Monoclonal Antibodies Demonstrate Limited Structural Homology between Myosin Isozymes from *Acanthamoeba*

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ABSTRACT We used a library of 31 monoclonal and six polyclonal antibodies to compare the structures of the two classes of cytoplasmic myosin isozymes isolated from *Acanthamoeba*: myosin-I, a 150,000-mol-wt, globular molecule; and myosin-II, a 400,000-mol-wt molecule with two heads and a 90-nm tail. This analysis confirms that myosin-I and -II are unique gene products and provides the first evidence that these isozymes have at least one structurally homologous region functionally important for myosin's role in contractility. Characterization of the 23 myosin-II monoclonal antibody binding sites by antibody staining of one-dimensional peptide maps and solid phase, competitive binding assays demonstrate that they bind to at least 15 unique sites on the myosin-II heavy chain. The antibodies can be grouped into six families, whose members bind close to one another. None of the monoclonal antibodies bind to myosin-II light chains and polyclonal antibodies against myosin-II light or heavy chain bind only to myosin-II light or heavy chains, respectively: no antibody binds both heavy and light chains. Six of eight monoclonal antibodies and one of two polyclonal sera that react with the myosin-I heavy chain also bind to determinants on the myosin-II heavy chain. The cross-reactive monoclonal antibodies bind to the region of myosin-II recognized by the largest family of myosin-II monoclonal antibodies. In the two papers that immediately follow, we show that this family of monoclonal antibodies to myosin-II binds to the myosin-II tail near the junction with the heads and inhibits both the actin-activated ATPase of myosin-II and contraction of gelled cytoplasmic extracts of *Acanthamoeba* cytoplasm. Further, this structurally homologous region may play a key role in energy transduction by cytoplasmic myosins.

Myosins from a variety of nonmuscle sources share many properties with muscle myosin (10, 34), yet there is little evidence regarding their physiological functions or the mechanisms by which they transduce chemical energy into mechanical force. We have taken an immunochemical approach to study myosin function and have produced polyclonal and monoclonal antibodies as probes for the structure and function of the myosin isozymes in *Acanthamoeba*.

Acanthamoeba is particularly favorable for such studies because this protozoa has, in addition to a conventional myosin called myosin-II (31, 39), unique globular myosin isozymes called myosin-I (37, 38; see reference 28 for review). Both classes of myosin bind to actin filaments and have Mg^{++}-ATPase activity stimulated by actin (6, 7, 30, 38), but they have distinctive physical and chemical properties. For example, the constituent polypeptides all have different molecular weights and one-dimensional peptide maps (13), and myosin-II has 32 cysteines (39) compared with none in myosin-I (37). Myosin-II (31, 39), like most conventional myosins, consists of two heavy chains and four light chains and has a molecular weight of 400,000 (39). There are two heads and a 90-nm tail that can aggregate laterally to form the backbone of bipolar filaments (35). Myosin IA and IB have molecular weights of ~150,000 and consist of a single heavy chain plus one or more light chains (37, 38). Finally, rabbit polyclonal antibodies recognize unique determinants on myosin-I and -II (12, 33). The precise relationship between these myosins and their roles in cell motility remain to be established.

In this paper, we elucidate the structural relationships between myosin-I and -II through the production, purification, and immunological and physical characterization of 31 monoclonal and six polyclonal antibodies that bind specifically to

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Acanthamoeba myosins. Antibody binding to one-dimensional peptide maps of myosin-II and competitive binding assays to intact myosin-II established that the 23 anti-myosin-II monoclonal antibodies bind at least 15 unique sites on the myosin-II heavy chain that are clustered spatially into six groups that we call families. We confirm that myosin-I and -II are the products of distinct myosin genes through studies of antibody cross-reactivity: none of the myosin-II monoclonal antibodies bind to myosin-I and two of the antimyosin-I monoclonals fail to cross-react with myosin-II. In addition, none of the antibodies recognize polypeptides larger than the 175,000-mol-wt, myosin-II heavy chain. This data shows that myosin-I and -II each contain unique antigenic determinants and their heavy chains are not produced from a common high molecular weight precursor. In addition, we demonstrate for the first time that these two morphologically distinct myosin isozymes are, to some extent, structurally related: two monoclonals against myosin-I bind strongly to myosin-II and four others bind weakly. One of two polyclonal sera raised against purified myosin-I heavy chains also react with myosin-II. The most cross reactive myosin-I antibodies bind to a constellation of myosin-II peptides identical to the set stained by the major family of myosin-II antibodies. This family of antibodies binds to the proximal end of the myosin-II tail, near its junction with the globular heads (23). These studies show for the first time that the globular myosin-I molecule has domains structurally related to the filamentous myosin-II tail.

In a second paper (23), we localize the binding sites of some of the monoclonal antibodies on myosin-II by electron microscopy of individual myosin-II molecules and myosin-II filaments and evaluate antibody effects on the assembly of myosin-II filaments. We use the electron microscope data in conjunction with the relationships ascertained by competitive binding studies and antibody staining of one-dimensional peptide maps to localize the binding sites of all but six myosin-II antibodies. The electron microscopy also extends our understanding of the relationship between some of the antibodies and demonstrates that certain antibodies that bind to the myosin-II tail are potent inhibitors of myosin-II filament formation.

