

Expression and Characterization of a Functional Myosin Head Fragment in *Dictyostelium discoideum*

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The isolated head fragment of myosin is a motor protein that is able to use energy liberated from the hydrolysis of adenosine triphosphate to cause sliding movement of actin filaments. Expression of a myosin fragment nearly equivalent to the amino-terminal globular head domain, generally referred to as subfragment 1, has been achieved by transforming the eukaryotic organism *Dictyostelium discoideum* with a plasmid that carries a 2.6-kilobase fragment of the cloned *Dictyostelium* myosin heavy chain gene under the control of the *Dictyostelium* actin-15 promoter. The recombinant fragment of the myosin heavy chain was purified 2400-fold from one of the resulting cell lines and was found to be functional by the following criteria: the myosin head fragment copurified with the essential and regulatory myosin light chains, decorated actin filaments, and displayed actin-activated adenosine triphosphatase activity. In addition, motility assays in vitro showed that the recombinant myosin fragment is capable of supporting sliding movement of actin filaments.

MYOSINS CONSTITUTE A FAMILY of diverse proteins that bind to actin and have Mg^{2+} -dependent adenosine triphosphatase (ATPase) activity that is stimulated by actin at low ionic strength. Interaction of myosins with actin in the presence of adenosine triphosphate (ATP) results in the conversion of chemical energy into mechanical force and displacement. Myosins occur in nearly every eukaryotic cell examined, where they participate in many fundamental cellular processes ranging from muscle contraction to cytokinesis. Two subgroups of myosins are known. The first group consists of the double-headed or conventional myosins, generally referred to as myosin or sometimes as myosin II. The second group consists of the single-headed or unconventional myosins, referred to as myosin I. In this report we will only refer to

conventional myosins, all of which show the same structural pattern. They consist of two heavy chains (~200 kD each) and two pairs of light chains (15 to 20 kD each). The NH_2 -terminal half of the myosin heavy chain (MHC) forms the globular head, which contains the binding sites for the myosin light chains, and the $COOH$ -terminal half forms the extended coiled-coil rod. The globular head fragment of the myosin molecule, also called subfragment-1 or S1, can be released as a soluble fragment by proteolytic cleavage of myosin and has the catalytic and actin-binding properties of the myosin molecule (1, 2). It has been shown that S1 alone is sufficient to cause sliding movement of actin filaments in vitro (3).

Understanding the mechanism by which myosin catalyzes the transduction of energy stored in chemical bonds into mechanical work will require knowledge of the high-resolution structure of S1 and the ability to manipulate the protein in specific manners at the molecular level. The S1 crystals ob-

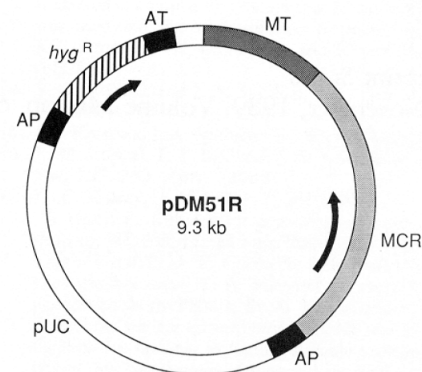


Fig. 1. Physical map of the myosin head fragment expression vector pDM51R. The expression vector is a derivative of the hygromycin resistance vector pDE102 (6) carrying 2.6 kb of 5' coding region of myosin (MCR). The MHF expression cassette lies in the opposite orientation (shown by arrows) to the hygromycin phosphotransferase gene (*hyg^R*), and the two genes are separated in this construct by 1.1 kb of *mhcA* terminator sequence (MT) and 0.3 kb of *act-15* terminator sequence (AT). Both the MCR and *hyg^R* sequences are controlled by the *act-15* promoter (AP). The white segments represent pUC119 sequences (pUC).

tained by Rayment and Winkelmann (4) should help in determining the high-resolution structure of the molecule. However, a source of S1 without the inherent heterogeneity of preparations made by proteolytic digestion and that allows the introduction of specific alterations into the molecule by molecular genetic approaches would be desirable. We have addressed this problem by exploring the use of the cellular slime mold *Dictyostelium discoideum* as an expression system for myosin fragments.

