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Myosin Subfragment-1 Is Fully Equipped with Factors Essential for Motor Function

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The sliding velocity of actin filaments propelled by chicken skeletal myosin subfragment-1 (S1) was measured when the tail end of S1 was specifically bound to the glass surface. To achieve the specific binding, a regulatory light chain was replaced by a recombinant fusion protein of biotin-dependent transcarboxylase (BDTC) and chicken gizzard smooth muscle regulatory light chain (cgmRLC). The BDTC-cgmRLC of S1 was then attached to the glass surface using a biotin-avidin system. The velocity of actin filaments caused by S1 bound to the surface in this manner was 6.8+/−0.6 μm/sec at 29 °C, which was 3.5-fold greater than that (1.9+/−0.3 μm/sec) when bound directly to the surface as in previous studies, but similar to that caused by native chicken skeletal myosin (6.5+/−0.6 μm/sec). The actin-activated Mg-ATPase activity was similar to that of S1 before the RLC of S1 was exchanged for BDTC-cgmRLC. The results indicate that S1 can produce a normal fast movement of actin filaments as well as hydrolyse ATP and generate force.

Myosin, a motor protein that converts chemical energy driven by ATP hydrolysis to mechanical work, is responsible for muscle contraction, cell locomotion and cytoplasmic streaming. Myosin consists of two globular domains (S1) and a rod like portion. The globular domain contains the ATPase and actin binding sites, and the rod like portion is responsible for formation of the filament (1). S1 can be separated from the rod like portion by proteases such as trypsin and papain (2). S1 is convenient to use because it is highly soluble in aqueous solutions of low ionic strength and shows similar ATPase activity to that of native myosin (1). S1 has been therefore widely used in both biochemical and biophysical studies. It was, however, unknown whether S1 could produce movement and force until the development of *in vitro* motility assays that enabled the actomyosin motor to be studied in a simple and highly controlled way using only purified proteins.

Novel *in vitro* motility assays have been developed based on a technique whereby single actin filaments labeled with fluorescent phalloidin are imaged using fluorescence microscopy (3). The sliding movement of single actin filaments along myosin bound to the surface of a coverslip can be observed using fluorescence microscopy (4). Using this assay, S1 was shown to move actin filaments (5). Furthermore, manipulation of single actin filaments by a fine glass needle showed that S1 could generate forces as large as those obtained for native myosin (6). Thus, S1 was found to sustain movement of actin filaments and produce force as well as hydrolyze ATP. The movement of actin filaments propelled by S1, however, was several-fold slower than that exhibited by native myosin. This result suggests that S1 may lack some component present in the intact molecule important for generating normal fast movement.

Recently, the mechanical elementary events of myosin and its subfragments have been directly measured at a molecular level using techniques involving the manipulation of a single actin filament with microneedles (7,8) and a laser trap (9,10) with nanometer accuracy. The atomic structure of S1 has been resolved (11), allowing the mechanism of energy transduction in the actomyosin motor to be extensively studied by relating the atomic structure of S1 to the function of myosin (12). Thus it is of great importance to examine whether the slower movement of S1 observed using an *in vitro* motility assay is a genuine effect. It is possible that the slow movement of S1 observed in the previous *in vitro* motility assays may be due to damage of S1 during its interaction with the surface of the artificial substrate. To examine this possibility, we have measured the

Abbreviations: LC, light chain; RLC, regulatory LC; cgmRLC, chicken gizzard myosin RLC; BDTC-cgmRLC, biotin dependent transcarboxylase-fused cgmRLC; S1, myosin subfragment-1; BDTC-cgmRLC-S1, S1 with BDTC-cgmRLC instead of wild type RLC; *E.coli, Escherichia coli.*

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movement of actin filaments propelled by S1 when its tail end was specifically bound to the surface of a coverslip in order to minimize the damage during interaction with the surface.

