

# Identification of Functional Regions on the Tail of *Acanthamoeba* Myosin-II Using Recombinant Fusion Proteins. I. High Resolution Epitope Mapping and Characterization of Monoclonal Antibody Binding Sites

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**Abstract.** We used a series of COOH-terminally deleted recombinant myosin molecules to map precisely the binding sites of 22 monoclonal antibodies along the tail of *Acanthamoeba* myosin-II. These antibodies bind to 14 distinguishable epitopes, some separated by <10 amino acids. The positions of the binding sites visualized by electron microscopy agree only approximately with the physical positions of these sites on the alpha-helical coiled-coil tail. On the other hand, the epitope map agrees precisely with competitive binding studies: all antibodies that share an epitope compete with each other for binding to myosin. Antibodies with adjacent epitopes can compete with each other at linear distances up to 5 or 6 nm, and many antibodies that bind 3–7-nm apart can enhance the binding of each other to myosin. Most of the antibodies that bind to the distal 37 nm of the tail disrupt assembly of octameric minifilaments and, depending upon the exact location of the binding site, stop assembly at specific steps yielding, for example,

monomers, antiparallel dimers, parallel dimers or antiparallel tetramers. The effects of these antibodies on assembly identify sites on the tail that are required for individual steps in minifilament assembly. Experiments on the assembly of truncated myosin-II tails have revealed a complementary group of sites that participate in the assembly reactions (Sinard, J. H., D. L. Rimm, and T. D. Pollard. 1990. *J. Cell Biol.* 111:2417–2426). Antibodies that bind to the distal tail but do not affect assembly appear to have a low affinity for myosin-II. Antibodies that bind to the proximal 50 nm of the tail do not inhibit the assembly of minifilaments. Many antibodies that bind to the tail of myosin-II, even some that have no obvious effect on minifilament assembly, can inhibit the actomyosin ATPase activity and the contraction of an actin gel formed in crude extracts. An antibody that binds between amino acids 1447 and 1467 inhibits the phosphorylation of serine residues distal to residue 1483.

**M**ONOCLONAL antibodies have been valuable probes of myosin structure and function (Kiehart et al., 1984a,b; Kiehart and Pollard, 1984a,b; Peltz et al., 1985; Flicker et al., 1985; Winkelman and Lowey, 1986; Pagh and Gerisch, 1986; Citi et al., 1989; Trybus 1989; Trybus and Henry, 1989), but the full potential of this approach has been limited by the lack of precision in the localization of the antibody binding sites along the polypeptide chain. To overcome this limitation we used recombinant fusion proteins with a series of 47 different deletions from the COOH

terminus of *Acanthamoeba* myosin-II to map the binding sites of 22 monoclonal antibodies on the tail of the molecule. Similar studies of recombinant fragments from the proximal part of the tail established the locations of five other monoclonal antibody binding sites (Rimm et al., 1989). When combined with the effects of these antibodies on minifilament assembly, ATPase activity, and gel contraction, the locations of the epitopes have defined a number of functionally important parts of the tail.

*Acanthamoeba* myosin-II is favorable for such an analysis of structure and function, because so much is already known about the molecule. It has two heads at one end of a tail 87 nm long and is composed of two 172-kD heavy chains and four light chains (Maruta and Korn, 1977; Pollard et al., 1978; Hammer et al., 1987). It has many properties in common with other cytoplasmic myosins (reviewed by Warrick

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and Spudich, 1987; Korn and Hammer, 1988; and Kiehart, 1990). Its actin-activated ATPase activity is regulated by phosphorylation of the heavy chain (Collins and Korn, 1980) near the tip of the tail (Coté et al., 1984; Hammer et al., 1987), but the mechanism of this regulation is not yet understood. Myosin-II assembles into bipolar minifilaments by three rapid dimerization steps (Sinard et al., 1989; Sinard and Pollard, 1990).

The antibodies used in this study come from an extensive collection of monoclonal antibodies to *Acanthamoeba* myosin-II (Kiehart et al., 1984a). Initial characterization of 23 of these monoclonal antibodies included a rough localization of some of the epitopes by electron microscopy and peptide mapping (Kiehart et al., 1984a,b) as well as an analysis of the effect of each on the assembly, actomyosin ATPase activity, and contractility (Kiehart et al., 1984b; Kiehart and Pollard, 1984a,b). At low resolution the effects on function could be correlated with general regions of the molecule such as the head, head-tail junction, or distal part of the tail, but the subtle differences could not be explained by effects on specific parts of the primary structure.

## Materials and Methods

### Production and Characterization of Monoclonal Antibodies

The isolation, purification, and characterization of 23 monoclonal antibodies to myosin-II were described by Kiehart et al. (1984a,b). An additional 27 antibodies were produced in the same way. These antibodies are named M2.x where x is 1 through 50. Briefly, mice were immunized and boosted with 200 µg of native myosin-II. Hybridoma cells secreting antibody detectable by a solid phase binding assay were cloned twice and grown as ascites tumors in mice. For ELISA solid phase binding assays and immunoblotting, antibodies were obtained from cell culture medium without further purification. For myosin ATPase, gel contraction, electron microscopy, antibody isotyping, and antibody isoelectric focusing, antibodies were purified from ascites fluid by low ionic strength precipitation (for IgMs) or ion exchange chromatography (for IgGs) according to Kiehart et al. (1984a, 1986). For competitive binding assays radioactive antibodies were labeled biosynthetically with [<sup>35</sup>S]methionine or chemically with <sup>125</sup>I (Kiehart et al., 1984a, 1986). Purified antibodies were used as cold competitors in competitive binding assays.

### Isolation and Expression of the Myosin-II Tail Fragment cDNA Clone

The cDNA clone 3.9.3 encodes the COOH-terminal 567 amino acids of myosin-II, the stop codon, the 3' untranslated region, and the poly A tail (Rimm et al., 1989). Clone 3.9.3 was subcloned into two different expression vectors, pATH-11 and pRX-1 (Rimm and Pollard, 1989). Fusion proteins purified from *Escherichia coli* transformed with either vector form bipolar minifilaments (Sinard et al., 1990), showing that the recombinant tail is similar to native myosin-II tail.

### Construction of the COOH-Terminal Deletion Studies

The 3.9.3 clone was subcloned into Bluescript (Stratagene, La Jolla, CA) and its orientation was determined by restriction mapping. The plasmid was then linearized with Hind III, a restriction enzyme that cuts adjacent to the 3' end of the 3.9.3 insert. The linearized plasmid was divided into aliquots and digested with the exonuclease Bal 31 (Promega Biotec, Madison, WI) as described in Maniatis et al. (1982) for 9 or 15 min at room temperature. The ends left by the exonuclease were polished with Klenow polymerase and ligated to Xba I multiple frame stop codon linkers (Pharmacia Fine Chemicals, Piscataway, NJ). The ligated DNA was digested with Eco RI and Xba I for 2 h at 37°C, electrophoresed on 1% agarose gels, and the appropriate sized smear of DNA fragments was cut out of the gel and electrocluted. These fragments were subcloned into Eco RI/Xba I-cut pATH-11s

plasmid, a derivative of the pATH-11 plasmid described below. The ligations were transformed into *E. coli* strain HB101 and screened for inserts by colony hybridization (Maniatis et al., 1982). There were 150 colonies with deleted 3.9.3 cDNA inserted. Plasmid minipreps (Holmes and Quigley, 1981) were done on colonies with inserts and the resultant DNA was restriction mapped and the 3' ends of the inserts were sequenced (Sanger et al., 1977) using a synthetic primer complementary to a region just 3' to the insertion site of pATH-11s. All of the fusion proteins consist of 37 kD of tryptophan synthetase followed by 31 amino acids coded by linker DNA and then myosin-II amino acids beginning at residue 942. We named each clone after its carboxy-terminal amino acid. Myosin-II consists of 1,509 residues with the COOH terminus at the tip of the tail (Hammer et al., 1987).