The plasmid pDM51R (Fig. 1), which was constructed to achieve expression of a myosin head fragment (MHF) nearly equivalent to S1, carries a translational fusion of the eighth codon of the *Dictyostelium act-15* gene to the second codon of the *Dictyostelium mhcA* gene. The polypeptide encoded by this fusion has 871 amino acids and extends 46 amino acids beyond the proline residue that marks the region of proteolytic cleavage in muscle myosin. The vector also contains a bacterial hygromycin resistance gene for selection in *Dictyostelium* (5) as well as pUC119 sequences (6) for selection and autonomous replication in *Escherichia coli*. Transformations of *Dictyostelium* axenic strain AX2 by the calcium phosphate precipitate technique were carried out according to the modified protocol described by Egelhoff *et al.* (5). Transformation efficiencies of $\sim 10^{-6}$ were observed. As pDM51R cannot replicate autonomously in *Dictyostelium*, all stable transformants must result from an integration event. The relative amounts of MHF produced by the transformants were estimated from immunoblots of whole-cell

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lysates probed with antibodies directed against the myosin head, in conjunction with Coomassie blue-stained SDS-polyacrylamide gels (7). For the immunoblotting, we used both a polyclonal antibody (P-5020) to a peptide corresponding to amino acids 600 to 616 in the *Dictyostelium* myosin sequence, a region that corresponds to the junction of the 20- and 50-kD myosin head domains (8), and a monoclonal antibody (M II.42) to the head region of *Acanthamoeba* myosin II. Expression of a polypeptide of the appropriate size was only detected in cells transformed with the expression vector. Cell line HS2210 produced the largest amounts of MHF and was used for the purification and characterization of the recombinant protein. These cells show normal morphology and growth rate and upon starvation undergo a complete developmental cycle. No changes in the rate of MHF expression have been observed in 8 months of maintaining this cell line.

MHF was purified from HS2210 cells that had been starved for 4 hours to induce development (9). Development was found to enhance the expression of MHF and to decrease the activity of proteases in crude cell extracts. The purification procedure re-

sulted in an approximately 2400-fold enrichment of the recombinant MHF. An average of 0.05% of the total cell protein was recovered as purified MHF, which corresponds to a yield of 2.5 mg of MHF from 100 g of cells.

The expression of a functional MHF in *Dictyostelium* relies in part on the fact that this organism can supply the expressed fragment of the MHC with its endogenous essential (ELC) and regulatory (RLC) myosin light chains. Analysis of the purified MHF by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of all three polypeptides. The relative mobility of the expressed fragment corresponded to that of a 95-kD polypeptide. The two bands corresponding in size to the *Dictyostelium* light chains had molecular masses of 18 kD (RLC) and 16 kD (ELC) (Fig. 2A). Furthermore, the presence of the RLC was confirmed by immunoblotting with a monoclonal antibody, My 8 (10), to the *Dictyostelium* RLC (11).

One of the most distinctive properties of a functional myosin head is the ATP-dependence of its interaction with actin. In the absence of ATP, the head portion of the myosin molecule forms a rigor complex with

actin filaments, giving rise to a typical arrowhead structure that can be detected by electron microscopy (12). The expressed MHF formed an arrowhead structure with F-actin (Fig. 2B). Similar to the situation found for muscle myosin and endogenous *Dictyostelium* myosin, the expressed MHF had ATPase activity at low ionic strength in the presence of Mg^{2+} that was greatly stimulated by actin (13). The maximal actin-activated ATPase activity measured with rabbit skeletal muscle actin was 480 nmol of inorganic phosphate (P_i) per minute per milligram of MHF. This corresponds to a turnover rate of one ATP molecule per second, which matches the turnover rate of 0.9 ATP molecules per second per head observed for *Dictyostelium* myosin (14). In the absence of actin and at high ionic strength, the expressed MHF had a high ATPase activity in the presence of Ca^{2+} (950 nmol of P_i per minute per milligram of MHF) and a considerably lower ATPase activity in the presence of K^+ and EDTA (65 nmol of P_i per minute per milligram of MHF), as is the case for native *Dictyostelium* myosin. Further evidence for the expression of a functional MHF was obtained when we tested the ability of the purified protein to move actin filaments labeled with tetramethylrhodamine-phalloidin in an in vitro assay system (3). Fragmentation and sliding movement of filaments occurred after infusion of the magnesium salt of ATP into the flow cell. The MHF supported actin movement at a mean rate of 130 nm/s (Fig. 2C). Similar to the situation observed with muscle myosin (3), the rate of movement of the head fragment is slower than the rate of 1 $\mu\text{m/s}$ observed with intact *Dictyostelium* myosin (15).

The expression and purification of an MHF capable of creating motive force demonstrates that it should now be possible to dissect the motor function of myosin by molecular genetic approaches. Indeed, we have successfully expressed a myosin head fragment that includes approximately 50 nm of coiled-coil rod. This myosin fragment, equivalent in size to the proteolytically defined muscle heavy meromyosin heavy chain, binds both light chains, decorates actin filaments to produce arrowheads, and has a maximal actin-activated ATPase activity of 880 nmol of P_i per minute per milligram of protein (16).

