

Monoclonal Antibodies against Seven Sites on the Head and Tail of *Dictyostelium* Myosin

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ABSTRACT Ten monoclonal antibodies (My1–10) against *Dictyostelium discoideum* myosin were prepared and characterized. Nine bound to the 210-kD heavy chain and one (My8) bound to the 18-kD light chain. They defined six topographically distinct antigenic sites of the heavy chain. Five binding sites (the My1, My5, My10 site, and the My2, My3, My4, and My9 sites) are located on the rod portion of the myosin molecule. The position of the sixth site (the My6 and My7 site) is less certain, but it appears to be near the junction of the globular heads and the rod. Three of the antibodies (My2, My3, and My6) bound to myosin filaments in solution and could be sedimented in stoichiometric amounts with the filamentous myosin. In contrast, My4, which recognized a site on the rod, inhibited the polymerization of monomeric myosin into filaments. A single antibody (My6) affected the actin-activated ATPase of myosin. The nature of the effect depended on the valency of the antibody and the myosin. Bivalent IgG and F(ab')₂ fragments of My6 inhibited the actin-activated ATPase of filamentous myosin by 50% whereas univalent Fab' fragments increased the activity by 50%. The actin-activated ATPase activity of the soluble chymotryptic fragment of myosin was increased 80–90% by both F(ab')₂ and Fab' of My6.

Dictyostelium serves as a model system for the investigation of many complex cellular processes (see reference 9 for review). It is useful in the study of the molecular basis of cell motility because many complex cellular movements (such as pseudopod extension, phagocytosis, cytokinesis, and karyokinesis) which are typical of eukaryotic cells occur in amoebae of *Dictyostelium*. Several cytoskeletal proteins of *Dictyostelium* have been purified and characterized (see reference 18 for review). *Dictyostelium* myosin (*D.* myosin)¹ was first characterized by Clarke and Spudich (1) and is like skeletal muscle myosin in its structure and function. The molecule is composed of two heavy chains of 210 kD each and two each of two classes of light chains of 18 and 16 kD. The globular head region exhibits ATP-dissociable binding to actin and an actin-activated ATPase activity. The coiled-coil tail region, called the rod, is involved in the formation of filaments at low ionic strength. The 18-kD light chain and the rod portion of the heavy chain of *D.* myosin are phosphorylated in vivo (7, 16). Phosphorylation of the myosin heavy chain inhibits the formation of thick filaments in vitro (7).

Monoclonal antibodies that bind to different sites on *D.*

myosin can provide specific probes for studying the structural and functional properties of this myosin. Claviez et al. (2) used electron microscopy to map the binding sites of five monoclonal antibodies against *D.* myosin. All five bind to the rod. The preparation and characterization of ten additional anti-myosin monoclonal antibodies, one of which binds to the 18-kD light chain, is presented here. Their effects on the assembly of myosin and its actin-activated ATPase is discussed. An accompanying paper by Flicker et al. (3) describes the positions of the antigenic determinants as visualized by electron microscopy and the effects of the antibodies on in vitro myosin-mediated motility.

MATERIALS AND METHODS

Myosin and Actin: RNA-free myosin was isolated and purified from *Dictyostelium discoideum* strain Ax-3 as previously described (11) with minor modifications (Griffith, L. M., and J. A. Spudich, manuscript in preparation). The chymotryptic soluble fragment of *D.* myosin was prepared as previously described (16). Actin was purified from rabbit skeletal muscle (19). Rabbit skeletal muscle myosin was purified by the procedure of Kielley and Harrington (5). *Acanthamoeba* myosin and turkey gizzard myosin were gifts from Dr. D. Kiehart (The Johns Hopkins University) and Dr. Z. Cande (University of California at Berkeley), respectively.

Monoclonal Antibody Production: Production of hybridomas was according to the method of Parham (12). BALB/c mice received three

¹ Abbreviations used in this paper: *D.* myosin, *Dictyostelium* myosin; RAM, rabbit anti-mouse IgG; RIA, radioimmunoassay.

intraperitoneal injections of 20–25 μg of purified *D. myosin* in Freund's adjuvant at 3-wk intervals. The first immunization was with complete adjuvant and the other two were with incomplete adjuvant. 1 wk after the third immunization, plasma was tested for antibody binding to purified *D. myosin* by solid-phase radioimmunoassay (RIA). Specific binding at titers of 1/625 was obtained. Three mice were selected and each received one intravenous injection of 20 μg of *D. myosin* in phosphate-buffered saline (PBS) (Gibco Laboratories, Grand Island, NY) per day for 4 d prior to fusion. Spleen cells were fused to the myeloma cell line, Sp2/0-Ag14 (17). Hybridomas were screened for production of antibodies binding to purified *D. myosin* by solid-phase RIA. Hybridomas were cloned by fluorescence-activated cell sorting and then passaged as ascites tumors in BALB/c mice. 50–200 ml of ascites fluid was obtained for each and the immunoglobulin was purified according to procedures described by Parham et al. (14).

Solid-phase RIA: All assays were performed in Dynatech microtiter plates (Dynatech Precision Sampling Corp., Baton Rouge, LA) at 23°C. PBS with 0.5% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) was the assay buffer used for dilutions and washing. Proteins were iodinated using chloramine-T (4, 10).

25 μl of myosin (5–10 mg/ml) in PBS was placed in a well of a microtiter plate for 2–4 h. After washing the wells three times and incubating them with assay buffer for 0.5 h, 25 μl of the monoclonal antibody solution was added for 1–2 h. The wells were then washed three times, and 25 μl of ^{125}I -F(ab')₂ of rabbit anti-mouse IgG (RAM) containing 250,000 cpm was added. After 1 h, the wells were again washed three times before separation with a hot-wire cutting device. γ -Counting for bound radioactivity was performed using a Beckman γ -4000 (Beckman Instruments, Inc., Palo Alto, CA). The level of nonspecific binding was determined by counting microtiter plate wells coated with BSA and wells coated with myosin incubated with a nonspecific control antibody of the same isotype, X63 (6).

Blocking studies were performed by adding 12.5 μl of the appropriate dilution of unlabeled blocking antibody to a myosin-coated microtiter well for 45 min. Then 12.5 μl of ^{125}I -labeled antibody was added for an additional 30 min. The supernatant was removed without addition of assay buffer before washing three times with assay buffer and γ -counting.