### Construction of the Expression Vector, pATH-11s

The plasmid expression vector used is a derivative of pATH-11, a vector used successfully for expression of this clone in previous work (Rimm et al., 1989). The derivative, pATH-11s, was made to insure that the newly deleted 3' ends would contain a minimum of linker-coded amino acids by insertion of a stop codon in all three reading frames. This was achieved by cutting pATH-11 DNA at its only Xba I site, filling in the ends with Klenow polymerase and ligating to Xba I multiple frame stop codons as described above. They were then recut with Xba I, electrophoresed to remove unincorporated linkers, and religated. The resultant vectors were transformed into HB101 and sequenced to confirm the insertion of the multiple frame stop fragment.

### Expression of Recombinant Myosin Fragments

The clones were grown, induced, and harvested as described in detail elsewhere (Rimm and Pollard, 1989). Briefly, cells are diluted from stationary phase in 3–5 vol of M9+CA medium and grown 3 h at 30°C, induced with 10 mM indolyl acrylic acid, and grown three more hours. Gel samples are made by pelleting 1 ml of a cell suspension for 10 s in a microfuge, washing with 1 ml of 10 mM Tris-Cl (pH 7.5), resuspending in 100 µl of Laemmli (1970) sample buffer, and boiling for 3 min before loading 10 µl on a 7.5% SDS-polyacrylamide gel.

### Gel Electrophoresis and Western Blot Technique

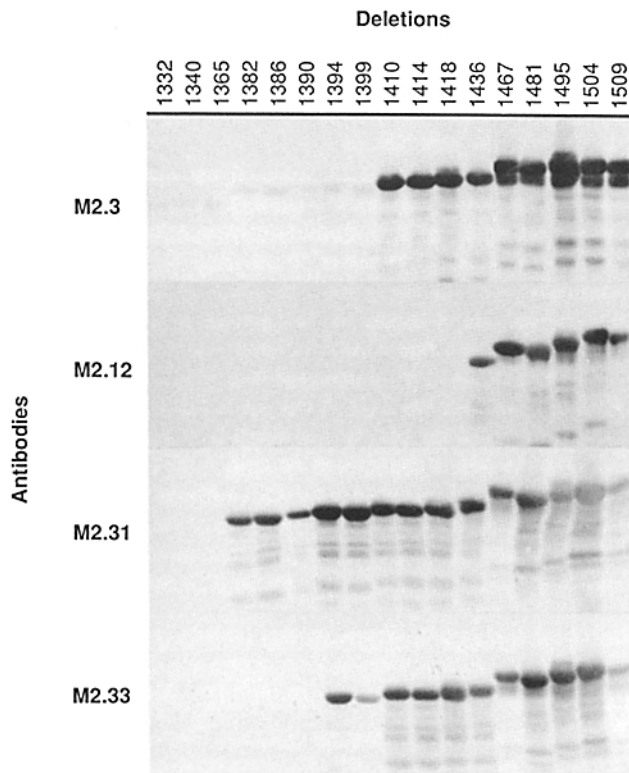
Gel electrophoresis and blotting onto nitrocellulose was done by minor modifications of the methods of Kiehart et al. (1984a). The nitrocellulose filter was removed from the apparatus, stained with 0.2% Ponceau S in 3% trichloroacetic acid, blocked in Tween-BSA buffer (100 mM NaCl, 10 mM Tris-Cl, pH 7.5, 0.1% Tween 20, 0.005% thimerosol, 1% bovine serum albumin) and incubated for 1 h at room temperature in a Tween-BSA buffer with 10 µg/ml of purified monoclonal antibody or 1:1–1:2 dilution of secreted monoclonal antibody in cell culture medium. After three washes for 10 min each in Tween buffer without the BSA, the blot was incubated in 10 µg/ml peroxidase-linked goat anti-mouse antibody (HyClone Laboratories, Logan, UT) or 1 × 10<sup>5</sup> cpm/ml iodinated goat anti-mouse antibody in Tween-BSA buffer. After a 1 h incubation and two Tween buffer washes, the blots with peroxidase-linked antibodies were washed twice with TBS buffer (10 mM Tris-Cl, 150 mM NaCl) and then developed using 0.03% hydrogen peroxide and 0.6 mg/ml 4 chloro-naphthol in TBS containing 20% methanol for 5–15 min. If <sup>125</sup>I-second antibody was used, the blots were washed three times for 10 minutes each in Tween buffer, air dried, and exposed overnight in cassettes with Kodak XAR-5 film.

### Other Biochemical Methods

Myosin-II was purified according to Sinard and Pollard (1989) and kindly provided by Dr. John Sinard of Johns Hopkins Medical School. Assays for the contraction of gelled cytoplasmic extracts and the actomyosin ATPase activity were done according to Kiehart and Pollard (1984a). Myosin-II heavy chain kinase activity was measured with a partially purified kinase and a method described by Coté et al. (1981).

### Electron Microscopy

Samples of myosin-II alone or with monoclonal antibodies were negatively stained with 1% uranyl acetate (Pollard, 1982) or mixed with glycerol and sprayed onto mica for rotary shadowing (Kiehart et al., 1984b). To test for effects of a monoclonal antibody on the assembly of minifilaments, a 1.3–2-fold molar excess of antibody was mixed with monomeric myosin-II in 400 mM



**Figure 1.** Immunoblots used to localize the binding site for monoclonal antibodies M2.3, M2.17, M2.31, and M2.33 on bacterial lysates containing a series of myosin-II fusion proteins with deletions at the COOH terminus of the heavy chain. The numbers at the top of each gel lane indicate the last residue present in the fusion protein. After PAGE the fusion proteins were transferred to nitrocellulose, stained lightly with amido black, and then reacted with the monoclonal antibody. In every case there is a sharp distinction between the reaction of the antibody with the pair of fusion proteins at the boundary of the epitope. For example M2.3 binds to the fusion protein terminating at 1,410 but not that terminating at 1,399. This establishes that residues between 1,399 and 1,410 are essential for binding this antibody.

KCl, 3% sucrose, 12 mM imidazole, pH 7 for 1 h on ice and then diluted to give final concentrations of 0.1  $\mu$ M myosin-II, 0.15% sucrose, 40 mM KCl, 10 mM imidazole, pH 7. Under these conditions more than 95% of myosin-II forms octameric minifilaments (Sinard and Pollard, 1989). Electron micrographs of well preserved areas were recorded and the results tabulated on prints by an observer uninformed about the nature of the experimental manipulations.

