicates that there are myosin and actin at high concentrations in the endoplasm. *Chara* myosin can crosslink actin filaments through vesicles or by myosin itself in the endoplasm, so that the endoplasm becomes very viscous and streams as a whole.

DISCUSSION

Chara myosin is characterized by the fast sliding of 60 μm/s, the fastest of all myosins observed so far (5). The sequence of the heavy chain of this fast motor protein of *Chara* myosin we obtained is reliable, because of the following four reasons: (1) first we obtained the cDNA clone containing more than 80% of the coding region by immunological screening; (2) only two cDNA clones with 798 bp overlapping were enough to obtain the full length of the coding region; (3) the aa sequences of seven polypeptide fragments of *Chara* myosin hc exactly matched the aa sequence deduced from the cDNA; and furthermore (4) the antibody used for screening of the cDNA library inhibited the *in vitro* movement on *Chara* myosin. Taken together, the deduced aa sequence is really that of the fastest *Chara* myosin.

The coiled-coil structure predicted by the structural search is consistent with the molecular image reported in (8). But the total length of the predicted coiled-coil structure (707 aa residues) is calculated to be ~100 nm, which is much longer than the stalk length of 50 nm observed (8). It is uncertain if our *Chara* myosin is the same as myosin in (8). The discrepancy may come from interruptions of the coiled coil structure three times by the random coil (Fig. 2C). Presumably, the

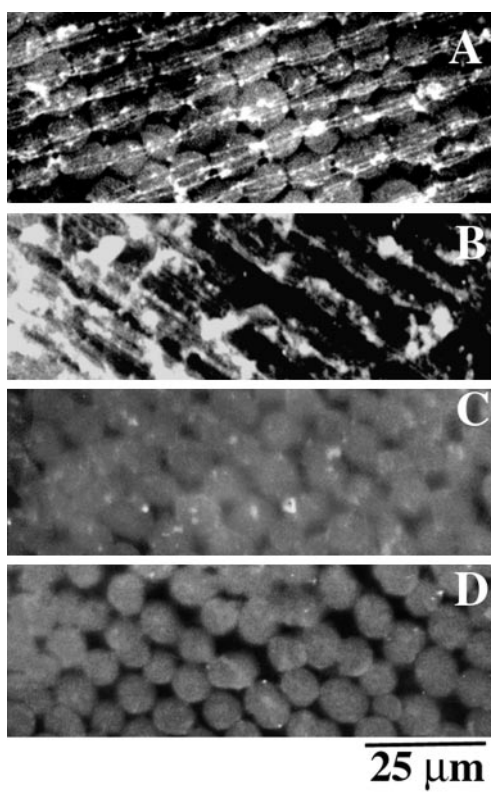

FIG. 3. Immunofluorescence microscopy of the internodal cell of *Chara corallina.* (A and B) The cells stained with the antibody against *Chara* myosin hc. (C) The cell stained with the antibody against *Chara* myosin hc after washing out the endoplasm with solution A containing 10 mM ATP. (D) The cell stained with nonimmunized serum.

coiled-coil structure may bend and stack at these flexible random coils, or may be included partly in the tail.

Since *Chara* myosin has six IQ motifs for calmodulin binding in the neck region, light chains like calmodulin are expected to be associated with *Chara* myosin hc. Myosin V has six IQ motifs and is sensitive to Ca^{2+} (17). In a living cell of *Characeae*, endoplasmic streaming is stopped by membrane depolarization accompanied by the entry of Ca^{2+} to the cytoplasm (18), but in the *in vitro* motility assay, *Chara* myosin does not show Ca^{2+} sensitivity (5). It is unknown whether motility of *Chara* myosin is regulated by direct binding of Ca^{2+} to the light chain(s) such as calmodulin, or not.

The actin binding site and the actin-myosin interface are highly conserved in *Chara* myosin hc. The sequences of variable regions of loop 1 and loop 2 vary from myosin to myosin and seem to characterize each myosin. Loop 1 is located near the ATP binding site and loop 2 is near the actin binding site. When chimera myosins were constructed by replacing loop 1 or loop 2 with those of other myosins, their mechanochemical properties greatly changed (19, 20). In *Chara* myosin, loop 1 and loop 2 are short. Especially loop 2 is much shorter than those of other myosins. Consequently, short loop 2 would be one of the significant structural bases for the fast sliding of *Chara* myosin. MYA1 belonging to class XI has also short loop 2, but at present, there is no report on its biochemical and mechanochemical properties.

The other characteristics is found in the actin-myosin interface. In chicken skeletal myosin 552N–556H can be in contact with the 40-50 loop of the below actin in a filament (15). Next to this actin-myosin interface four aa residues are deleted in *Chara* myosin hc, comparing with that of chicken skeletal myosin. Enzymatic cleavage at the 40–50 loop of actin greatly impairs the *in vitro* movement on skeletal muscle myosin (21). On *Chara* myosin, however, cleaved actin can slide at the same speed as that of intact actin (5). This phenomenon may be explained by four aa deletion at the actin–myosin interface mentioned above. This deletion may contribute to fast sliding of *Chara* myosin.

Myosin V was reported to move by a long step (22). On the other hand, muscle myosin S1 moves by multisteps during a single ATPase cycle (23), as suggested by the idea of loose coupling between the chemical reaction and the mechanical event (24). It is interesting whether *Chara* myosin moves fast by a long step, or by multisteps. Knowledge about the primary structure of *Chara* myosin reported here will provide us a clue to investigate the molecular mechanism of the fastest sliding movement by using various techniques such as molecular biology and molecular manipulations.

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