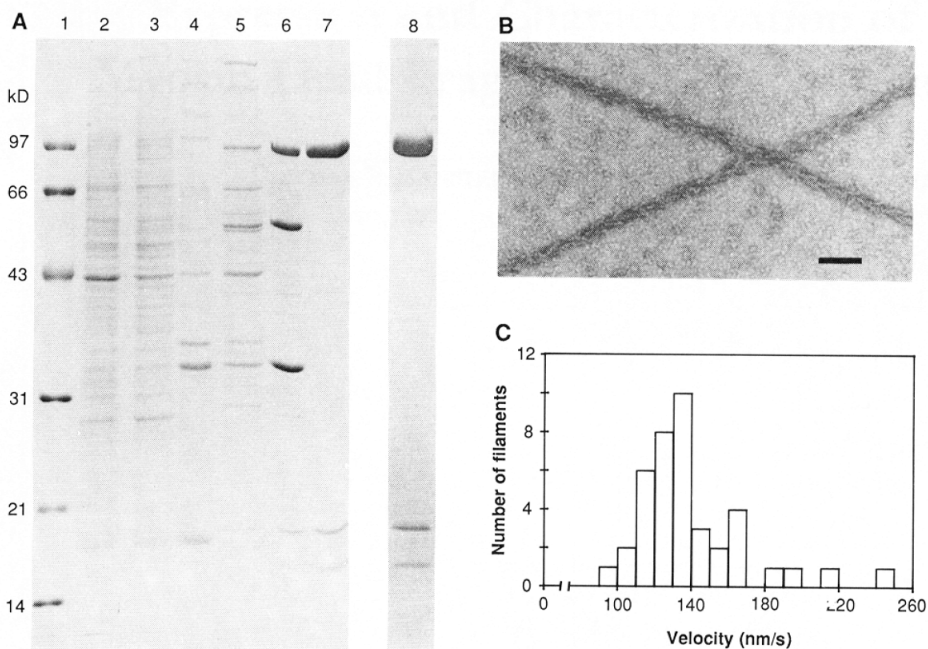


Fig. 2. (A) SDS-polyacrylamide electrophoresis of *Dictyostelium* MHF at various stages of purification. Samples subjected to SDS-PAGE (12.5% separating gel) were molecular mass markers (lane 1), crude cell lysate (lane 2), supernatant after actomyosin precipitation (lane 3), actomyosin pellet after Mg-ATP extraction (lane 4), Mg-ATP-extracted protein (lane 5), flow-through after first DEAE column (lane 6), pool of MHF-containing fractions after second DEAE column (lane 7), pool of MHF-containing fractions after gel filtration column (lane 8). In each case, 3 μg of protein was loaded, except for lane 8, where 5 μg was loaded. The gel was stained with Coomassie brilliant blue R. (B) Electron micrograph of filamentous actin from rabbit muscle decorated with the expressed MHF. The arrowhead pattern is like that of filamentous actin decorated with S1 obtained by proteolytic cleavage of muscle myosin. Because of the decoration, the 9-nm-wide actin filament appears much wider (17). Scale bar, 100 nm. (C) Histogram showing the sliding speed at 25°C of 40 actin filaments moving on a nitrocellulose surface covered with *Dictyostelium* MHF. MHF was applied to the flow cell at a concentration of 100 $\mu\text{g/ml}$.