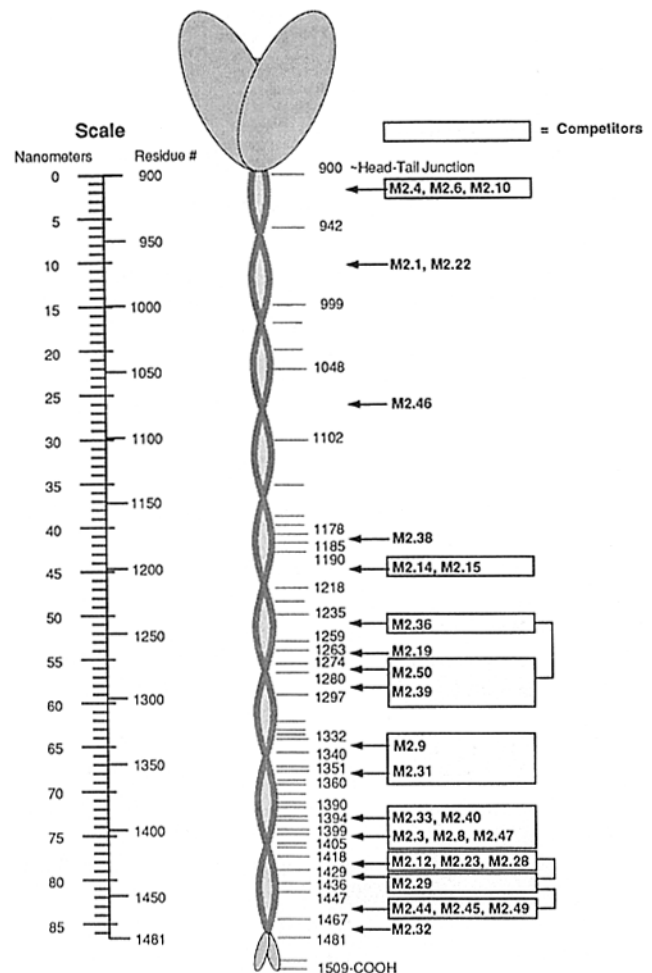
## Results

### High Resolution Epitope Mapping of Monoclonal Antibodies That Bind the Tail of Myosin-II

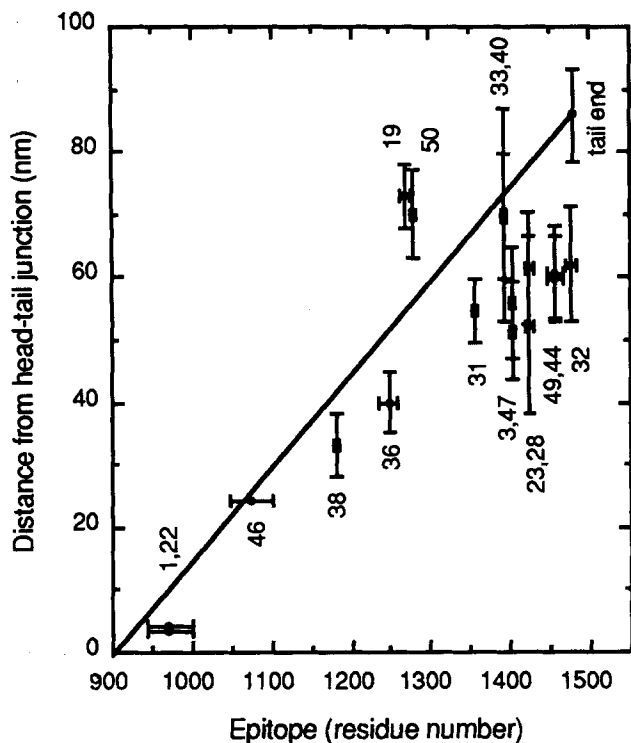
We determined the binding sites on the tail of *Acanthamoeba* myosin-II for 22 different monoclonal antibodies by reacting each with a series of fusion proteins that differed in length at their COOH termini (Fig. 1). The fusion proteins in crude bacterial lysates were separated by SDS-PAGE and then blotted onto nitrocellulose. Representative examples of this procedure are shown for four monoclonal antibodies (Fig. 1). In every case, the epitope was identified clearly because a given antibody reacted with all of the fusion proteins longer than

the minimal peptide containing the epitope and with none of the shorter fusion proteins. By this criterion, an epitope was located between the end of the smallest fusion protein that bound the antibody and the longest fusion protein that did not bind the antibody. As thus defined, the epitopes have a mean length of only 12 residues (or 3.5 turns of the helix) with a range of 4–28 residues. We realize, of course, that the physical binding sites may actually extend into the sequence of the longest fusion protein that did not bind the antibody.

This procedure produced a detailed map of the 14 distinguishable epitopes (Fig. 2). Note that no monoclonal antibody bound at more than one site and that all antibodies that bound denatured myosin-II on blots also bound to fusion proteins. Together with three epitopes mapped previously near the head (Rimm et al., 1989), we have identified 17 epi-



**Figure 2.** Epitope map of the monoclonal antibodies that bind to the tail of myosin-II. The scales on the left give the length in residues and nanometers from the head tail junction. The horizontal bars to the right of the tail indicate the COOH-terminal ends of fusion proteins that were used to localize the binding sites for the groups of monoclonal antibodies listed on the far right. In addition to the 22 antibodies mapped distal to residue 1,178 in this study, the figure includes six additional antibodies mapped on the proximal part of the tail by Rimm et al. (1989). The boxes around groups of antibodies indicate those that compete with each other for binding to myosin-II.



**Figure 3.** Comparison of the position of antibody binding sites on the tail of myosin-II defined by deletion mapping with the positions measured by electron microscopy of shadowed antigen-antibody complexes. The vertical bars represent the mean positions from electron microscopy  $\pm 1$  SD. The horizontal bars represent the boundaries of the antibody binding site determined by deletion mapping. The numbers of the antibodies are indicated near the bars.

topes on the tail. They are distributed from one end to the other, but are biased toward the COOH terminal half of the tail. However, none have been identified on the last 27 residues that are presumably not alpha-helical and include the three heavy chain phosphorylation sites (Hammer et al., 1987). Single antibodies bind to 10 of these sites. Two antibodies bind to each of three of these sites and three antibodies bind to each of four sites.

The sequences of the minimal epitopes defined by the borders of the deletions do not have any features that strongly differentiate them from the rest of the myosin-II tail. Most of these epitopes are hydrophilic according to the criteria of Hopp and Woods (1983). 7 of 14 epitopes have a net negative charge, 4 are neutral, and 3 have a net positive charge. Overall the epitopes have a net negative charge of 0.105 per residue, substantially higher than the tail as a whole which has a net negative charge of 0.045 per residue.

#### **Comparison of Electron Microscopic Localization of Binding Sites with Epitope Position**

Since the tail of myosin-II is an alpha-helical coiled-coil, the epitope map defines precisely the physical position of each antibody binding site and provides an opportunity to evaluate the accuracy of antibody localization by electron microscopy. In addition to data reported earlier for M2.1, M2.3, M2.4, M2.9, M2.10, M2.12, and M2.46 (Kiehart et al., 1984b; Rimm et al., 1989), we have localized M2.19,

M2.22, M2.23, M2.28, M2.31, M2.33, M2.36, M2.38, M2.40, M2.44, M2.47, M2.49, and M2.50 (Fig. 3). Complexes of some antibodies with myosin-II do not survive the drying and shadowing procedure, including M2.5, M2.14, M2.15, M2.20, and M2.24.

The apparent positions of some of the antibody binding sites on the tail of myosin-II visualized by electron microscopy of sprayed and shadowed molecules differ from the actual epitope positions by up to 30 nm (Fig. 3). In many cases, especially near the tip of the tail, electron microscopy underestimates the distance of the epitope from the head-tail junction, but in other cases electron microscopy overestimates this distance. All antibodies that bind to the last 20 nm of the tail obscure the part of the tail distal to the antibody. This is true even for M2.50 that binds to residues almost 30 nm from the end of the helical domain; <10% of these complexes had tail exposed beyond the antibody. These results show the limitations of relying solely on electron microscopy to localize antibody binding sites on the tails of myosin molecules, especially for sites near the end of the tail.

#### **Effect of Epitope Position on the Ability of Monoclonal Antibodies to Compete with Each Other for Binding to an Alpha-Helical Coiled-Coil**

There is complete agreement between the high resolution epitope map and the results of competitive binding experiments (Table I and Figs. 2 and 4). Almost all antibodies that share a given epitope as demarcated by the method in Fig. 1, compete with each other for binding to myosin-II (Figs. 2 and 4) in a solid phase assay. Given the size of the boundaries of these epitopes, the binding sites of competing antibodies are generally separated by <5 nm (or  $\sim 10$  turns of the alpha-helix) along the coiled-coil (Fig. 2).

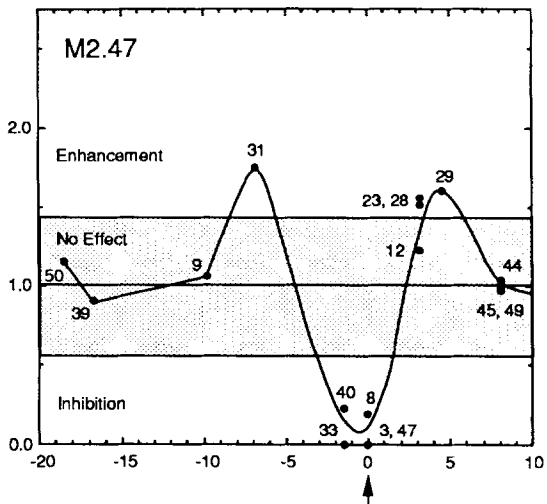
Antibodies that bind to different epitopes can either inhibit, enhance or have no effect on the binding of each other to myosin-II as illustrated for test antibodies M2.47 and M2.9 in Fig. 4, *top* and *center*. Fig. 4, *bottom* summarizes the results of competition between 161 pairs of antibodies with epitopes located <13 nm apart. Antibodies that bind >7 nm apart do not compete with each other (Fig. 4, *bottom* and extensive data not illustrated).

