Site-specific Inhibition of Myosin-mediated Motility In Vitro by Monoclonal Antibodies

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ABSTRACT Monoclonal antibodies directed against seven different sites on Dictyostelium myosin (Peltz, G., J. A. Spudich, and P. Parham, 1985, J. Cell Biol., 100: 1016–1023) were tested for their ability to inhibit movement of myosin in vitro, using the Nitella-based myosin-mediated bead movement assay (Sheetz, M. P., R. Chasan, and J. A. Spudich, 1984, J. Cell Biol., 99: 1867–1871). To complement this functional assay, we located the binding sites of these antibodies by electron microscopy, using the rotary shadowing technique. One antibody bound to the 18,000-dalton light chain and inhibited movement completely. All of the remaining antibodies bound to various positions along the rod portion of the myosin molecule, which is ~1,800 Å long. Antibodies that bound to the rod about 470, 680, and 1400 Å from the head-tail junction did not alter myosin movement. One antibody appeared to bind very close to the head-tail junction and to inhibit movement 50%. Surprisingly, three antibodies that bound about 1,200 Å from the head-tail junction inhibited movement completely. This inhibition did not depend on using intact IgG, since Fab’ fragments had the same effect.

The interaction of myosin and actin is fundamental for a wide variety of cellular movements (17). The function of myosin has been best studied in muscle where development of tension or speed of shortening can be measured to probe the effects of varying conditions on contraction of myofibrils. Myosin movement has not been measured in nonmuscle cells. However, the direct visualization of movement of myosin-coated beads along polar arrays of actin filaments from the alga Nitella (33) has provided a quantitative assay for myosin movement in vitro (32). We report here studies in which we have used this assay to assess the effects on movement of binding monoclonal antibodies to different probe sites on the myosin molecule.

Anti-myosin monoclonal antibodies provide valuable probes for regions of the myosin molecule important for motility. Antibodies specific for particular muscle myosin isoforms provide important tools for examination of the distribution of myosin isoforms during development and in adult fibers. During early development muscle fibers destined to become either fast or slow twitch fibers both react with antibodies directed against slow muscle myosin and fast muscle myosin (16). These findings have been extended using monoclonal antibodies specific for adult myosin to show that structural changes occur in widely separated regions of the myosin molecule during myogenesis (4, 38). Immunocytochemistry using polyclonal antibodies has been used to identify individual fiber types and demonstrate the presence of more than one isoform of myosin in a single muscle (14, 15). Nonmuscle myosins have been localized in the contractile ring and in stress fibers using immunofluorescence microscopy (30, 37). Further studies that used myosin-specific antibodies have probed the correlation between the structure and function of the myosin molecule. Polyclonal antibodies specific for the regulatory light chains or the essential light chains of scallop myosin abolished calcium regulation in myofibrils and myosin by elevating the actin-activated ATPase activity in the absence of calcium (36). These antibodies were then used to localize the light chains on the myosin heads using electron microscopy. Fab’ fragments specific for the regulatory light chains and Fab’ fragments specific for the essential light chains bind preferentially to scallop myosin in the narrow region of the myosin head near its junction with the tail (13). A monoclonal antibody specific for the LC2 light chain of fast skeletal muscle myosin has been mapped by electron microscopy on the myosin head at the head-rod junction; binding of this antibody to LC2 light chain does not affect the enzymatic activity of skeletal muscle myosin (38). Monoclonal antibodies specific for several epitopes in the 25-kilodalton and 50-kilodalton tryptic fragments of the myosin head (S1) have been mapped (39). Monoclonal antibodies specific...
for nonmuscle myosins are beginning to be used to examine structure and function of myosin in nonmuscle cells. Binding sites of several antibodies directed against Dictyostelium myosin (D. myosin) have been localized by Claviez et al. (7), but the effect of these antibodies on myosin function has not been reported as yet. Kiehart and Pollard (23) mapped binding sites of antibodies specific for Ac.thamoeba myosin II and made the interesting finding that a monoclonal antibody specific for the tail inhibits myosin ATPase activity by filament disassembly.

As described in the previous paper by Peltz et al. (29), antibodies specific for seven distinct sites on Dictyostelium myosin have been prepared. Ten monoclonal antibodies were isolated and designated My1 to My10. The affinity of those antibodies for chymotryptic fragments of Dictyostelium myosin divided the antibodies into two groups: those that bind to the tail portion of the molecule and those that bind to the head portion. Localization at higher resolution was essential to interpret the effects of the antibodies on movement. We therefore report here a quantitative analysis of these antibodies on the rate of myosin movement in the functional in vitro assay described by Sheetz et al. (32).