Isotype Determination: Determination of the isotype of antibodies was performed by solid-phase RIA as described above using affinity-purified goat antibodies that are specific for binding to mouse IgG of isotype IgG1 or IgG2 as iodinated second step reagents (15). For each of the 10 monoclonal anti-myosin antibodies the amount of iodinated anti-IgG1 bound was 80% or more of the amount of iodinated RAM bound, whereas the amount of iodinated anti-IgG2 bound was similar to the amount of binding in microwells containing nonspecific control IgG.

PAGE and Immunoblotting: SDS PAGE was carried out as described (8). Slab gels were scanned with a Transidyne RFT scanning densitometer (Transidyne General Corp., Ann Arbor, MI) at 600 nm. The relative amount of protein present in a single band was quantitated by cutting out and weighing the corresponding peak. The absolute amount of protein present was determined by comparing the weight of a peak with the weight of peaks generated from electrophoresis of known amounts of the same protein.

Immunoblotting was performed according to Towbin et al. (20). After SDS PAGE, proteins were electrophoresed out of the gel and onto nitrocellulose paper. The nitrocellulose paper was incubated in a 3% BSA solution to saturate protein-binding sites. Then the nitrocellulose papers were placed in a solution containing 10 $\mu\text{g}/\text{ml}$ of the monoclonal antibody, 5% heat-inactivated rabbit serum, 3% BSA, 0.02% SDS and 0.2–1.0% Triton X-100. After a series of washings, the nitrocellulose papers were placed in solutions containing 5×10^5 cpm ^{125}I -RAM/ml. Subsequently, the nitrocellulose papers underwent another series of washings and were dried prior to autoradiography using Kodak X-Omat X-Ray film with a Dupont Cronex Intensifying screen (DuPont Co., Wilmington, DE).

Sedimentation Assays: Binding to myosin filaments was tested by incubating 12 μg myosin and 14.4 μg IgG in 150 μl of 25 mM PIPES (pH 6.8), 5 mM MgCl₂, 0.1 mM CaCl₂, and 15 mM KCl at 23°C for 30 min. The samples were then centrifuged in a Beckman airfuge (Beckman Instruments, Inc.) at 17 pounds per square inch (psi) for 17 min. The pelleted material was resuspended in 30 μl of buffer for SDS PAGE and densitometric quantitation. As a control, the same volume of buffered solution containing either myosin or antibody alone was centrifuged at the same time. For the three monoclonal antibodies that bound to filamentous myosin, additional sedimentation experiments were performed under the same conditions except the amount of immunoglobulin added was changed or 25 μg of actin was added to the reaction mixture.

Sedimentations with the soluble fragment of *D. myosin* were performed in 100 μl of solution containing 3 μg of soluble fragment, 7 μg of antibody, 25 μg of actin, and the same buffer as above. The samples were incubated at 23°C for 30 min and then centrifuged in a Beckman airfuge (Beckman Instruments,

Inc.) at 8 psi for 10 min to pellet the F-actin with its associated proteins. The pelleted material was resuspended in 30 μl of buffer prior to SDS PAGE and densitometric quantitation.

To examine the inhibition of filament formation, we performed sedimentation assays by incubating 14.2 μg of IgG with 12 μg of myosin in 15 μl of 0.5 M KCl, 10 mM PIPES (pH 6.8), 1 mM dithiothreitol, and 1 mM EDTA for 40 min at 23°C. The solution was then diluted 10-fold with buffer containing 20 mM PIPES (pH 6.8), 5 mM MgCl₂, and 1 mM CaCl₂, and then incubated for 20 min to allow filaments to form. The samples were centrifuged in a Beckman airfuge (Beckman Instruments, Inc.), and the pelleted material was resuspended in 30 μl of buffer prior to SDS PAGE and densitometric quantitation. The amount of myosin heavy chain sedimenting in the presence of IgG is expressed as a percentage of the amount sedimenting in the absence of IgG.

ATPase Assay: ATPase activity was measured using [γ -³²P] ATP, as described (1). The assay was in 100 μl of solution containing 25 mM PIPES (pH 6.8), 5 mM MgCl₂, 0.1 mM CaCl₂, 15 mM KCl, 1 mM ATP, either 6 μg of myosin or 1–3 μg of soluble fragment, and the indicated amount of actin or monoclonal antibody. The solutions were incubated at 25°C for 15–20 min. The actin-activated ATPase activity was calculated by subtracting the activity found for myosin or soluble fragment alone (2–5% of the actin-activated activity with excess actin).

Preparation of F(ab')₂ and Fab' Fragments of My6: Antibody fragments were made from My6 IgG as described (13). IgG was digested with pepsin (25 $\mu\text{g}/\text{ml}$) in 0.1 M citrate (pH 3.5) for 12 h at 37°C to produce F(ab')₂. The reaction was stopped by addition of 1 M Tris-HCl (pH 8.0) to a final concentration of 0.2 M. L-cysteine was added to one-half of the F(ab')₂ preparation to a concentration of 15 mM, and incubated at 37°C for 4 h. Iodoacetamide was then added to a concentration of 30 mM and incubated for 12 h at 4°C to produce Fab'. The Fab' and F(ab')₂ preparations were then dialyzed against 10 mM KCl, 5 mM Tris-HCl (pH 7.5).

RESULTS

Monoclonal Anti-Dictyostelium Myosin

Ten hybridomas (My1–10) which secrete IgG1 antibodies against myosin were produced. They were passaged as ascites tumors and the immunoglobulin purified from the ascitic fluid by sequential ammonium sulfate fractionation, Sephadex G-200, and DEAE column chromatography (14).

Indirect solid-phase RIA showed that My1–10 bind to purified *D. myosin* (Fig. 1). All except My7 and My8, which appear to have a lower affinity for myosin, exhibited a plateau of binding extending to immunoglobulin concentrations of 1 $\mu\text{g}/\text{ml}$ (Fig. 1). The antibodies bound poorly to myosins purified from other cells (Fig. 2). The strongest cross-reaction was seen with My4 binding to smooth muscle myosin. Even in this case more than 100 $\mu\text{g}/\text{ml}$ of My4 was required to bind an amount of radioactivity equivalent to that bound to *D. myosin* with a 1,000-fold lower antibody concentration.