S1 can be divided into two main domains; a catalytic domain that interacts with actin and hydrolyses ATP, and a neck domain that consists of a long α-helix and two different light chains, called essential and regulatory light chains, which wrap around the α-helix. In this study, S1 was bound to the surface at its tail end by attaching the regulatory light chain (RLC) to the artificial surface using a biotin-avidin system. In order to biotinylate RLC, a recombinant fusion protein consisting of biotin-dependent transcarboxylase (BDTC) and RLC was prepared. BDTC is a 123 amino acid peptide sequence of the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase (13). The biotin moiety was attached to the lysin residue (located 34 residues from the carboxyl terminus) by biotin holoenzyme ligase in *Escherichia coli* (*E.coli*) *in vivo* (14). In this study the RLC of chicken gizzard smooth muscle myosin (cgmRLC) has been used. The nucleotide sequence homology between the regulatory light chains of chicken skeletal muscle myosin and chicken gizzard smooth muscle myosin is 65% (15) and the cgmRLC has higher affinity for skeletal muscle myosin than the endogenous one (16,17). The RLC of skeletal muscle myosin can be relatively easily exchanged for cgmRLC while still maintaining the function of the myosin heads (16,18). Fusion proteins of BDTC and cgmRLC were expressed in *E.coli* and the RLC of chicken skeletal myosin S1 was then replaced by the fusion protein. S1 could then be bound to the glass surface by attaching the BDTC-cgmRLC to the surface using a biotin-avidin system. BDTC was fused at the N terminus of RLC that is located at the end of the neck domain of S1 (11). Thus, S1 is expected to attach to the surface by its tail end. The velocity of actin filaments caused by S1 bound to the surface in this manner was as fast as that obtained when using native myosin. Thus, this result indicates that S1 is fully equipped with the components essential for motor function.

MATERIALS AND METHODS

Recombinant fusion protein of BDTC and cgmRLC. CgmRLC cDNA was synthesized by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using specific oligonucleotide primers (primer1: 5′>CCCAGCTGCTCATATGTCCAGC<3′, 22 mer, primer 2: 5′> CGGCCTGCAGCTCTAATCGTCC <3′, 22 mer) in a chicken gizzard 5′-STRETCH cDNA Library (CLONTECH). Primer 1 has a Nde I recognition sequence (shown by an underline) at the point of start codon, methionine. Primer 2 has a CTA sequence (complementary for the codon of TAG) providing the stop codon. Primers 1 and 2 were synthesized based on the cDNA sequences previously described by Messen and Kendrick-Jones (15). The full-length clone for cgmRLC was subcloned into pUC119 and sequenced using a Sequencing High chemiluminescent DNA sequencing Kit (TOYOBO). The length of the coding region, excluding the initiation (ATG) and termination (TAG) codons, was 516 base pairs. The amino acid sequence was identical to that reported previously (15) and to the chemically determined chicken gizzard RLC sequence (19). The cDNA was blunted and then constructed in-frame gene into pinpoint-Xa1 protein expression vector (Promega Corporation) carrying a segment encoding a part of the 1.3 S subunit of *Propionibacterium shermanii* transcarboxylase peptide. BDTC was post-translationally biotinylated in *E.coli* (14).

The expression plasmid of the BDTC-cgmRLC fusion protein was introduced into *E.coli*, JM109, which overproduces the $lacI^q$ protein. A single transformed colony of JM109 was grown overnight in 10 ml of Super Broth medium (12 % Bacto-Trypton, 24 % Bacto-Yeast Extract, 0.5 % Glycerol, 50 mM potassium phosphate(pH 7.2), 100 μg/ml ampicillin). The overnight culture was inoculated into 1L of Super Broth medium and incubated until OD_{600}=0.2. To express the fusion protein, IPTG was added into the culture at a final concentration of 100 μM and incubated for a further 6 hours. All growth procedures were carried out at 37°C with constant vigorous agitation. The cells were centrifuged (6,000 ×g, 10 min) and stored at −80°C. All subsequent steps were performed at 0-4 °C. The BDTC-cgmRLC fusion protein was mostly present in the insoluble inclusion fraction in the cells. The recombinant protein from the inclusion fraction was obtained according to the method of Trybus and Chatman (17) without hydroxylapatite column. The crude recombinant protein (approximately 300 mg/ 6 ml in 50 mM Pipes(pH 7.0), 0.6 M NaCl, 2 M guanidine hydrochloride) was obtained from a 1L culture. A portion(5 mg) of the recombinant protein was further treated with NAP-5 (Pharmacia Biotech: Sephadex G-25 column) to exchange for 50 mM Pipes(pH 7.0), 0.5 M NaCl, 10 mM EDTA, 10 mM DTT.