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9. Cell line HS2210 was grown in 6-liter Erlenmeyer flasks, each containing 2.5 liters of HL5 medium. Cells were shaken on a gyratory shaker at 200 rpm at 22°C. MHF was typically purified starting with 40 liters of culture medium, which yielded about 250 g of cells. Cells were harvested at a density of 5×10^6 cells per milliliter by centrifugation for 7 min at 2500 rpm in an IEC PR-6000 centrifuge. To initiate development, cells were suspended at a density of 2×10^7 cells per milliliter in a solution containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8), 0.2 mM CaCl₂, and 2 mM MgSO₄ and shaken at 140 rpm for 4 hours. All remaining manipulations were performed at 4°C. The developed cells were washed once with a solution containing 10 mM tris (pH 8.0) and 1 mM EDTA and suspended in 1 ml of lysis buffer [50 mM Hepes (pH 7.5), 20 mM sodium pyrophosphate, 2 mM EDTA, 1 mM dithiothreitol (DTT), 30% (w/v) sucrose, 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF), 5 mM benzamidine, *N*-tosyl-L-phenylalanine chloromethyl ketone (20 µg/ml), *N*- α -p tosyl-L-lysine chloromethyl ketone (20 µg/ml), and leupeptin (10 µg/ml)] per gram of cells. Cells were lysed by sonication with a Heat-System Ultrasonic model W 220-F sonicator equipped with a 0.75-inch flat tip. Unbroken cells and most of the cell debris were removed by centrifugation for 15 min at 40,000g. The supernatant was adjusted to 0.1M KCl using a 3M stock solution and then centrifuged at 100,000g for 3 hours. A key step in the purification scheme, resulting in a 140-fold enrichment in MHF, was the formation of an actomyosin pellet by removal of the sucrose from the resulting supernatant [M. Clarke and J. A. Spudich, *J. Mol. Biol.* **86**, 209 (1974)] and the specific release of MHF from this pellet by addition of the magnesium salt of ATP (Mg-ATP). The supernatant was dialyzed for 48 hours against 10 mM 1,4-piperazine-diethanesulfonic acid (pH 6.9), 50 mM KCl, 2 mM EDTA, 0.5 mM DTT, 0.2% NaN₃, 1 mM benzamidine, and 0.5 mM PMSF. The actomyosin precipitate formed during dialysis was collected by centrifugation at 27,000g for 15 min and washed with HKE buffer [10 mM Hepes (pH 7.5), 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, and 1 mM benzamidine]. The MHF was solubilized by extracting the actomyosin pellet with HKE buffer containing 10 mM Mg-ATP in a Dounce-type homogenizer. The suspension was centrifuged at 48,000g for 15 min. To induce the formation of actin paracrystals, we increased the magnesium concentration of the supernatant to 12 mM. After incubation on ice for 30 min, 1 mM ATP was added to the now turbid solution, and the actin paracrystals were removed by centrifugation at 100,000g for 1 hour. A further fourfold enrichment in MHF was achieved by passing the resulting clear supernatant through a column (1.5 by 20 cm) containing a weak anion-exchanger matrix (Toyopearl DEAE-650 S) equilibrated with HKE buffer. The flow-through, containing the MHF, was collected and dialyzed overnight against HE buffer [20 mM Hepes (pH 7.5), 0.5 mM EDTA, 0.5 mM DTT, and 1 mM benzamidine]. The dialysate was then loaded onto a column (1.5 by 20 cm) containing a strong cation-exchanger matrix (Toyopearl SP-650 S) connected to a DEAE-650 S column (1.0 by 18 cm). Washing with HE buffer was continued as long as the flow-through showed absorbance at 280 nm. The SP-650 S column was then removed, and MHF was eluted from the DEAE-650 S column with a 75-ml gradient from 0 to 500 mM KCl in HE buffer. This combined cation-anion exchanger step led to a further fourfold enrichment for MHF. After this step the MHF could be used successfully both for the decoration of actin filaments and in an in vitro movement assay. Traces of impurities could be removed by passing the MHF over a Superose 12 gel filtration column.
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13. Actin-activated ATPase activity was measured by adding 10 µg of MHF to 0.5 ml of assay solution containing 17 mM KCl, 2.7 mM MgCl₂, 0.7 mM EGTA, 0.7 mM DTT, 17 mM Hepes (pH 7.4), and actin. The final concentrations of actin in the assay solution were 0, 1, 2.5, 5, 10, and 20 µM. All assays were performed at 30°C. Samples (100 µl) were removed after 5, 10, 15, and 20 min, added to 50 µl of stop-buffer containing 13.3% (w/v) SDS and 0.12M EDTA, and mixed vigorously. The release of phosphate was then determined by addition of 350 µl of freshly prepared developer solution [0.5M H₂SO₄, 0.5M ammonium molybdate, and 0.5% (w/v) FeSO₄]. The absorbance of 550 nm was measured [H. D. White, *Methods Enzymol.* **85**, 698 (1982)]. ATPase activity in the presence of Ca²⁺ was measured with 10 µg of MHF in a 0.5-ml assay volume. The assay solution contained 0.6M KCl, 5 mM CaCl₂, 4 mM ATP, and 10 mM Hepes (pH 7.4). ATPase activity in the presence of EDTA was measured in a similar way except that 25 µg of MHF was used and the assay solution contained 0.6M KCl, 5 mM EDTA, 4 mM ATP, and 10 mM Hepes (pH 7.4).
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18. We thank E. Kubalek for providing micrographs showing MHF-decorated actin filaments; J. W. La-Claire II and T. Q. P. Uyeda for help with the in vitro motility assay; T. A. Mietzner for the gift of peptide antibody P5020; D. A. Kaiser and T. D. Pollard for the gift of antibody M II.42; and T. T. Egelhoff for his help in finding suitable expression vectors for *Dictyostelium*. Supported by NIH grant GM 33289 (J.A.S.). D.J.M. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (Ma 1081-2/2).

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