Localization of the Sites of Antibody Binding by Immunoblotting

We used immunoblotting to determine to which subunit of *D. myosin* the monoclonal antibodies bind. Nine antibodies bound to the 210-kD heavy chain (Fig. 3), and one antibody, My8, bound to the 18-kD light chain (Fig. 4). None bound to the 16-kD light chain. When tested against a crude cytoplasmic extract of *Dictyostelium*, all nine antibodies bound specifically to a polypeptide that co-migrated with the appropriate subunit of purified *D. myosin*. In addition, My4 and My5 bound to a 35-kD protein in the cytoplasmic extract that may be a fragment of the myosin heavy chain.

We used immunoblotting to further localize the binding sites of the nine antibodies against the heavy chain. Chymotrypsin cleaves *D. myosin* into a soluble, globular, NH₂-terminal head fragment (soluble fragment), and into a filamentous, COOH-terminal tail fragment, both of which mi-

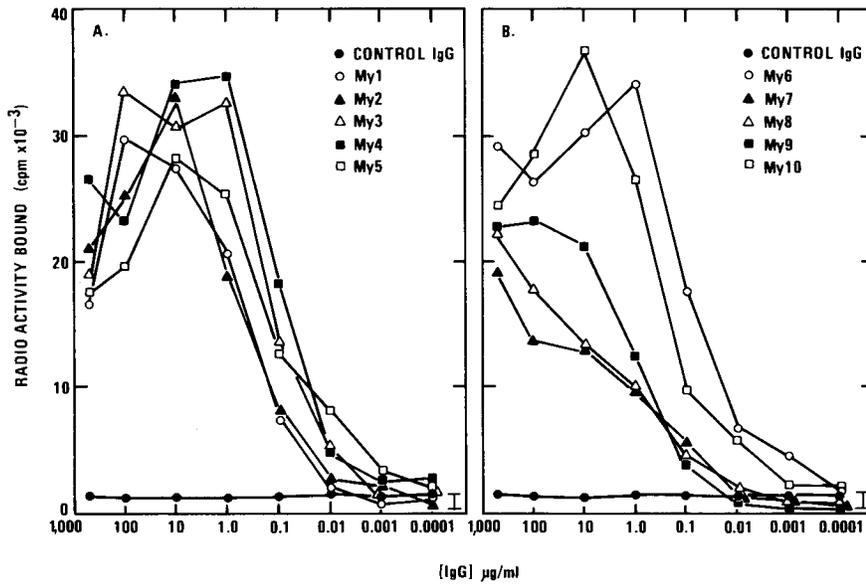


FIGURE 1 Titration of My1-10 against *D. myosin* in solid-phase RIA (My1-5 are in A; My6-10 in B; control IgG was X63). The concentration on the abscissa is that of the monoclonal antibody in the first incubation of the assay. The bar in the bottom right hand corner of each panel indicates the range of values obtained when the highest concentration of monoclonal IgG was incubated in microtiter wells coated with BSA.

grate as 105-kD on denaturing gels (16). These fragments were made, separated by ultracentrifugation and tested for binding with the antibodies. Seven of the antibodies, My1-5, My9, and My10, bound to the sedimentable tail fragment (Fig. 5). My6 and My7 bound to both the soluble fragment and the tail fragment. The relative intensities of the autoradiographic signals indicated that My6 and My7 bind to the soluble fragment to a greater extent than to the tail fragment. Electron microscopy of these fragments described by Flicker et al. in this issue (3) showed heterogeneity in the fragment preparations and binding of My6 to a position on the rod close to the globular heads. Thus, heterogeneity in the position of chymotryptic cleavage probably explains why My6 reacts with both the soluble fragment and the tail fragment preparations. Similar analysis was not possible with My7. However the blocking analysis described below indicates they are directed against a closely related site.

Blocking Experiments Reveal Seven Distinct Antibody Binding Sites on Myosin

Antibodies that bind to topographically related sites on the myosin molecule were identified by competition experiments (Fig. 6). If an unlabeled monoclonal antibody prevents the binding of another iodinated antibody to myosin, this indicates the binding of antibodies to sites on the myosin molecule that are close to each other. No blocking indicates the sites are topographically distinct. Eight of the antimyosin antibodies were iodinated and shown to retain their immunological specificity. Unlabeled My1, My5, and My10 inhibited the binding of iodinated My1 to the same extent, which indicates that they bind to related sites. In fact, the binding sites of all three antibodies are indistinguishable by direct visualization in the electron microscope, as shown by Flicker et al. (3). The binding of iodinated My7 was inhibited by unlabeled My6

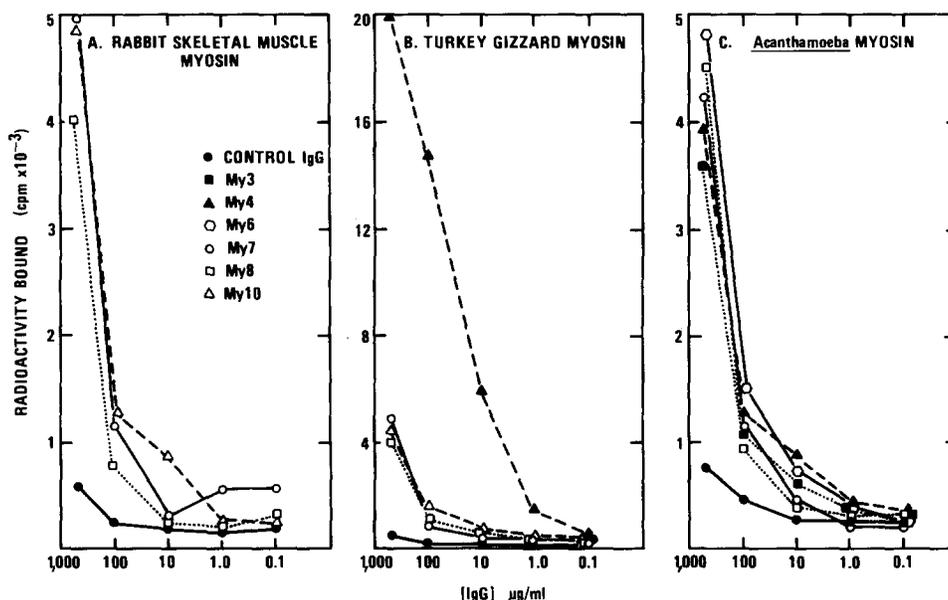


FIGURE 2 Binding of monoclonal antibodies made against *D. myosin* to myosins from other species. Results are of solid-phase RIA with (A) rabbit skeletal muscle, (B) turkey gizzard, and (C) *Acanthamoeba* myosins.