Antibodies that are located <7 nm apart on the coiled-coil can compete with each other, but this is not obligatory. The probability of competition is high below 3 nm (six turns of the alpha-helix) and drops off with the distance between the epitopes (Fig. 4, *bottom*). Inhibition of binding is frequently (21 cases), but not always (31 cases) reciprocal, most likely depending upon the relative affinities of the two antibodies for myosin-II. In the rare cases where antibodies bind close together but do not compete, they may simply bind at different azimuthal positions.

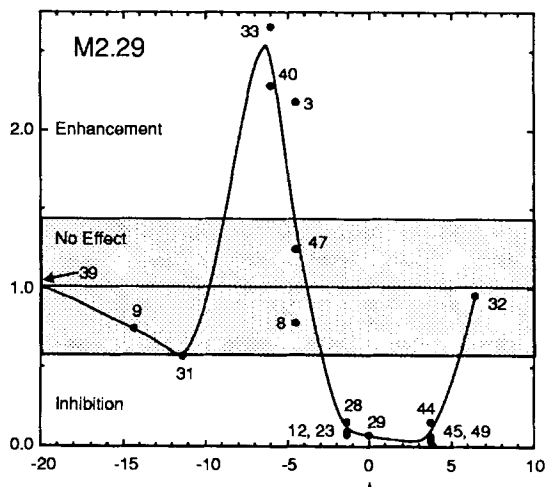
Some antibodies enhance the binding of others to myosin-II by 150–300% (Fig. 4, *top* and *center*, and other examples not illustrated). The highest probability of enhancement occurs at epitopes separated by 3–7 nm along the tail (Fig. 4, *bottom*). The enhancement is reciprocal in only 4 out of 31 cases.

#### **Effects of Tail Binding Monoclonal Antibodies on the Assembly of Myosin-II**

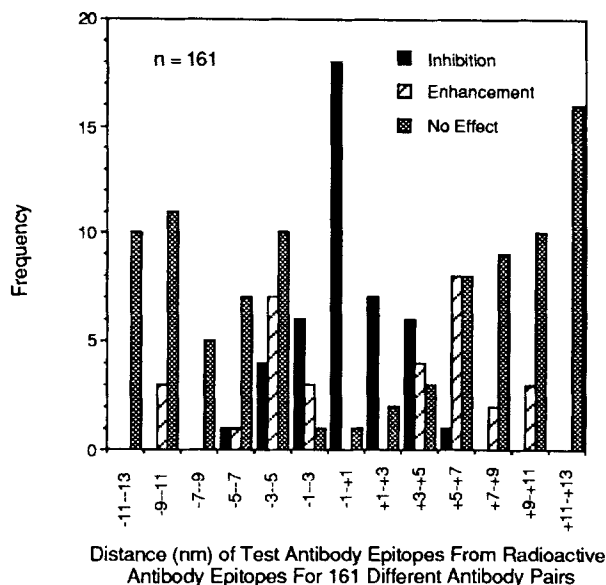
Having mapped the binding sites of the library of tail binding



Distance (nm) of Test Antibody Epitopes From M2.47 Epitope



Distance (nm) of Test Antibody Epitopes From M2.29 Epitope



Distance (nm) of Test Antibody Epitopes From Radioactive Antibody Epitopes For 161 Different Antibody Pairs

antibodies, we completed an analysis of their effects on the assembly of myosin-II minifilaments that was initiated by Kiehart et al. (1984b). Assembly was analyzed by electron microscopy of both negatively stained and rotary-shadowed specimens. Rotary shadowing provided much additional information not revealed in the earlier study with negative staining alone. This analysis was also facilitated by recent advances in our understanding of the assembly mechanism and the demonstration that rotary shadowing is a valid method to visualize assembly intermediates present in solution (Sinard and Pollard, 1989, 1990; Sinard et al., 1989). The normal pathway of assembly involves three very rapid dimerization steps: two monomers form an antiparallel dimer ( $K_d < 10^{-10}$  M); two dimers form an antiparallel tetramer ( $K_d < 10^{-10}$  M); and two tetramers form an antiparallel octamer ( $K_d = 2 \times 10^{-8}$  M) (see Fig. 7, below).

The effects of the antibodies on the assembly of minifilaments depend on both the position of the binding site and the affinity of the antibody (Table I and Fig. 5). In general, antibodies that bind to the proximal 50 nm of the tail do not prevent assembly of minifilaments, while most antibodies that bind to the distal 37 nm of the tail disrupt the normal pathway of assembly with a wide variety of consequences. The antibodies that bind to the distal 37 nm of the tail but fail to inhibit assembly appear to have lower affinity for myosin-II than the antibodies that disrupt assembly. They will be presented separately at the end of this section.

All antibodies tested that bind to the proximal 50 nm of the tail allow assembly of bipolar minifilaments (Fig. 5 for M2.38 and M2.46; Kiehart et al. [1984b] for M2.1, M2.4, and M2.10). Those that bind near the head-tail junction decorate the ends of the minifilaments (Kiehart et al., 1984b for M2.1, M2.4, and M2.10). Those that bind in the middle of the tail may decorate the bare zone (Fig. 5 for M2.46), but some do not (Fig. 5 for M2.38). Electron microscopy (especially negative staining) does not provide reliable quantitative data on the extent of the assembly reaction, so we cannot rule out some inhibition of the assembly process by one or more of these antibodies.

The antibodies that bind tightly to the distal 37 nm of the myosin-II tail can sequester the myosin-II in the monomeric state or allow the assembly of higher order structures including dimers and tetramers (Fig. 5, Table I). Some of these are intermediates along the normal assembly pathway, while others are abnormal intermediates arising from the inhibition of a preceding step in the pathway. We will consider these antibodies in order of their binding sites from proximal

**Figure 4.** Competition between monoclonal antibodies for binding to myosin-II. (Top) Effects of various antibodies on the binding of radiolabeled M2.47 to myosin-II in a solid phase binding assay. A large excess of each competing antibody was tested separately. The graph illustrates the zones that we have defined as having no effect, enhanced binding or inhibition of binding. These definitions are used in the summary of results in the bottom panel. (Center) An experiment similar to the top panel with M2.29 as the labeled antibody. (Bottom) A plot of the frequency of inhibition, no effect, and enhancement of antibody binding as a function of the physical distances defined by deletion mapping between the binding sites of the labeled antibody and each test antibody.

**Table I. Monoclonal Antibodies to the Tail of *Acanthamoeba Myosin-II***

Antibody name	Isotype	Competitive binding group	Epitope location (deletion analysis)	Effect on CaATPase	Effect on actin-activated MgATPase	Effect on gel contraction	Effect on polymerization	Effect on phosphorylation
M2.1	IgG2A	M2.1	942-999	No effect	Inhibits	Inhibits	No effect	
M2.3	IgG2B	M2.3,8,33,40,47	1400-1405	No effect	Inhibits	Inhibits	1>4>2*	No effect
M2.4	IgG2B	M2.4,6,10	881-942	No effect	Inhibits	Inhibits	No effect	
M2.6	IgG2B	M2.4,6,10	881-942	No effect	Inhibits	Inhibits	No effect	
M2.8	IgM	M2.3,8,33,40,47	1400-1405	No effect	Inhibits			
M2.9	IgG1	M2.9,31	1333-1340	No effect		Inhibits	8>4>1>2	
M2.10	IgG1	M2.4,6,10	881-942	No effect	Inhibits	Inhibits	No effect	
M2.12	IgG2B	M2.12,23,28,29,(8)	1419-1429	No effect	Inhibits	Inhibits	1>4>2*	No effect
M2.14	IgG1	M2.14,15	1191-1218					
M2.15	IgG1	M2.14,15	1191-1218	No effect	No effect	Inhibits	No effect	
M2.19	IgG1	M2.19	1264-1274	No effect	Inhibits	Inhibits weakly	8>16>4, 1	
M2.22	IgG1	M2.22	942-999	No effect	No effect	No effect	No effect	
M2.23	IgG2B	M2.12,23,28,29,(8)	1419-1429	No effect	No effect	Inhibits		No effect
M2.28	IgG2B	M2.12,23,28,29,(8)	1419-1429	No effect	No effect	Inhibits	1>2>4*	No effect
M2.29	-	M2.12,23,28,29,44,45,49	1430-1436					
M2.31	IgG2B	M2.9,31	1352-1360	No effect	No effect	Inhibits	8>4>2>1	
M2.32	-	M2.32	1468-1481					
M2.33	IgG1	M2.3,8,33,40,47	1391-1394	No effect	Inhibits	Inhibits	1>2>8>, 4*	
M2.36	IgG2B	M2.36,39,50	1236-1259	No effect	No effect	Inhibits	1>2>4*	
M2.38	IgG1	M2.38	1179-1185		No effect	Inhibits weakly		
M2.39	IgM	M2.36,39,50	1281-1297			No effect		
M2.40	IgG2B	M2.3,8,33,40,47	1391-1394	No effect	No effect		8>4>1	
M2.44	IgG2B	M2.29,44,45,49	1448-1467	No effect	Inhibits weakly	Inhibits	1	Inhibits ~30%
M2.45	IgG2B	M2.29,44,45,49	1448-1467	No effect	No effect			
M2.46	IgG2B	M2.46	1048-1102	No effect	Inhibits weakly	Inhibits	8>16>4>1*	
M2.47	IgG1	M2.3,8,33,40,47	1400-1405	No effect	Inhibits	Inhibits	8, 4>1>2	
M2.49	IgG2B	M2.29,44,45,49	1448-1467	No effect	Inhibits weakly		1>2>4*	
M2.50	IgG1	M2.36,39,50	1275-1280	No effect	No effect	Inhibits weakly	8>4>16, 1>2*	