Preparation of myosin subfragment-1 (S1). Skeletal muscle myosin was isolated from chicken pectoral muscles (20). Approximately 800 mg of myosin was suspended with 40 ml of a digestion buffer (10 mM Pipes(pH 7.0), 40 mM NaCl, 2 mM $MgCl_2$) and digested by papain (Worthinton, final concentration of 10 μg/ml) which was activated by 5 mM cysteine, 2 mM EDTA(pH 6.2), for 10 min at 20 °C. The protease reaction was stopped by cysteine protease inhibitor, E64 ([L-3-trans-Carboxyoxiran-2-Carbonyl]-L-Leucyl-Agmatin · $1/2H_2O$: Peptide Institute, final concentration=50 μM) on ice, and immediately centrifuged (14K, 10 min) at 4 °C to remove light meromyosin (LMM) and filamentous myosin from S1. The skeletal myosin S1 fraction was dialyzed against 10 mM Pipes(pH 7.0) and 0.1 M KCl.

Exchange of the regulatory light chain of S1 for BDTC-cgmRLC. Exchange of the RLC of S1 was performed by methods previously described (21, 16, 17) with some modificatons. Skeletal myosin S1 (1.26 mg/ml , 9.52 μM heads) was incubated with a 10-fold molar excess of the recombinant light chains (95.2 μM) in an exchange buffer (50 mM Pipes(pH 7.0), 0.5 M NaCl, 10 mM EDTA, 10 mM DTT,10 mM ATP) for 20 min at 40°C. The reaction was terminated by the addition of $MgCl_2$ to a final concentration of 12mM and cooled on ice. The hybrid myosin S1 (BDTC-cgmRLC-S1) was subsequently separated from the excess BDTC-cgmRLC and the displaced RLC by fast protein liquid chromatography (FPLC) in 10 mM Pipes(pH 7.0), 0.1 M KCl, 1 mM $MgCl_2$ on a gel filtration, Superose 6 column at a flow rate of 0.5 ml/min. The degree of exchange was determined by densitometry of SDS-PAGE patterns visualized by Coomassie Brilliant Blue R-250. After transferring the sample to a nitrocellurose membrane, biotinylated proteins were detected with streptavidin-alkaline phosphatase conjugates and bromochroloindolyl phosphate / nitro blue tetrazolium (BCIP/NBT) as a substrate.

In vitro motility assay. The *in vitro* motility assay of BDTC-cgmRLC-S1 was carried out at 29 °C as follows (22) with some modifications. The surface of a silicone-coated coverslip (23) was coated with biotinylated Bovine Serum Albumin (BSA)s by applying a solution containing biotinylated BSAs at a concentration of 1 mg/ml. Biotinylated BSAs were prepared as described (22). After washing out unbound BSAs, the bathing solution was replaced by a solution

containing streptavidins at a concentration of 1 mg/ml. The coverglass was then placed in a humidity box for 10 mins to allow the streptavidins to bind. After thoroughly washing out free streptavidins, BDTC-cgmRLC-S1 molecules were added at a concentration of 0.2 mg/ml to the streptavidins-biotinylated BSAs coated surface. Thus S1 molecules were bound to the surface by their tail ends by biotinylated BDTC-cgmRLCs via streptavidin-biotinylated BSAs attached to the surface. For S1 and monomeric myosin, the conventional assay using a silicone-coated coverslip (23) was used. Actin filaments were labeled with Phalloidin-tetramethylrhodamine B isothiocyanate conjugate (Fluka) by incubating them over night at on ice (3). Motility assays were performed in a solution containing 20 mM Hepes(pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 1 mM ATP. To reduce photo-bleaching, 4.5 mg/ml glucose, 36 μg/ml catalase, 216 μg/ml glucose oxidase, and 0.5 % 2-mercaptoethanol were added as previously described (6,23).

ATPase assay. Mg-ATPase and actin-activated ATPase assays were carried out using a modified Malachite Green method (24) with the exception of a detergent. The ATPase activities (Pi release/sec/head) were measured in 20 mM Hepes (pH7.8), 1 μM S1, 25 mM KCl, 5 mM $MgCl_2$, 40 μM ATP at 25 °C for Mg-ATPase and in 20 mM Hepes(pH7.8), 0.1 μM S1, 10 μM actin, 25 mM KCl, 5 mM $MgCl_2$, 200 μM ATP at 25 °C for actin-activated ATPase.