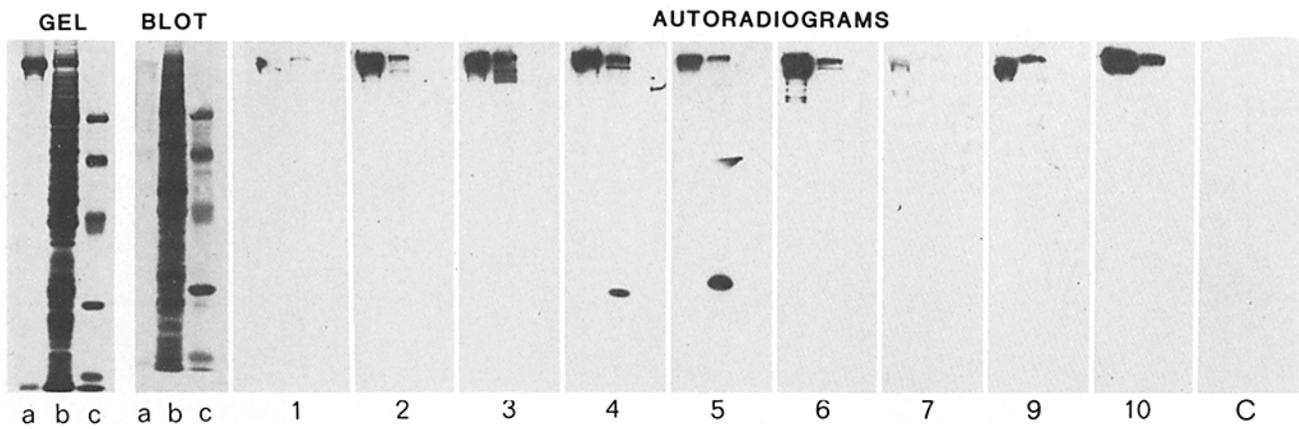


FIGURE 3 Immunoblots demonstrating the binding of nine monoclonal anti-myosin antibodies to the 210-kD *D. myosin* heavy chain. The GEL shows 10% SDS PAGE of (a) 2.5 µg of purified *D. myosin*, (b) 5 µl of a *Dictyostelium* cytoplasmic extract, and (c) protein standards. The BLOT shows nitrocellulose paper stained with Amido black after electrophoretic transfer of the GEL. Note that the myosin heavy chain transfers poorly. The AUTORADIOGRAMS are of nitrocellulose papers after incubation with the various My antibodies, indicated by their number, or (C) the control IgG and subsequent incubation with ¹²⁵I-RAM.

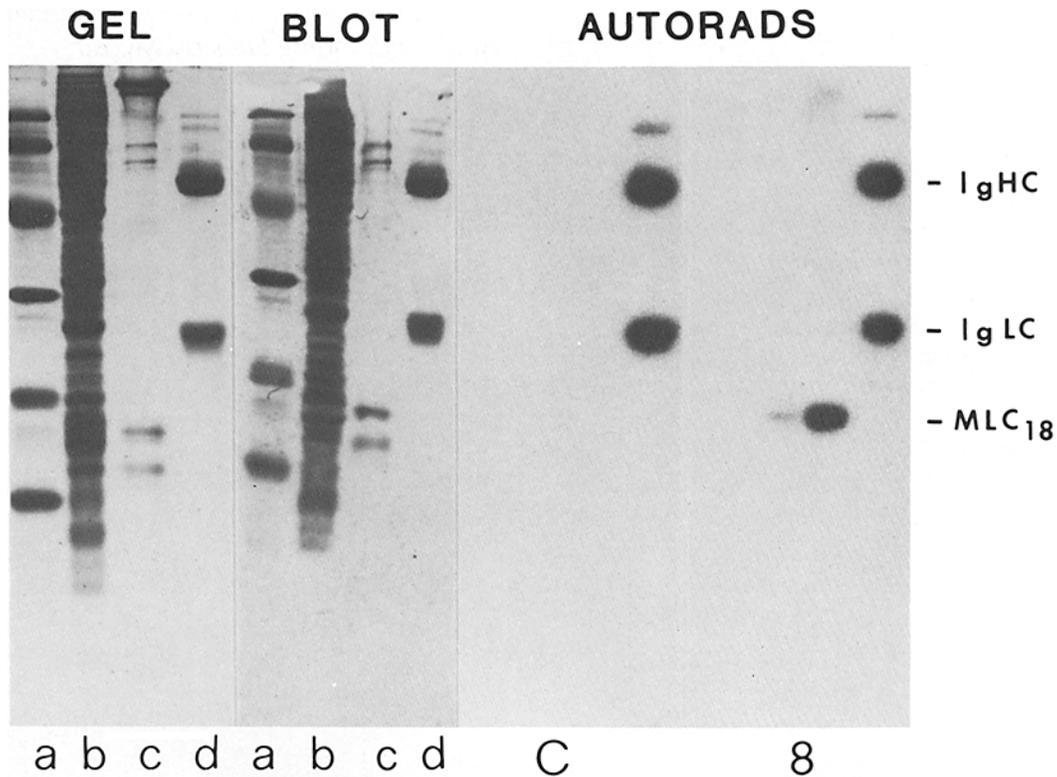


FIGURE 4 Immunoblot demonstrating the binding of My8 IgG to the 18-kD light chain of *D. myosin*. The GEL is 15% SDS PAGE of (a) protein standards, (b) 5 µl of a *Dictyostelium* cytoplasmic extract, (c) 3 µg of purified *D. myosin*, and (d) 4 µg of purified My1 IgG. The BLOT is nitrocellulose paper stained with Amido black after electrophoretic transfer of the GEL. The AUTORADIOGRAMS are of nitrocellulose papers after incubation with either My8 (8) or control IgG (C) and subsequent incubation with a solution containing ¹²⁵I-RAM.

but the binding of iodinated My6 was not inhibited by unlabeled My7. Thus My6 and My7 may identify closely related sites. The binding of the other heavy chain antibodies and the light chain antibody were not inhibited by any other antibody. These results show that these antibodies define a minimum of seven distinct antigenic sites on the myosin molecule (Table I).