Summary of the properties including isotype, competitive binding groups, binding sites, and effects on ATPase activities, gel contraction, polymerization, and phosphorylation of the heavy chain. The results of polymerization experiments are summarized by listing the size of the products in order of frequency with 1 = monomer, 2 = dimer, 4 = tetramer, and 8 = octamer. An asterisk (\*) indicates that the antibody remained bound to the myosin after shadowing. Blank cells, not tested.

to distal. In each case we note the binding site as a distance from the head-tail junction.

When assessed by negative staining, M2.36 (binding at 52 nm) appeared to allow the assembly of tiny filaments and to decorate the bare zone, but after spraying and shadowing, decorated monomers and antiparallel dimers were the major species (Fig. 5). This antibody may simply destabilize any filaments that form to an extent where they do not survive spraying and shadowing.

M2.50 (57 nm) inhibits the formation of octamers from antiparallel tetramers (Fig. 5). Compared with controls where octamers predominate, in the presence of M2.50 nearly half of the myosin-II molecules are in the form of tetramers with normal bare zone geometry. All of the tetramers and octamers have two or more antibodies bound to the bare zone.

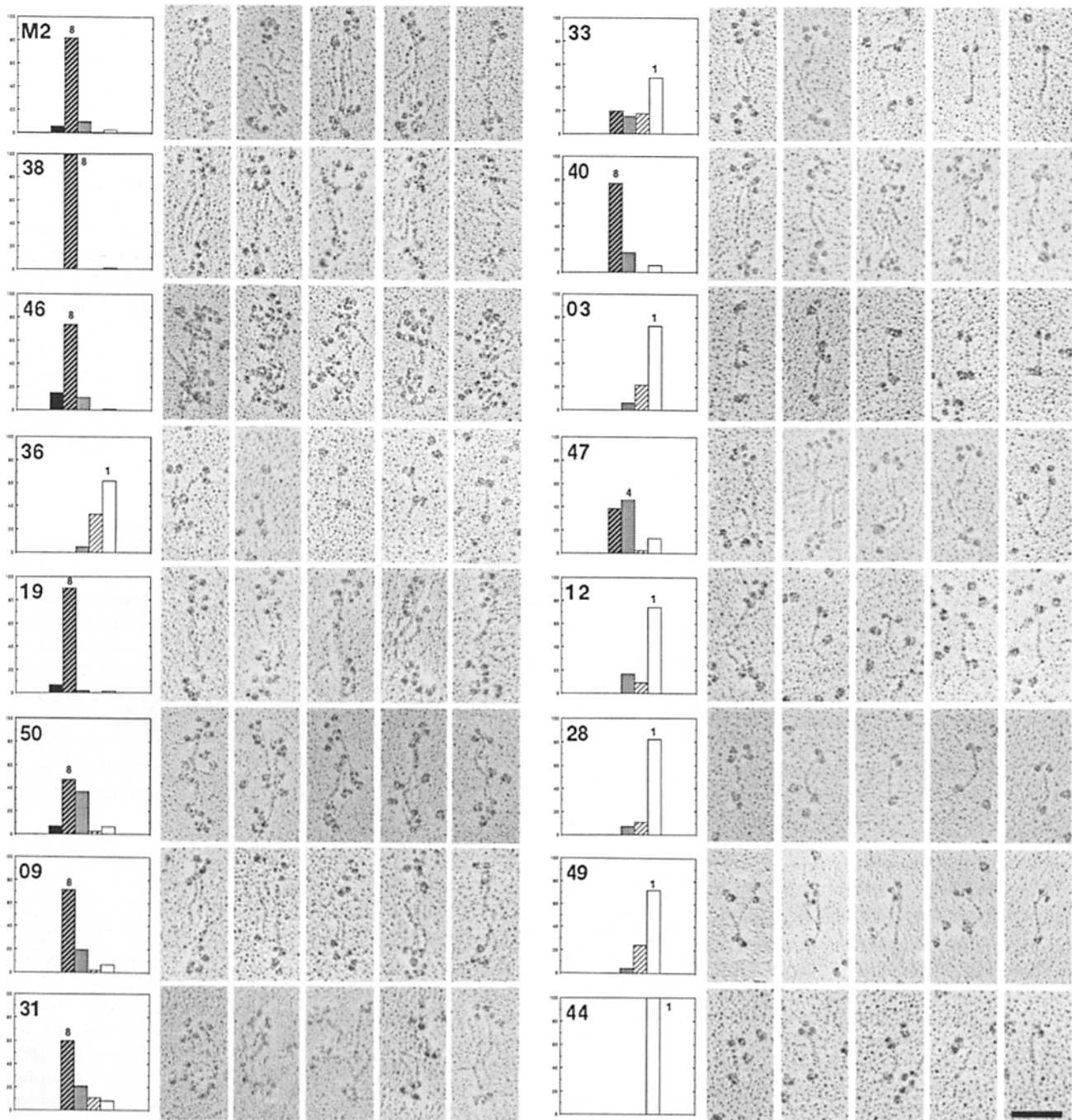
M2.19 (55 nm) does not inhibit assembly and does not decorate the minifilaments (Fig. 5). In high salt <5% of monomers have an M2.19 bound after shadowing, a further indication of low affinity.

M2.33 binds ~73 nm from the head-tail junction and inhibits assembly. Most of the myosin-II is monomeric or aggregated in clusters without defined geometry, but a few dimers and tetramers are also present (Fig. 5).

M2.3 and M2.47 bind to the same epitope (defined by deletions at residues 1,399 and 1,405) 75 nm from the head-tail

junction but have different effects on assembly. M2.3 partially inhibits the formation of dimers as well as the higher order assembly of these dimers (Fig. 5). This yields predominantly monomers along with a substantial number of antiparallel dimers. Most of the dimers and monomers have antibody bound near the tip of the tail (Fig. 5). Many of the "tetramers" may be myosin-II molecules cross-linked by bivalent antibodies near the tips of their tails. No filaments are present in negatively stained specimens. On the other hand, M2.47 partially inhibits assembly at the tetramer step and leaves a few monomers. Neither the tetramers or monomers have M2.47 bound, at least after drying and shadowing (Fig. 5). Short filaments (perhaps tetramers) are present in negatively stained specimens.

M2.12 and M2.28 bind between residues 1,418 and 1,429 ~78 nm from the head-tail junction. Although negative staining revealed some filaments in the presence of M2.12 (Kiehart et al., 1984b and confirmed by new experiments), there are very few complete minifilaments after rotary shadowing (Fig. 5). Instead there was a mixture of bare monomers, monomers with an antibody bound near the tip of the tail and a few tetramers. In the presence of M2.28 no filaments were observed by either method. The specimens were similar in appearance to those with M2.12 except for the presence of a few parallel dimers with a stagger of ~15 nm and an antibody bound near the tip of the tail.