MATERIALS AND METHODS

Proteins Used in This Study: RNA-free myosin was purified from Dictyostelium as described (26) with minor modifications (Griffith, L. G., and J. A. Spudich, manuscript in preparation). Ten monoclonal anti-Dictyostelium myosin IgG antibodies, referred to as My1 to My10 (or My1–10), were prepared as described in the previous paper by Peltz et al. (29). The Fab' fragments of these antibodies were prepared by pepsin digestion as described by Parham (27).

Preparation of Beads Coated with Dictyostelium Myosin: Purified Dictyostelium myosin was attached to fluorescent beads (Covalent Technologies, Inc., Ann Arbor, MI). 1 vol of the stock solution of beads was diluted 1:9 into a solution containing 5 mM potassium phosphate (pH 6.5), 0.2 mM dithiothreitol, 50 mM KCl and myosin at a concentration of 0.2–0.4 mg/ml at 0°C for at least 2 h. The beads were then pelleted using a microfuge and resuspended in 1 vol of assay buffer containing 20 mM Tris, 2 mM KCI, 2 mM EDTA, and 5% normal rabbit serum.

Control beads were prepared by diluting 1 vol of stock solution of beads with 9 vol of assay buffer at 0°C for at least 2 h. After centrifugation, the beads were resuspended in 1 vol of assay buffer.

Radioimmunoassay (RIA) for Binding of Anti-myosin Antibodies to Myosin-coated Beads: Monoclonal anti-myosin IgG was added to 20 μl of a myosin-coated bead suspension to a concentration of 0.5 mg/ml. After 2–4 h of incubation at 0°C, the beads were washed twice with 1 ml of assay buffer. The pelletled beads were resuspended in 50 μl of assay buffer containing 500,000 cpm of 125I-labeled Fab'2, rabbit anti-mouse IgG (RAM). After 1–2 h of incubation at 22°C, the beads were then washed twice using 1 ml of assay buffer. The amount of radioactivity bound to the pelletled beads was then measured. Experiments measuring the binding of Fab' fragments to myosin-coated beads were performed in the same manner except Fab' was added to a concentration of 0.325 mg/ml. In each experiment, nonspecific binding was measured as the amount of radioactivity bound when a nonspecific control IgG of isotype γ2 was added to the myosin-coated bead suspension. As an additional control, the amount of radioactivity bound when beads lacking myosin were incubated with each of the monoclonal IgG antibodies or Fab' fragments was measured.

Measurement of the Rate of Movement of Myosin-coated Beads In Vitro: Monoclonal IgG was added to suspensions of myosin-coated beads to a concentration of 0.5 mg/ml. After 3-h incubation at 4°C, the IgG-bead solutions were diluted 1:3 in 50 mM Tris, 25 mM KCl, 4 mM MgCl2, 4 mM EGTA, 1 mM ATP (pH 7.6), and then were pipetted onto the cytoplasmic surface of a dissected Nitella cell according to procedures described by Sheetz, Chasan, and Spudich (32). The dissected Nitella cell was immersed in 5 mM imidazole, 25 mM KCl, 4 mM MgCl2, 4 mM EGTA, 1 mM ATP (pH 7.0) containing 100 μCi/ml of monoclonal IgG. The beads settled on the rows of microtubules and their movement was monitored with a water-immersible 40X objective (Carl Zeiss, Inc., Thornwood, NY) for 10–30 min. All observations were recorded on videotape for analysis. Measurement of the effect of Fab' fragments on the rate of bead movement was performed by the same procedure, except Fab' was added to the myosin-coated bead solution to a concentration of 0.25 mg/ml.

The rate of bead movement was determined by following each of 10–50 distinct beads or bead aggregates over a distance of 40 μm. The maximum rate of migration of each particle measured over at least a 10 μm distance was used as the velocity of movement.

Incubation of Myosin with Antibodies and Rotary Shadowing: Typically, 0.03 mg of myosin (stored at 0.8–2 mg/ml) was incubated with IgG or Fab' at a 1:1 molar ratio in 0.5 M KCl, 10 mM Tris (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, and 0.02% azide at 22°C for 0.5 h. In some cases, myosin plus IgG was incubated at 4°C for 1–3 h. The antibodies were stored at −20°C in 5 mM Tris (pH 7.5), 30% NaCl, and 0.02% azide. Before incubation with myosin, a small aliquot was thawed and spun in a Beckman airfuge (Beckman Instruments, Inc., Palo Alto, CA) for 10 min at 30 pounds per inch square (psi) to remove aggregates. The concentration was determined spectroscopically (A = 15).