My2, My3, and My6 Bind and Sediment with Myosin Filaments

The ability of the antibodies to bind to filamentous myosin was tested by sedimenting myosin plus antibody at low ionic strength. Under these conditions *D. myosin* forms bipolar filaments similar to those formed by skeletal muscle myosin

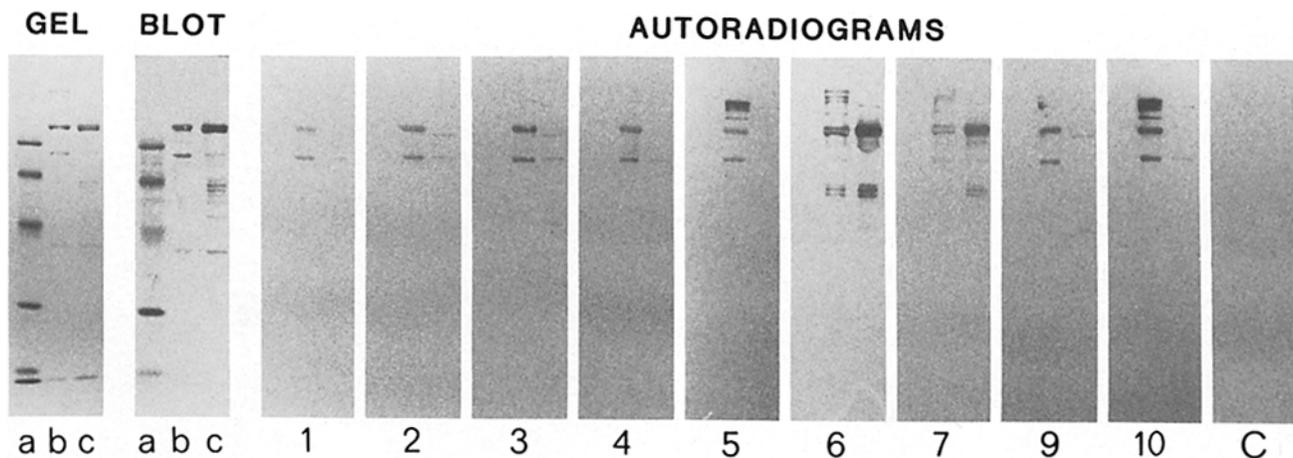


FIGURE 5 Immunoblot localization of the binding of monoclonal anti-myosin IgGs to chymotryptic fragments of *D. myosin*. The GEL is 10% SDS PAGE of (a) protein standards, (b) 2 µg of protein sedimenting after chymotryptic cleavage of purified *D. myosin*, and (c) 2 µg of protein that remains in solution after centrifugation of chymotryptic cleavage products of *D. myosin*. The BLOT is nitrocellulose paper stained with Amido black after electrophoretic transfer of the GEL. AUTORADIOGRAMS are of nitrocellulose papers after incubation with the My antibodies indicated by number or control IgG (C) and subsequent incubation with a solution containing ^{125}I -RAM.

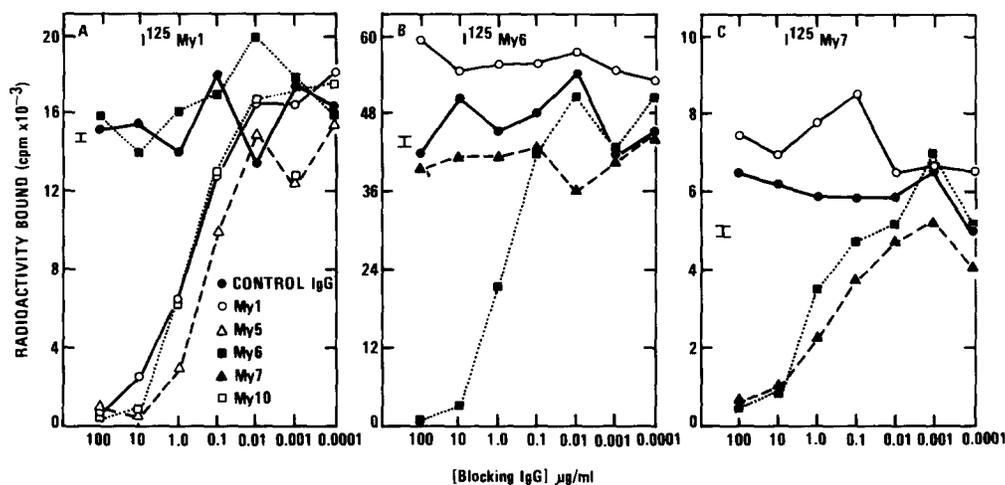


FIGURE 6 Solid-phase blocking RIA to identify antibodies that bind to spatially related sites on *D. myosin*. Representative results to show the blocking of ^{125}I -My1 (A), ^{125}I -My6 (B), and ^{125}I -My7 (C) binding to *D. myosin* are shown. Blocking antibodies are as shown in A. The bar indicates the amount of binding in the absence of blocking Ig.