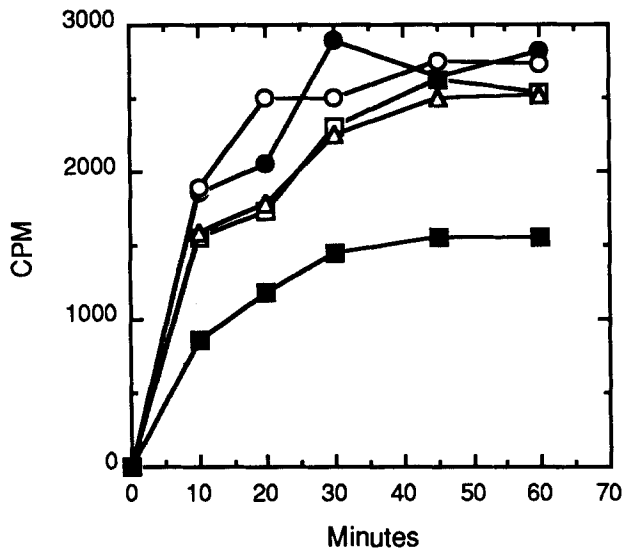


**Figure 5.** Effects of monoclonal antibodies on the assembly of myosin-II minifilaments assessed by electron microscopy of sprayed and shadowed specimens. The electron micrographs were selected to illustrate the range of structures present in each specimen. The histograms give the frequency distributions of myosin-II oligomers formed in the presence of each monoclonal antibody. Monomers, dimers, tetramers, octamers, and hexadecamers are indicated by different shading with the size of the major species indicated above its bar in each graph. The antibody numbers are indicated on each histogram. Bar, 100 nm.

Both M2.44 and M2.49 (84 nm) prevent the formation of filaments detectable by negative staining. With M2.44 only monomers are present in rotary shadowed specimens (Fig. 5). M2.49 produces an interesting mixture of monomers and parallel dimers (Fig. 5). The two molecules in these dimers are staggered by  $\sim 15$  nm with the two tails bound together at a site  $\sim 60$  nm from its head-tail junction of the leading molecule and  $\sim 45$  nm from the heads of the trailing mole-

cule. In these parallel dimers the trailing molecule usually has an antibody bound at the tip of its tail. M2.32 binds to the most distal epitope on the tail, 85 nm from the head-tail junction, and strongly inhibits assembly (not illustrated).

Several antibodies that bind to the distal part of the tail fail to inhibit assembly even when their epitopes are near those of an inhibitory antibody. For example, M2.33 and M2.40 both bind between residues 1,391 and 1,394, but only M2.33



**Figure 6.** Effects of 10-fold molar excess concentrations of monoclonal antibodies on the time course of myosin-II heavy chain phosphorylation. A background of 423 cpm was subtracted from each specimen. (□) antibody buffer; (△) M2.23; (●) M2.28; (○) Alice; (■) M2.44.

has an obvious effect on assembly yielding predominantly monomers (Fig. 5). One clear difference between these two antibodies is that M2.33 remains bound to many of the monomers and dimers while M2.40 does not bind to any of the products of assembly. Similarly M2.19 (55 nm) does not inhibit assembly and does not decorate the minifilaments (Fig. 5). In high salt <5% of monomers have an M2.19 bound after shadowing, a further indication of low affinity.

#### **Effects of Monoclonal Antibodies on Actomyosin ATPase Activity and Contraction of Gelled Cytoplasmic Extracts**

Many of the monoclonal antibodies that bind to the tail of myosin-II inhibit the actin-activated  $Mg^{2+}$ -ATPase and the contraction of gelled cytoplasmic extracts (see Table I for a summary of all antibodies tested; Kiehart and Pollard, 1984a,b for original data on M2.1, M2.3, M2.4, M2.6, M2.9, M2.10, M2.15, and M2.19). The contraction assay was done with high speed supernatants of sucrose extracts of *Acanthamoeba* cytoplasm that form a gel when warmed to room temperature and that then undergo an ATP-dependent contraction. The ATPase assay was done with dephosphorylated myosin-II in 7 mM  $MgCl_2$ , which causes the lateral aggregation of fully formed octameric minifilaments but not of tetramers or smaller assembly intermediates (Sinard and Pollard, 1989).

All of the antibodies tested (M2.3, M2.12, M2.28, M2.33, M2.36, and M2.44) that held myosin-II in the form of monomers or dimers under polymerizing conditions inhibited either or both the actin-activated  $Mg^{2+}$ -ATPase activity and the contraction of gelled extracts (Table I). Antibody M2.31, which has ambiguous effects on assembly, inhibited both functional assays. These extensive results are consistent with our previous conclusion that soluble complexes of monomeric myosin-II with antibody have low ATPase activity and cannot generate the force required to contract gelled ex-

tracts (Kiehart and Pollard, 1984a,b). These results also agree with the inhibition of both assembly and actomyosin ATPase activity by proteolytic removal of the COOH-terminal 66 amino acids from the tail of myosin-II (Kuznicki et al., 1985) and by polyclonal antipeptide antibodies to residues 1,463–1,481 (Atkinson et al., 1988).

The three antibodies that produce mixtures of tetramer and octamers, M2.9, M2.47, and M2.50, gave slightly different results. M2.9 and M2.47 inhibited both the actomyosin ATPase and contraction, while M2.50 inhibited contraction some, but not all of the time. Tentatively, we conclude that tetramers have low ATPase activity and are ineffective in the gel contraction assay, but this deserves closer scrutiny in the future.

The antibodies that bind to the proximal part (M2.4, M2.6, M2.10) or the middle (M2.15, M2.19, and M2.46) of the tail and do not prevent assembly of minifilaments can still interfere with both the actomyosin ATPase activity and gel contraction (Table I). Thus, inhibition of polymerization is not the only mechanism that accounts for loss of these activities.

#### **Effect of Monoclonal Antibodies on Phosphorylation of the Myosin-II Heavy Chain**

Although none of the 50 monoclonal antibodies bound to the nonhelical region at the tip of the tail where the three phosphorylated serines are located (Coté et al., 1984), one antibody, M2.44 that binds between residues 1447 and 1467, partially inhibited phosphorylation of the heavy chain (Fig. 6). In the presence of this antibody both the rate and extent of the phosphorylation were reduced by about one-third. Neither M2.23 nor M2.28, two antibodies that bind to an adjacent epitope between residues 1,418 and 1,429, inhibited the kinase.

## **Discussion**

### **Comparison of Epitope Mapping Methods**

Our characterization of 25 monoclonal antibodies that bind to the tail of myosin-II provides new insights about the accuracy and resolution of four different epitope mapping methods. Mapping on a deletion series prepared by expression of recombinant fusion proteins clearly has the highest resolution and accuracy, at least for linear epitopes like those characterized here. The method can easily and unambiguously define the edge of epitopes to a few amino acid residues. The resolution is limited only by the effort invested in the isolation of a collection of deleted cDNAs. Others have shown that mapping on two deletion series, one from each end of a protein, does not improve the resolution (Gross and Rohmann, 1990). The deletion map confirmed our previous groupings of antibodies by peptide mapping (Kiehart et al., 1984a). Epitope mapping on peptides was correct but limited in resolution by the size of the peptides and knowledge about their positions along the polypeptide chain.

Competitive binding experiments also provide very reliable data on the proximity of antibodies with neighboring epitopes along a linear structure (Fig. 4). Antibodies that bind close together have a high probability of inhibiting the binding of each other to the myosin tail. This probability falls to zero for epitopes with center to center spacing >6.2



nm. These data include both IgG and IgM isotypes. The mechanism of this inhibition is most likely steric hindrance between the antibodies bound to the myosin. Therefore, observation of inhibition in a competitive binding assay establishes that the two antibodies bind within 6.2 nm on a coiled-coil. On the other hand, a negative result cannot exclude binding even with 3 nm. Jackson et al. (1988) demonstrated that two monoclonal antibodies can bind simultaneously to two epitopes separated by only three amino acids.