RESULTS AND DISCUSSION

Exchange for recombinant BDTC-cgm RLC. In order to bind S1 to the surface of a coverslip by its tail end without damaging the molecule upon interacting with the artificial surface, an endogenous RLC of S1 was replaced by a biotinylated cgmRLC and the biotinylated cgmRLC was bound to the surface using a biotin-avidin system. The biotin-avidin system is very useful for specific and stable binding between proteins (25). Biotinylated cgmRLCs were obtained by preparing recombinant fusion proteins of biotin dependent transcarboxylase (BDTC) and cgmRLC (see Materials and Methods). Exchange for biotinylated fusion proteins of BDTC-cgmRLC was performed according to methods previously described (15,17,21) with slight modifications (see Materials and Methods). Fig. 1 shows SDS-PAGE patterns stained by CBB (lanes 1-3) and streptavidin-alkaline phosphatase conjugates and BCIP/NBT as a substrate (lanes 4-6). The line corresponding to RLC almost disappeared from the CBB-staining pattern (lane 3), and instead a new line corresponding to that expected from the molecular weight of BDTC-cgmRLC was present (lane 3). This line was stained by streptavidin-alkaline phosphatase conjugates and BCIP/NBT substrate that colors biotinylated proteins (lane 6). Densitometry of the gel patterns showed that more than 90% of RLC of S1s were exchanged for BDTC-cgmRLC (data not shown).

ATPase activity. Mg-ATPase activity of BDTC-cgmRLC-S1 (0.09 Pi/sec/head, at 25 °C) was similar to that of S1 (0.09 Pi/sec/head, at 25°C). In the presence of 10 μM actin, actin-activated ATPase activity of BDTC-cgmRLC-S1 (5.5 Pi/sec/head, at 25 °C) was also similar to that of S1 (6.0 Pi/sec/head, at 25 °C). This result agrees well with a previous study where

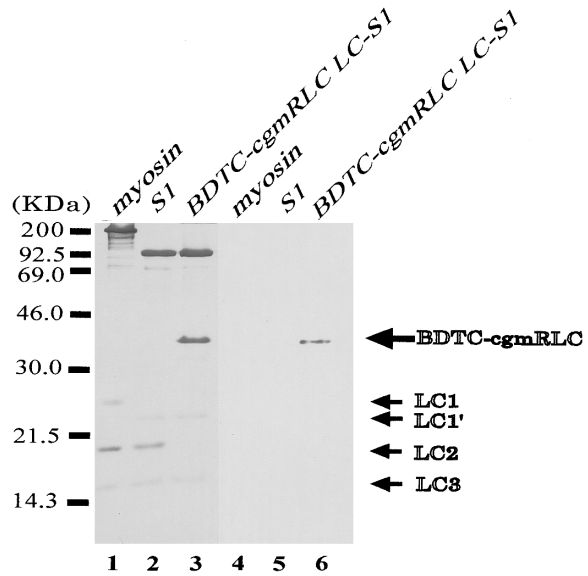

FIG. 1. SDS-PAGE of myosin (lanes, 1 and 4), S1 (lanes, 2 and 5) and S1 of which RLC was replaced by BDTC-cgmRLC (lane, 3 and 6). Gels were stained with Coomassie Brilliant Blue R-250 (lanes, 1-3) and biotinylated proteins were detected with streptavidin-alkaline phosphatase and bromochroloindolyl phosphate / nitro blue tetrazolium (BCIP/NBT) substrate (lanes, 4-6). The first lane is the standard molecular weight markers. An arrow marked by BDTC-cgmRLC points to the exchanged recombinant light chain (Calculated MW: 33,493). LC2 indicates RLC, and LC1 and LC3 indicate essential light chain (ELC). LC1′ indicates an ELC, of which N-terminal trimethylated peptide was cleaved by papain digestion. Approximately 5.5 μg, 2.0 μg, 2.0 μg, 1.4 μg, 0.4 μg, 0.4 μg protein were separated in lane 1, 2, 3, 4, 5, 6, respectively.

replacement of RLC of rabbit skeletal muscle myosin for cgmRLC did not change the actin-activated ATPase activities (17).