TABLE I
Identification of Monoclonal Anti-myosin Antibodies Binding to Spatially Related Sites on *Dictyostelium Myosin*

Iodinated IgG	Blocking IgG									
	My1	My2	My3	My4	My5	My6	My7	My8	My9	My10
My1	+	-	-	-	+	-	-	-	-	+
My2	-	+	-	-	-	-	-	-	-	-
My3	-	-	+	-	-	-	-	-	-	-
My4	-	-	-	+	-	-	-	-	-	-
My6	-	-	-	-	-	+	-	-	-	-
My7	-	-	-	-	-	+	+	-	-	-
My8	-	-	-	-	-	-	-	+	-	-
My9	-	-	-	-	-	-	-	-	+	-

Summary of solid-phase blocking RIA to identify monoclonal anti-myosin antibodies binding to spatially related sites on *Dictyostelium myosin*. (+) Indicates that the binding of the iodinated antibody was inhibited to background levels when the blocking antibody concentration was above 1 µg/ml. (-) Indicates the binding of the iodinated antibody to *Dictyostelium myosin* was not inhibited by the presence of the unlabeled blocking antibody.

in vitro (1). (In this paper, the term myosin filaments refers to those filaments formed in vitro by dilution of a *D. myosin* solution to low ionic strength.) My1, My4, My5, My7-10, and control IgG did not co-sediment with myosin filaments whereas My2, My3, and My6 did (Fig. 7). The stoichiometries of the binding of the three monoclonal antibodies were 1 mol

My2 per 2 mol myosin and 1 mol My3 or My6 per 1 mol myosin (Table II). This ratio did not change when the molar ratio of IgG to myosin in the starting solution was varied, nor did the binding of myosin to actin interfere with the binding of the monoclonal IgG (Fig. 8).

Of the antibodies that bind and sediment with myosin

filaments, My2 and My3 bound to the myosin tail fragment. The third antibody, My6, preferentially bound to the soluble fragment in immunoblotting but also showed some binding to the tail fragment. The soluble fragment of *D. myosin* is known to bind to actin filaments in an ATP-dissociable fashion and will sediment with actin filaments (16). When My6 was added to this system, it too sedimented with actin filaments, but only if the head fragment of myosin was present (Fig. 9). When binding of the head fragment to actin was inhibited with ATP, co-sedimentation of My6 with the actin

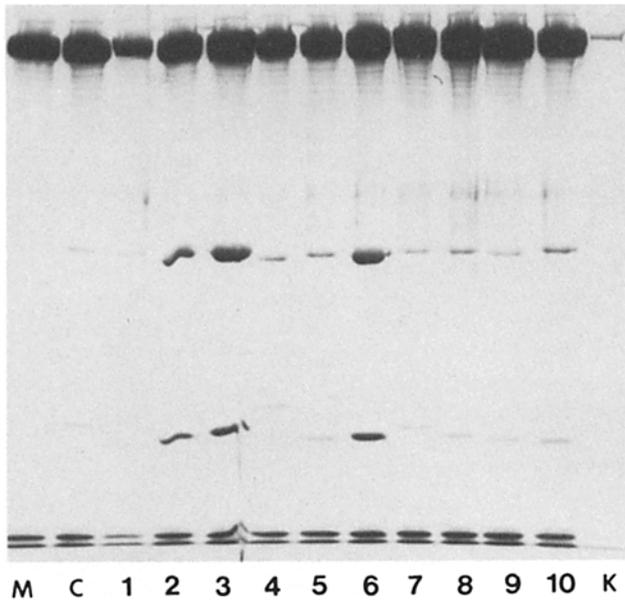


FIGURE 7 10% SDS PAGE analysis of sedimentation assay for the binding of monoclonal anti-myosin antibodies to filamentous *D. myosin* in solution. Antibody was incubated with myosin filaments which were then centrifuged. The pellet was resuspended in 30 μ l of buffer and 15 μ l analyzed by SDS PAGE. Lanes show the pelleted material from solutions containing: (M) myosin without IgG; (C) myosin and control IgG; (K) myosin in solution contain 0.5 M KCl; and (1-10) My1-10, respectively.

TABLE II

Stoichiometry of Anti-myosin Monoclonal Antibody Binding to *Dictyostelium* Myosin or Its Soluble Chymotryptic Fragment

Native myosin	Observed Ratio: $\left(\frac{\mu\text{g MHC}}{\mu\text{g IgHC}}\right)$	Indicated Ratio: $\left(\frac{\text{mol IgG}}{\text{mol myosin}}\right)$
IgG		
My2 (7)	7.25 ± 1.84	1/2
My3 (5)	3.67 ± 0.47	1/1
My6 (4)	3.96 ± 0.34	1/1
Soluble Chymo- tryptic Fragment	Observed Ratio: $\left(\frac{\mu\text{g Fragment HC}}{\mu\text{g IgHC}}\right)$	Indicated Ratio: $\left(\frac{\text{mol IgG}}{\text{mol fragment HC}}\right)$
My6 (3)	2.35 ± 0.35	1/2

Summary of densitometric quantitation of sedimentation assays to determine the stoichiometry of monoclonal anti-myosin antibodies binding to *Dictyostelium* myosin or the soluble chymotryptic fragment in solution. Sedimentation assays were performed as described in the legend to Figs. 7 and 8. The lanes were scanned with a densitometer and the amount of protein in a peak was determined by comparison with standards as described in Materials and Methods. The molar ratio of IgG to the heavy chain of native myosin or the soluble chymotryptic fragments was then determined. The ratio did not vary with the ratio of IgG to myosin initially present in solution, nor did the addition of actin alter the ratios for native myosin. The ratios remained constant for the number of different determinations indicated in parentheses.

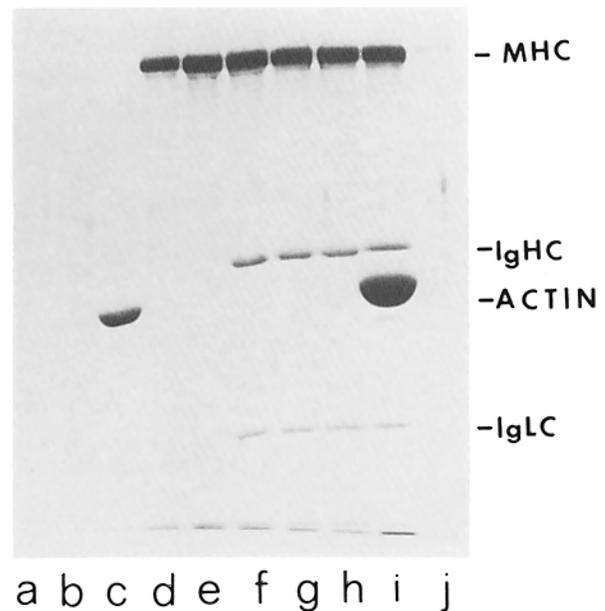


FIGURE 8 10% SDS PAGE analysis of sedimentation assay to determine the stoichiometry of My6 binding to filamentous *D. myosin* (12 μ g) in solution. Sedimentation assays were with (f) 3.6 μ g of My6, (g) 7.2 μ g of My6, (h) 14.4 μ g of My6, or (i) 7.2 μ g of My6 with 25 μ g of actin. Centrifugation of solutions containing (a) 7.2 μ g My6, (b) 7.2 μ g control Ig, or (c) 7.2 μ g of My6 plus 25 μ g of actin does not result in sedimentation of antibodies. Pelleted material from solutions containing (d) myosin, (e) myosin and 7.2 μ g of control Ig, and (j) myosin plus 0.5 M KCl are also shown. MHC, Myosin heavy chain; IgHC, IgG heavy chain; IgLC, IgG light chain.