Electron microscopy of antibody/antigen complexes on the tail of myosin-II has provided rough estimates of the epitope locations, but it has two limitations for high resolution epitope mapping that are unrelated to the resolution of the microscope. First, a large fraction of the antibody/antigen complexes do not survive preparation for electron microscopy. Second, some antibodies appear to bind as much as 30 nm away from their epitopes defined by deletion mapping. This problem is minimal near the heads but is severe near the end of the myosin tail. Many antibodies that bind within 30 nm of the COOH terminus appear to be localized at the tip of the tail and in most cases the tail appears to be foreshortened. Binding of the antibody somehow obscures or distorts the distal part of the tail.

### *Characterization of Epitopes*

The combined results of deletion mapping and competitive binding assays lead to the conclusion that few residues contribute to high affinity binding of antibodies to the tail of myosin-II, but that the region of antigen-antibody contact is likely to be extensive. Many monoclonal antibodies show a profound difference in binding to two fusion proteins differing in length at their COOH termini by <10 amino acids (1.5 nm or three turns of the alpha-helix) (Fig. 2). In one case such a difference was observed with the deletion of only four residues (Fig. 2). Our immunoblot assay is not quantitative, but we expect that positive and negative results (Fig. 1) represent differences of orders of magnitude in the binding constants. On the other hand, the competitive binding experiments (Fig. 4) show that the linear extent of the tail in contact with each antibody is much greater than 1.5 nm, since the probability of competition between two monoclonal antibodies is high when they bind to adjacent sites <5 nm apart. Given the width of a coiled-coil (2 nm), contact sites are likely to be on the order of 10 nm<sup>2</sup>. This is about the same size as the 7-8 nm<sup>2</sup> contacts between lysozyme and three different monoclonal antibodies that have been studied at atomic resolution (see Davies et al., 1988).

Although we have resolved 17 different epitopes on the tail of myosin-II and although 2 or more independently derived monoclonal antibodies bind to 7 of these sites, we doubt that we have identified all of the antigenic sites on the tail. First, we continued to find new epitopes in each new group of monoclonal antibodies tested. Second, the tail is structurally homogeneous and the side chain chemistry of the known epitopes does not distinguish them from the other parts of the tail with no known epitope. This leads us to favor the conclusion of Benjamin et al. (1984) that the whole exposed surface is potentially antigenic.

Further definition of the epitopes circumscribed by the deletion map might be obtained by testing the effect of amino acid substitutions on antibody binding or by testing a panel of synthetic peptides overlapping the epitope for their ability

to inhibit antibody binding (see for example, Schoofs et al., 1988; Tan et al., 1990). Substitution of amino acids in fusion proteins by *in vitro* mutagenesis or in synthetic peptides might provide information about the azimuthal orientations of the epitopes. On the other hand, detailed studies of lysozyme have established that there is no simple, quantitative relationship between variations in amino acid side chain chemistry of an antigen and antibody affinity (reviewed by Davies et al., 1988), so more insight about most of the epitopes described here would require x-ray crystallography of the antigen-antibody complex.

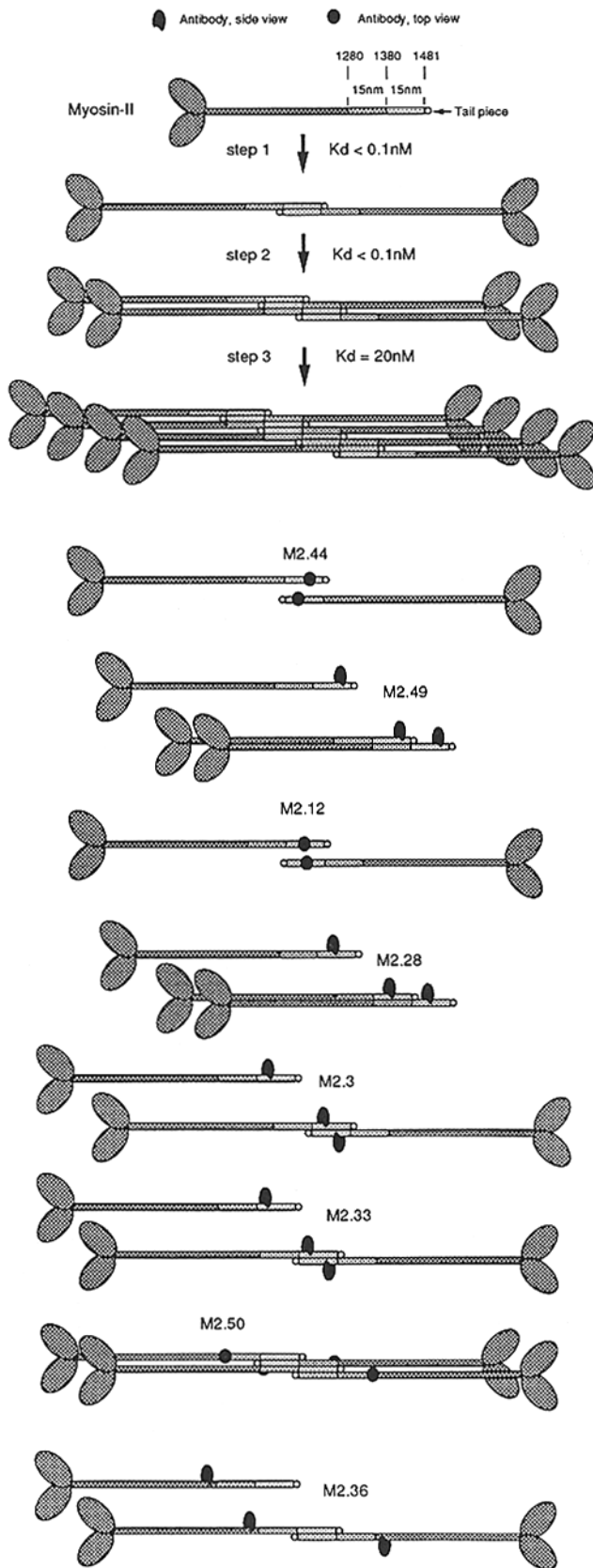
### *Enhancement*

Our competitive binding experiments provide a new insight into the process by which one monoclonal antibody can enhance the binding of another antibody to an antigen. Such enhancement has been observed many times with both protein (Ehrlich et al., 1982; Holmes and Parham, 1983) and polysaccharide (Greenspan et al., 1987) antigens, but there has never been a detailed assessment of the role of epitope proximity on the probability of enhancement. Since the epitopes on the tail of myosin-II are present in a stereotyped, linear array, and since we tested a large collection of antibodies, we learned that enhancement is most likely when two epitopes are separated by 3-7 nm (Fig. 4).

This new information is consistent with two different mechanisms that have been suggested for enhancement, both of which require local effects. (We think that we can rule out formation of cyclic antigen-antibody complexes as invoked for other systems [Ehrlich et al., 1982], since our antigen is immobilized on a surface.) The first local mechanism is that the enhancing antibody alters the conformation of the antigen and increases the affinity of a neighboring epitope for its antibody. This has been suggested for systems where Fab fragments of immunoglobulins enhance the binding of other monoclonal antibodies (Heinz et al., 1984; Parham et al., 1986). In support of this mechanism, x-ray crystallography has revealed small changes in the structure of lysozyme bound to an antibody (see Davies et al., 1988). One would expect that antibody-induced conformational change in a coiled-coil to propagate over a limited distance, and this could explain the limited zone of enhancement adjacent to any epitope. The second local mechanism is that the Fc domains of one antibody might interact with an antibody bound to an adjacent site and enhance its binding to the antigen. This has been suggested in cases where intact antibodies are more effective enhancers than Fab's (Ehrlich et al., 1982; Greenspan et al., 1987). Such a mechanism would require adjacent epitopes as we observed. Our results cannot distinguish these two alternatives (or some trivial explanation for enhancement), but do define for the first time the linear distance over which such enhanced binding can occur.