Samples were diluted to 10–20 μg/ml myosin in 70% glycerol and 0.3 M ammonium acetate (1 mM dithiothreitol and 1 mM EDTA were present in some experiments) and immediately sprayed onto freshly cleaved mica as described by Tyler and Branton (35). Samples were mounted on a rotary platform in an Edwards evaporator (Edwards High Vacuum, Inc., Grand Island, NY) and dried in vacuo. A 6° shadow angle was used for rotary shadowing with platinum (0.008 inch wire, 3 cm) vaporized from a heated tungsten filament (13). The resulting replicas were coated with a supporting film of carbon, floated on distilled water, and picked up on bare 400-mesh grids. Micrographs were recorded with a Philips EM201 (Philips Electronic Instruments, Inc., Mahwah, NJ) operating at 80 kV accelerating voltage. The magnification was calibrated using negatively stained tropomyosin paracrystals (395 Å periodicity) (8). Antibody binding sites were estimated by measurement on enlarged images (×2.6) using a digital data tablet connected to a microcomputer.

RESULTS

Monoclonal Anti-myosin Antibodies Bind to Myosin Immobilized on Beads

Anti-myosin antibody binding to myosin-coated beads was measured for each of the IgG monoclonal antibodies. They all showed substantial binding above control levels (Table I).

| Table I | Binding of Monoclonal IgG and Fab' Fragments to Myosin-coated Beads |
| --- | --- |
| IgG | Radioactivity bound (cpm) |
| Control IgG | 1,300 | 3,500 |
| My1 | 2,200 | 13,300 |
| My2 | 5,400 | 41,800 |
| My3 | 1,200 | 31,000 |
| My4 | 2,000 | 25,900 |
| My5 | 1,400 | 22,200 |
| My6 | 1,500 | 21,300 |
| My8 | 3,100 | 20,900 |
| My9 | 2,900 | 14,000 |
| My10 | 600 | 18,000 |

| Fab' | |
| My1 | 400 | 12,600 |
| My6 | 1,000 | 20,800 |
| My8 | 1,800 | 5,600 |
| My10 | 1,400 | 18,800 |

The amount of 125I-RAM bound to control or myosin-coated beads after incubation with monoclonal IgG or Fab' fragments was determined as described in Materials and Methods.

Abbreviations used in this paper: D. myosin, Dictyostelium myosin; RAM, rabbit anti-mouse IgG; RIA, radioimmunoassay.
Binding of Fab' fragments of four of the antibodies was also measured. Fab' fragments of My1, My6, and My10 showed substantial binding, whereas the Fab' fragment of My8 bound very weakly to myosin-coated beads.

**Some of the Monoclonal Anti-myosin Antibodies Inhibit the Rate of Movement of Myosin-coated Beads**

Beads coated with D. myosin moved along Nitella actin cables with a velocity of about 1 μm/s (Fig. 1, Table II). The presence of a nonspecific control IgG did not alter this rate. My8, which binds to the 18-kD light chain of D. myosin (29), completely inhibited bead movement. Monoclonal antibodies directed against four different sites in the tail portion of D. myosin (My2, My3, My4, and My9) did not affect the rate of bead movement. However, four of the monoclonal antibodies known to bind to the myosin heavy chain (29) did inhibit myosin-bead movement. My6, which appears to bind to the rod portion of the myosin heavy chain very near the head-tail junction (see below), inhibited the rate of bead movement by 50%. My1, My5, and My10, which bind to a spatially related site in the tail portion of the heavy chain of myosin (29), completely inhibited bead motility.

To minimize possible steric blocking due to the size of an intact IgG molecule, we assayed the effects of Fab' fragments on myosin-coated bead motility (Table II). Fab' fragments of My1 and My10 completely inhibited bead movement. The Fab' fragment of My6, which was shown to bind weakly to myosin-coated beads, partially inhibited the rate of bead movement, and no inhibition was observed with the Fab' fragment of My8.