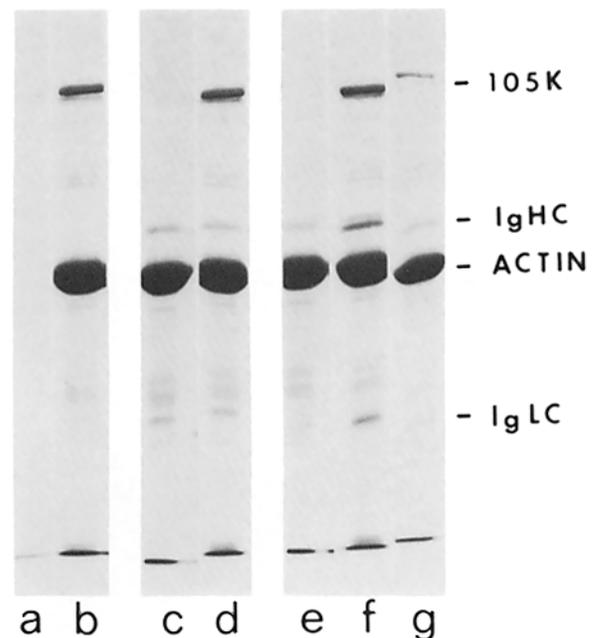


FIGURE 9 10% SDS PAGE of sedimentation assay to determine the stoichiometry of My6 binding to the chymotryptic soluble fragment of *D. myosin*. Sedimentation assays contained 3 μ g myosin fragment, 7.2 μ g antibody, and 25 μ g of actin. Lanes show the pellet after centrifugation of solutions containing: (a) My6; (b) myosin fragment and actin; (c) control IgG and actin; (d) control IgG, actin, and myosin fragment; (e) My6 and actin; (f) My6, actin, and myosin fragment; and (g) My6, actin, myosin fragment, and 1 mM ATP. The heavy and light chains of My6 are indicated by IgHC and IgLC.

TABLE III

Effect of Monoclonal Anti-myosin Antibodies on the Formation of Filaments by *Dictyostelium Myosin*

IgG	% Filaments Sedimenting
X63	95 ± 1
My1	89 ± 3
My2	121 ± 17
My3	116 ± 15
My4	21 ± 7
My9	99 ± 13

Effect of monoclonal anti-myosin IgG on the formation of filaments by *Dictyostelium myosin*. Solutions containing 4 mol of IgG per mol of myosin and 0.5 M KCl were incubated for 40 min before dilution to lower the salt concentration to allow filaments to form. The amount of filaments formed was determined by sedimentation as described in Materials and Methods. The values are recorded as a percentage of the amount of myosin filaments sedimenting from a control solution that did not contain any IgG. All values are the average of three determinations.

TABLE IV

Effect of Monoclonal Anti-myosin Antibodies on the Actin-activated ATPase Activity of Native *Dictyostelium Myosin*

IgG	Mean % control	Range
Control IgG	100	96-100
My1	98	97-100
My2	96	89-100
My3	93	82-100
My4	88	86-95
My5	85	73-95
My6	50	42-62
My7	80	75-100
My8	83	73-97
My9	95	89-100
My10	77	70-100

Effect of monoclonal anti-myosin IgG on the actin-activated ATPase activity of *Dictyostelium myosin*. All ATPase activities were determined as described in Materials and Methods in the presence of 4 mol of IgG per mol of myosin. The control value was the actin-activated ATPase activity of a sample containing myosin without any added IgG. The data summarized in this table are the averages of 4 to 6 duplicate determinations.