Sliding velocities of actin filaments propelled by S1 and myosin. Fig.2a shows the velocities of actin filaments propelled by papain digested chicken skeletal muscle S1 when directly bound to the surface of a silicone-coated coverslip. The velocity was 1.9+/−0.3μm / sec (+/−SD, n=68) at 29 °C, which was 3.5-fold smaller than that obtained by native chicken skeletal muscle myosin (6.5+/−0.7 μm/sec (+/−SD, n=152)) (Fig.2c). This result is consistent with that reported for rabbit skeletal muscle S1 (5). Fig.2b shows the velocity of actin filaments propelled by S1 containing a biotinylated BDTC-cgmRLC when the S1 was bound at its tail end to the surface by reacting a biotinylated BDTC with a streptavidin-BSA adsorbed onto the surface. The velocity was 6.8+/−0.6 μm/sec (+/−SD, n=104), which was faster than that caused by S1 when directly bound to the surface (Fig.2a) but similar to that produced by native myosin (Fig.2c). It has been reported previously that papain digested S1 can produce similar force to that by myosin *in vitro,* even when S1 was directly bound to the surface (6). Thus, this result indicates that S1 contains all essential factors for motor function.

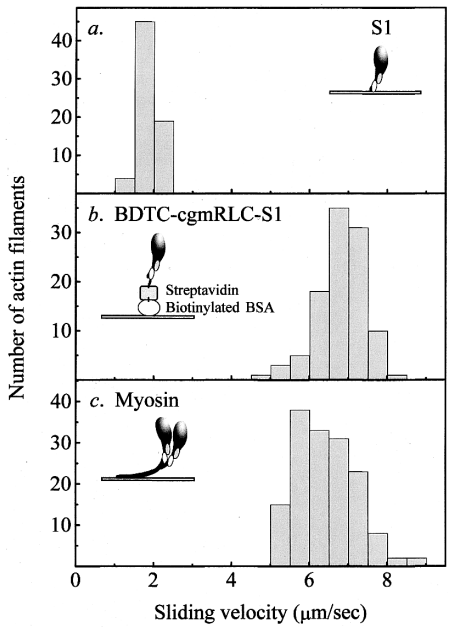

FIG. 2. Histograms of the sliding velocities of actin filaments propelled by S1s when directly bound to the silicone-coated surface (a) and by BDTC-cgmRLC-S1s when specifically bound to the surface at its tail end using the biotin-avidin system to minimize the damage caused upon interacting with the surface (b), and by whole myosins when bound to the silicone-coated surface in monomeric form (c). Each histogram shows the sliding velocities of >50 individual actin filaments. Assay medium, 20mM Hepes(pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 1 mM ATP. Temperature, 29 °C.

The slow velocities produced by S1 reported previously are probably due to the direct interaction of S1 with the glass surface. Recently, Waller and colleagues (26) reported that the velocity of actin filaments produced by S1 of rabbit skeletal muscle obtained by papain digestion increased from 2.5 to 4 μm/sec at 30 °C when S1 was attached to a nitrocellulose-coated coverslip via a specific antibody for the N terminus of RLC. Although the velocity of sliding was increased, it was still approximately one-half of that obtained when using native myosin (7-8 μm/sec at 30 °C). The N terminus of RLC is located in the end of the S1 molecule (11), so S1 would be attached to the surface by its tail end, and neither the catalytic or neck domains of S1 should have been damaged during the interaction with the surface. The velocity, however, was smaller than that produced by native myosin. Our study showed that when S1 was bound to the surface by its tail end the sliding velocity of actin filaments propelled by S1 was not different to that produced by myosin. So how we can account for this discrepancy? When the endogenous RLC of S1 was exchanged for BDTC-cgmRLC, no change in the sliding velocity was observed when these molecules were directly bound to the silicone-coated surface without using the biotin-avidin system (data not shown). It is therefore unlikely that BDTC-cgmRLC accelerated the movement of S1. Recently, it has been suggested that the mobility of the neck domain of myosin is important in producing efficient actin filament movement (27). The slow velocity obtained by Waller and colleagues (26) may be due to differences in the flexibility of the joint between the RLC of S1 and the surface, when S1 is attached with an antibody for the N terminus of RLC.

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