**The Antibody Binding Sites were Localized by Electron Microscopy**

D. myosin molecules have the characteristic shape exhibited by myosin from a number of muscle and nonmuscle sources (Fig. 2) (12). Images of myosin molecules contrasted for electron microscopy by rotary shadowing showed two pear-shaped heads attached to a long narrow tail. The tail of D. myosin (1,800 Å; Fig. 2) (7) is ~250 Å longer than that of muscle myosin (1,560 Å) (11). IgG or Fab' fragments can be distinguished, in general, from the myosin molecule itself in replicas of antibody-myosin complexes (7, 13, 38), and binding of My1-10 to D. myosin was examined by this method. The binding sites of six antibodies have been localized using this approach. Examples of myosin molecules with three of the antibodies which do not affect movement are shown in Fig. 2. My3 binds 470 Å from the heads, about one-fourth of the total tail length from the head-tail junction. My2 binds ~680 Å distal from the heads, and My4 binds about three-fourths of the tail length (1,350 Å) from the heads.

**Table II**

| IgG               | Number of Beads | Velocity (μm/s) |
|-------------------|-----------------|-----------------|
| none              | 24              | 1.0 ± 0.2       |
| Control IgG       | 42              | 1.0 ± 0.3       |
| My1               | 25              | 0               |
| My2               | 27              | 1.0 ± 0.2       |
| My3               | 20              | 1.1 ± 0.2       |
| My4               | 22              | 1.1 ± 0.3       |
| My5               | 25              | 0               |
| My6               | 25              | 0.5 ± 0.1       |
| My8               | 25              | 0               |
| My9               | 21              | 1.2 ± 0.2       |
| My10              | 25              | 0               |

Velocity of Dictyostelium myosin-coated bead movement along Nitella actin cables in the presence of monoclonal anti-myosin IgG or Fab' fragments. The rate of bead movement was measured as described in Materials and Methods.
FIGURE 2 Rotary-shadowed *D. myosin* molecules with bound IgG showing binding sites of antibodies which do not affect motility. Myosin molecules without bound antibody (row 1), with antibody-bound My3 (row 2), with antibody-bound My2 (row 3), or with antibody-bound My4 (row 4). ×125,000.

FIGURE 3 *D. myosin* molecules with bound My10, an antibody which inhibits movement. (Top row) Myosin molecules with My10 IgG; (bottom row) myosin molecules with My10 Fab’. ×125,000.

agree with blocking experiments which suggested that these antibodies, My1, My5 and My10, bind to spatially related sites (29).

Measurements of the location of the antibodies along the tail were made on 30–50 examples of each antibody mapped. These results are summarized in Fig. 4. Each antibody bound to a distinct narrowly defined site on the myosin tail, and no binding to other sites on the molecule was observed. The
precision of the measurement of the binding site was limited by the size of the IgG.

In any given field of molecules, not all myosin molecules clearly exhibited bound antibody. Some myosin molecules had no antibody bound. Aggregates of myosin cross-linked by antibody were often seen. The percentage of myosin molecules with bound antibody is shown in Table III. Three of the monoclonal antibodies, My7, My8 and My9, did not show any binding to myosin in the electron microscope. Two of these antibodies, My7 and My8, have been shown to have lower affinity than the other anti-myosin antibodies by solid-phase RIA (29). Although the frequency of molecules with bound My6 was low, this antibody consistently appeared close to or at the junction of the head with the tail (Fig. 5).

In light of the apparent low affinity of the My6 antibody for myosin under the conditions used for rotary shadowing, supportive evidence for the localization of the My6 antibody binding site was provided by examination of the soluble fragment generated from D. myosin by chymotrypsin cleavage (28). This fragment, which is soluble at low ionic strength and retains ATPase activity, binds My6 (29). Images of the soluble fragment prepared by rotary shadowing revealed that it was heterogeneous (Fig. 5). Many fragments appeared to be comprised of two heads and a short tail, similar to heavy meromyosin from skeletal muscle myosin but having less of the rod portion of the molecule. Individual heads and short rod fragments were also seen. In general, the appearance of the soluble fragment preparation is consistent with the apparent binding site of My6 on myosin (Fig. 5, top), i.e. close to the junction of the rod with the tail.

### DISCUSSION

These studies begin to provide a map of regions of the myosin molecule important for movement per se. Our preliminary map, presented in Fig. 6, shows the location of antibody binding sites along the myosin tail and the rate of movement of myosin-coated beads in the presence of each antibody. The sites important for motility shown here lie at widely different sites on the molecule.