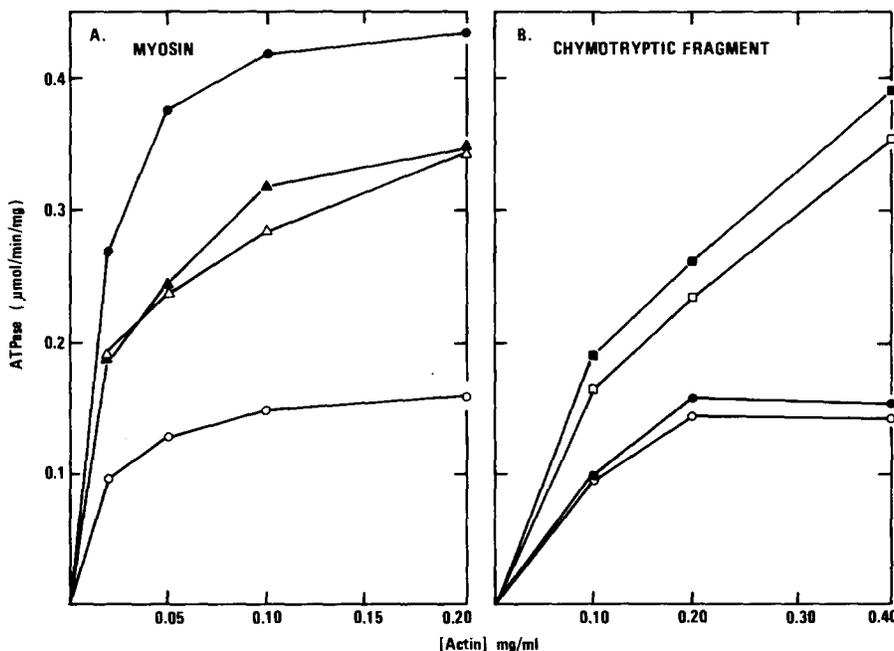


FIGURE 10 Effect of monovalent (Fab') and bivalent F(ab')₂ fragments of My6 on the actin-activated ATPase of *D. myosin* or of the soluble chymotryptic fragment. (A) 6 µg of myosin, the indicated actin concentration and either (Δ) no IgG, (▲) 1.4 µg control IgG; (○) 1.3 µg My6 F(ab')₂, or (●) 1.3 µg My6 Fab'. (B) 1.5 µg soluble chymotryptic myosin fragment, the indicated amount of actin, and either (○) no IgG, (●) 1.0 µg control IgG, (□) 0.5 µg My6 F(ab')₂, or (■) 0.5 µg My6 Fab'. The solutions were incubated for 1 h before the measurement of the actin-activated ATPase activity. Each point is the average of two determinations. Differences in the ATPase activities at the same actin concentration on a per active site basis of chymotryptic fragment versus myosin reflect the greater affinity of native myosin for actin than that of chymotryptic fragment (16).

filaments was also eliminated. The amount of control IgG sedimenting with actin filaments was independent of the presence of the myosin head fragment (Fig. 9). The stoichiometry of binding of My6 to the soluble fragment of myosin was 1 mol IgG per 2 mol soluble fragment heavy chain (Table II).

My4 Inhibits Filament Formation

Antibodies that bind to five different sites on the myosin rod were tested for their effect on filament formation. They were incubated with monomeric myosin in high salt and then diluted to lower the salt concentration and to allow filaments to form. Filament formation was quantitated by sedimentation. My4 inhibited filament formation by 80%, in contrast to My1 and My9 which had no effect. My2 and My3, which co-sediment with myosin filaments, caused a slight increase in the amount of sedimented myosin (Table III).

My6 Alters the Actin-activated ATPase of Myosin

Nine of the antibodies had little effect on the actin-activated ATPase activity of myosin in solution (Table IV). My6 inhibited the activity by ~50% (Table IV). To further investigate the effect of My6 on the ATPase activity, we prepared bivalent F(ab')₂ and monovalent Fab' fragments of My6. Over a range of actin concentrations, My6-F(ab')₂ inhibited the ATPase activity of myosin by ~50% as seen with the IgG, whereas My6-Fab' increased the activity by ~50% (Fig. 10). These effects were observed at ratios of 1 mol of F(ab')₂ added per mol of myosin and 2 mol of Fab' added per mol of myosin. To eliminate effects due to the formation of myosin filaments, we determined the actin-activated ATPase activity of the soluble fragment of myosin in the presence of the My6 fragments. Under these conditions F(ab')₂ and Fab' both increased the actin-activated ATPase activity by 80-150% above control values over a range of actin concentrations (Fig. 10). Control IgG had no effect on the actin-activated ATPase activities of either myosin or the soluble fragment of myosin. Monovalent and bivalent fragments of My6 did not alter the ATPase activity of myosin or the soluble fragment in the absence of actin.

DISCUSSION

D. myosin is composed of two structural and functional domains that can be separated by limited proteolysis. The carboxy-terminal tail domain is a coiled-coil involved in the assembly of myosin into filaments. The amino-terminal domain consists of the globular heads, which are involved in actin and nucleotide binding, plus a small part of the rod-like tail (3). Ten monoclonal antibodies were obtained that bind strongly to *D. myosin* and weakly to other myosins. The antibodies define seven distinct antigenic sites on *D. myosin*: one on the 18-kD light chain, one on the amino-terminal half of the heavy chain, and five on the carboxy-terminal half of the heavy chain. My6 and My7 show strong binding to the globular amino portion of the heavy chain and a weaker but significant binding to a 105-kD band in the pelletable material. This result with My6 and My7 may be explained in the following ways. Topographic mapping and electron microscopic analysis of My6-myosin complexes described in the following paper (3) show that the site recognized by these antibodies is in the region at which proteolytic cleavage takes place. Heterogeneity of proteolysis could lead to mostly head and some tail fragments having the My6 and My7 sites or, alternatively, the antigenic sites might be split by a unique chymotrypsin cleavage yielding fragments that both have affinity for the antibodies.

Although the monoclonal antibodies all bind strongly to myosin immobilized on the surface of a microtiter well or nitrocellulose paper, only three of the ten, My2, My3 and My6, co-sediment with myosin filaments. Since the stoichiometry of antibody binding is not altered when myosin is bound to actin, these antibodies and actin must bind to spatially distinct sites on the myosin molecule. Antibodies against three distinct sites on the carboxy portion of the myosin heavy chain do not co-sediment with myosin filaments. These antibodies may have low affinity for myosin or the site to which they bind on monomeric myosin may not be accessible when myosin filaments are formed. My6 and My7 bind to a spatially related site on the myosin molecule, yet My6 co-sediments with myosin filaments and My7 does not. It is most likely that this difference is because the affinity of My7 is much less than that of My6. The nonreciprocal blocking between My6 and My7 in the solid-phase blocking RIAs supports this interpretation.

My4 inhibits filament formation indicating that the site it recognizes on the tail of the myosin heavy chain is in a region essential for that process. My2 and My3 slightly enhanced filament formation; this may be a result of the antibody providing additional cross-linking of myosin filaments.

Nine of the antibodies did not alter the actin-activated ATPase activity of myosin. In the cases of My2 and My3, in which binding to myosin in solution is apparent by sedimentation, we can conclude that binding does not alter the actin-activated ATPase activity. In the other cases, the binding in solution may simply be too weak to have an effect on the ATPase activity. The inhibition by My6 could be due to the

formation of a large aggregate that is less accessible to actin filaments or a conformational constraint imposed on myosin by bivalent antibody binding. When cross-linking is eliminated by using monovalent My6-Fab' or reduced by using the soluble chymotryptic fragment of myosin, the actual effect of My6 binding was to increase the actin-activated ATPase activity of myosin. Thus none of the monoclonal antibodies compete with actin or nucleotides for binding to myosin. This is not surprising as the actin and nucleotide binding sites probably represent the most conserved parts of the molecule whereas the antibodies all show considerable specificity for *D. myosin* and are therefore against sites on the myosin molecule that have been less conserved in evolution.

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