### *Identification of Sites on the Myosin-II Tail That Are Critical for Minifilament Assembly*

Antibody affinity appears to be particularly important in the effects of antibodies on myosin-II assembly, since the myosin-II molecules have such a high affinity for each other (Sinard and Pollard, 1990). This may lead to a situation where an antibody binds to a site essential for polymerization but fails to inhibit assembly since it has a lower affinity for myosin-II



**Figure 7.** Diagrammatic summary of the effects of monoclonal antibodies on the assembly of myosin-II minifilaments. This scale drawing with the antibodies precisely positioned at their binding sites illustrates one or two major species of oligomers formed in the presence of each antibody. Two different antibody shapes are

than the myosin-II molecules have for each other. M2.3 and M2.47 are examples of antibodies that bind to the same site but differ in affinity. By both electron microscopy and immunoblotting M2.3 appears to have a much higher affinity for the tail than M2.47 and is a much stronger inhibitor of assembly. M2.33 (strong) and M2.40 (weak) are another example of this behavior. It is probably not coincidental that all of the antibodies (M2.9, M2.19, M2.31, M2.40, and M2.47) that inhibit assembly poorly, compared with antibodies on the same or adjacent sites, do not bind to myosin-II in shadowed specimens. On the other hand, antibodies that strongly inhibit one or more steps are nearly always visible on the blocked intermediates after shadowing (Fig. 5).

Since the minifilaments are held together by specific interactions between the tails of the myosin-II molecules, the effects of a monoclonal antibody depend on several geometrical factors, including the size of the antibody and the longitudinal and azimuthal position of the epitope. Although only a few amino acids contribute to high affinity binding, the region of the myosin-II tail covered by the antibody is substantially larger,  $\sim 5$  nm long. Thus antibody binding can sterically block interactions that occur over a relatively large area,  $\sim 10$  nm<sup>2</sup>. The longitudinal location is obviously important for interfering with molecular associations. The sensitivity to azimuthal position was not anticipated because we thought that a bulky antibody bound to the slender tail of myosin-II might block interactions in all directions. However, given that antigens bind in a shallow groove on one end of the Fab domain (Davies et al., 1988; Stanfield et al., 1990), at least part of the coiled-coil is likely to be exposed even at the antibody binding site. Such exposed surfaces of the tail can apparently participate in binding to another myosin-II molecule. If the azimuthal positions of the epitopes were known, it might be possible to deduce the packing of the tails in the minifilaments.

With one exception, the effects of the monoclonal antibodies on assembly can be explained by steric hindrance of the associations required for one or more of the three dimerization reactions required to form minifilaments (Fig. 7). The figure illustrates our interpretation of all of the results, so only the main points are discussed in the following paragraphs. For each antibody we have included the most abundant species. In several cases, we also show second most abundant species, when its structure is revealing of the mechanism of assembly. When viewing this figure, keep in mind that it is drawn in two dimensions, since nothing is known about the packing of the tails in the third dimension. The effects of these IgGs are remarkably discrete with clear differences apparent even between two antibodies that bind to the same epitope. We have not compared these effects of IgGs with the effects of Fab fragments of these antibodies, but expect that even more subtle differences will be revealed by the smaller probes.

used to illustrate the different azimuthal positions postulated to account for the observed assembly intermediates. The normal assembly pathway is illustrated at the top. Note that two different antibodies such as M2.44 and M2.12, M2.49 and M2.28, and M2.3 and M2.33 can give the same products even though they bind to different sites.

M2.44 blocks steps 1–3, while M2.49 blocks step 1 but allows step 2 to a limited extent. Since these two antibodies bind to the same epitope, the difference is likely to be due to the azimuthal positions of the epitopes, with M2.49 leaving open laterally a site required for the parallel association of the last 15 nm of coiled-coil with the second 15 nm of the trailing myosin-II molecule. Both blocked sites are required for step 3.

The pair, M2.12 and M2.28, have effects that closely parallel those of M2.44 and M2.49, even though the M2.12/M2.28 epitope is at least 20 residues closer to the head than the M2.44 epitope. The formation of these parallel dimers in the presence of M2.49 and M2.28 shows that the second assembly step is not absolutely dependent upon prior formation of antiparallel dimers. This conclusion is confirmed by experiments with myosin-II fusion proteins (Sinard et al., 1990). Molecules lacking the tail piece cannot form antiparallel dimers (step 1) but can still make parallel dimers staggered by 15 nm (step 2).

M2.3 and M2.33 bind to adjacent epitopes and have similar effects on assembly. Both completely inhibit steps 2 and 3 but only partially inhibit step 1. Both epitopes are just COOH-terminal to the site at residue 1,380 postulated by Sinard et al. (1989) to be the initial binding site on the coiled-coil for the nonhelical tail piece during step 1, so these antibodies must bind in an azimuthal position that does not block the binding site.

M2.50 binds outside the region of 15 nm overlap in antiparallel dimers and allows steps 1 and 2 but not step 3. The antibody binds within the overlap used in step 2. Since at least two different intermolecular interactions are required to bind together two antiparallel dimers, we cannot argue persuasively that the azimuthal position fortuitously allows both of these interactions as illustrated in Fig. 7. Rather, we suspect that the essential associations for step 2 are largely or entirely in a narrow ~20 nm zone in the middle of the tetramer. The importance of this central region is supported by the formation of myosin-II fusion protein tetramers and octamers that are held together only near the tips of their tails (see Fig. 7 in Sinard et al., 1990). Associations outside the central region required for step 3 are blocked by M2.50.

The effect of M2.36 on assembly provides the only example that cannot be explained simply by steric hindrance. This antibody binds outside the regions of the distal tail that overlap in steps 1 and 2 and yet it inhibits both of these steps. Therefore, it must have some long range effect that remains mysterious. It might be related to the fact that M2.36 binds near proline 1,244 where the tail frequently bends (Hammer et al., 1988).

The results with monoclonal antibodies complement our analysis of the assembly of myosin-II fusion proteins with COOH-terminal deletions (Sinard et al., 1990). The COOH-terminal deletions have identified three very short sequences in the last 15 nm of the tail that are essential for each of the three steps (see Figs. 1 and 13 in Sinard et al., 1990). Considering these to be donor sites, there must be acceptor sites on adjacent myosin-II molecules in minifilaments. The antibodies have been particularly useful for identifying these acceptor sites (Fig. 7), because the binding properties of these acceptor sites cannot be tested by reconstitution for two reasons. First, neither the donor nor acceptor sites are self-sufficient for assembly, and second, there are extensive

regions of interaction and no individual residues are likely to contribute much binding energy. In addition, the antibodies have revealed one site where they may exert long-range effects on the conformation of essential binding sites.

### **Identification of a Site on the Coiled-Coil that Affects Phosphorylation**

M2.44 binds to an epitope defined by residues 1,447–1,467, near the end of the coiled-coil at 1481 and inhibits by one third phosphorylation in the nonhelical COOH-terminal domain. We speculate that this represents nearly complete inhibition of phosphorylation at one of three serines located at positions 1,484, 1,489, and 1,494 (Coté et al., 1984). This antibody binds 17–37 residues (3–5 nm) away from the proximal phosphorylation site. At this distance ~30% of antibodies inhibit binding of a neighboring antibody (Fig. 4), so the most likely mechanism of inhibition of the kinase is steric hindrance of enzyme binding to its substrate. Other less likely possibilities include the folding back of the nonhelical tail piece onto the helical part of the tail to make direct contact with the M2.44 epitope or that the heavy chain kinase has a recognition site that extends into the helical region of the tail.

### **Comparison with Previous Work**

Monoclonal antibodies have probably not reached their full potential for revealing how myosin assembles and functions, because our results and each of the earlier studies have all provided unique insights. For example, there are antibodies to vertebrate myosin-II that can stabilize filaments (Trybus and Henry, 1989), stabilize folded monomers or extended monomers (Citi et al., 1989), or inhibit assembly (Citi et al., 1989). All of these antibodies were useful in elucidating the relationships among conformation, assembly, phosphorylation, and ATPase activity. Antibodies to *Dictyostelium* myosin-II have a wide range of effects on assembly, motility, and actin-activated ATPase activity (Peltz et al., 1985; Flicker et al., 1985; Pagh and Gerisch, 1986). These results generally parallel our results with *Acanthamoeba* myosin-II in establishing the importance of the distal tail in assembly and of assembly on enzyme activity. One of these monoclonal antibodies to *Dictyostelium* myosin-II even inhibited antiparallel associations but not parallel associations of the tails (Pagh and Gerisch, 1986), an effect similar to M2.49 and M2.28. When the precise location of these epitopes and the details of the *Dictyostelium* myosin-II assembly mechanism become available, it will be interesting to see whether there are any general principles governing the assembly and functions of these two myosin-II's that differ considerably in the length of their tails.

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