One site required for motility, identified by My8, is on the 18-kD light chain. This light chain appears analogous to the regulatory light chains found in smooth muscle and other nonmuscle myosins (see reference 2 for review). Phosphorylation of these light chains regulates the actin-activated ATPase activity (1, 3, 6, 10) and assembly state of the myosin (31). The D. myosin 18-kD light chain is phosphorylated in vivo during growth of the amoebae (24), suggesting that light chain

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**Table III**

| Antibody | Number of myosin molecules scored | Percent of myosin with bound antibody |
|----------|----------------------------------|---------------------------------------|
| My2      | 850                              | 43                                    |
| My3      | 850                              | 47                                    |
| My4      | 785                              | 68                                    |
| My10     | 1,015                            | 41                                    |
| My10 Fab' | 290                              | 35                                    |
| My1      | 125                              | 44                                    |
| My1 Fab' | 75                               | 28                                    |
| My5      | 185                              | 48                                    |

Percentage of myosin molecules with bound antibody. The number of molecules which were unclear (10–20% of total molecules counted), because they lacked some characteristic feature of myosin, were poorly contrasted, or were aggregated, was subtracted before the calculation of percentages.
phosphorylation also is involved in regulating myosin activity in Dictyostelium. Although the location of this 18-kD light chain was not directly visualized by electron microscopy, it can be inferred from the position of the binding sites of antibodies specific for regulatory light chains of other myosins. Polyclonal antibodies specific for scallop myosin regulatory light chains (13) and monoclonal antibodies specific for vertebrate skeletal myosin LC2 light chains (38) bind to the narrow "neck" portion of the myosin head near its junction with the tail.

The mechanism for regulation requiring phosphorylation of light chains may involve changes in the conformation or position of the light chain and its contacts with the heavy chain. Cross-linking experiments have shown that the regulatory light chain in scallop myosin, a regulated myosin that binds calcium directly, moves relative to the essential light chain upon activation (18). My8 binding to the Dictyostelium 18-kD light chain may block some analogous movement. My8 IgG could also inhibit myosin motility by intramolecular cross-linking of myosin heads. Alternatively, My8 binding may stabilize or enhance formation of a folded myosin conformation (discussed below) which is unable to act in force generation. The lack of complete inhibition of bead movement by the Fab' fragment of My8 is probably due to its low affinity for binding to myosin.

Another site required for motility is defined by the binding of My6. This antibody binds to the soluble fragment of D. myosin (29), and images of myosin molecules with bound My6 antibody suggest that this antibody binds on the rod near the junction of the heads with the tail. This region of the myosin molecule near the head-tail junction may be involved in movements of the head relative to the tail important for motility. Myosin heads are thought to change their angle of attachment to actin in the course of the crossbridge cycle (5, 21, 22). Such changes may be restricted in the presence of My6.

The third interesting region that is revealed by these studies is on the myosin rod approximately two-thirds of the distance from the head-tail junction to the tip of the tail. Cross-linking of molecules by bivalent intact antibody cannot explain inhibition of myosin movement by My1, My5, and My10 since Fab' fragments of My1 and My10 also inhibit the movement. One intriguing possibility is that this region is critical for motive force production by some mechanism such as the helix-coil transition proposed by Harrington (19, 20). Another possible explanation, based on recent studies of other myosins, is that this region of the myosin tail might fold back and physically interact with the head region of the molecule. Smooth muscle myosin and thymus myosin assume such a folded "hair-pin" structure under certain conditions (9, 34).

There are several binding sites in the D. myosin tail that do not appear to be important for myosin movement. However, one of these sites, specified by My4, does appear to be important for myosin filament formation. In the presence of My4, myosin filament formation is inhibited (29). The region identified by My4 is located about three-fourths of the total tail length from the junction of the heads with the tail. The My4 binding site is only ~150 Å away from the My1, My5, My10 binding site. It is therefore remarkable that these antibodies have such different effects on the myosin function. The monoclonal antibodies provide the ability to resolve particular functional regions within the tail.

To further understand the effects of these antibodies on myosin motility in the bead assay, we must determine the arrangement of myosin on the beads. It will be important, for example, to assess whether thick filament formation is required. The low ionic strength of the myosin-bead mixture would favor thick filament formation, but adherence to the bead surface may disrupt filaments or inhibit their formation. Indeed, My1, My4, My5, and My10 are all incapable of binding to myosin filaments in solution (29) and yet they bind to myosin on beads and, except for My4, inhibit bead movement. This suggests that the myosin responsible for bead movement may not be in a filamentous form. Future studies directed at a better definition of the topology of the myosin-coated bead surface are necessary.

These studies represent the first attempts to define the portions of the myosin molecule critical for its ability to move. These findings are being extended using other approaches to modify the myosin molecule, such as proteolytic fragmentation. Assays of the ability of myosin fragments or other modified myosins to move will further define the portions of the myosin molecule involved in generating the motive force.

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