In a third paper (25), we evaluate the effect of the antibodies on the actin activated ATPase activity of purified myosin-II and the contraction of gelled extracts of amoeba cytoplasm. Some of the antibodies inhibit mechanochemical energy transduction and confirm a role for myosin-II in the contraction of a cell free model system. The results from the three papers identify some parts of myosin-II that contribute to its major functions. Most of the conclusions agree with accepted ideas about the function of myosin but some are completely unpredicted. These features might relate to the special functional requirements of contractility in nonmuscle cells but could be general properties of myosin that have not been revealed by previous studies.

Portions of this work were presented at Meetings of the American Society for Cell Biology and the Biophysical Society (20–22, 33).

MATERIALS AND METHODS

Acanthamoeba Cell Culture: *Acanthamoeba castellanii* were cultured by the methods of Pollard and Korn (37), except that one half of the protease peptone in the culture medium was replaced by an equivalent weight of yeast extract (Difco Laboratories, Inc., Detroit, MI).

Protein Purification: *Acanthamoeba* myosin-I was purified by the method of Maruta et al. (30) and myosin-II was purified by the method of Pollard et al. (39).

Protein Concentrations: Protein concentration was measured by absorbance at 280 nm. Extinction coefficients were 0.56 cm^2/mg for myosin-II and 1.4 cm^2/mg for mouse Ig. In some cases protein concentrations were estimated with the Bradford assay (3) or the Hartree assay (14), using ovalbumin as a standard.

Monoclonal Antibody Production and Physical Characterization: The production and characterization of monoclonal antibodies are described in detail in the appendix. The specificity of each antibody was established by reaction with purified myosins, partial proteolytic and chemical digests of purified myosins, and whole cell extracts that were electrophoresed on polyacrylamide gels in the presence of SDS, then transferred to nitrocellulose paper (43). Certain physical properties of each antibody and the apparent affinity of each antibody for binding to myosin were determined as detailed in the appendix.

Rabbit Antibodies to Myosin Subunits: The heavy and light chains of purified myosin-I and myosin-II were isolated by preparative gel electrophoresis in SDS (40). Their purity was established by analytical gel electrophoresis (36). Two rabbits were immunized with 15 to 50 μg of each antigen (myosin-I heavy chain, myosin-II heavy chain and myosin-II light chains) as described by Fujiwara and Pollard (11) and ~75 ml of blood was obtained every other day during the second week after boosting. All of the rabbits produced specific binding. Gamma-globulins were obtained from serum by ammonium sulfate precipitation (11) and were evaluated by the same methods used for the monoclonal antibodies.

Reaction of Antibodies with Polypeptides Separated by Gel Electrophoresis: Polypeptides were resolved according to their molecular weight by SDS gel electrophoresis (36), then stained with antibodies either directly (1, 4), or after electrophoretic transfer to nitrocellulose paper (43). To facilitate the transfer of polypeptides, we added 0.1% SDS to the transfer buffer. After transfer, peripheral lanes with molecular weight standards and a small fraction of the test sample were stained for protein with amido black (32). A 7 cm wide central lane with the remainder of the test sample was incubated in STTAB[1] buffer (150 mM NaCl, 10 mM Tris-Cl, pH 7.7, 3 mM NaN_3, 0.1% Triton X-100, 0.1% bovine serum albumin) for ~15 min, then cut with a microtome knife into 40 strips, each of which displayed the entire spectrum of resolved polypeptides. Individual strips were incubated with specific antibody in STTAB or in culture medium with 0.1% Triton X-100 added. They were incubated at 4°C with gentle agitation for 4–48 h. The strips were washed with three changes of STTAB and reacted with approximately $1–10 \times 10^5$ cpm/ml ^{125}I-goat antimouse $F(ab')_2$ antibody (for monoclonal antibodies) or with ^{125}I-Protein A (for rabbit antibodies) in STTAB at 4°C with gentle agitation for 3–24 h. After washing with three changes of STTAB, the strips were mounted on Whatman 3MM paper with double stick tape and dried for 15–30 min under vacuum. Autoradiograms were made on Kodak X-Omat AR X-ray film and processed in an X-Omat processor. Exposures ranged from 15 min to 10 d.

Competitive Binding Assays: We used a competitive, solid-phase binding assay to evaluate the ability of each unlabeled monoclonal antibody to block the binding of directly labeled homologous and heterologous antibodies to native myosin-II. DEAE-purified antibodies were labeled in vitro with ^{125}I or biosynthetically with [^{35}S]methionine as described in the appendix. The assay was similar to the solid-phase binding assay, except that test antibodies were directly labeled, so no second, labeled antimouse antibody was required. Wells containing myosin-II were washed once with STTAB, then incubated with $0.4–2.5 \times 10^{-9}$ M ^{125}I-labeled antibody ($0.5–3 \times 10^5$ cpm/well) in the presence of 10^3- to 10^4-fold molar excess of unlabeled DEAE-purified monoclonal antibody. Incubation with labeled antibody in the absence of unlabeled antibody provided a measure of labeled antibody binding without competitor present. Alternately, competitive binding was analyzed with biosynthetically labeled antibody. Wells were incubated with ^{35}S-labeled antibody ($1–10 \times 10^{-8}$ M, 2×10^4 cpm/well) in the presence of a 2- to 100-fold molar excess of cold antibody. After 1–2 h of incubation, wells were rapidly washed three times with STTAB and counted. In the case of ^{35}S-labeled antibodies, the proteins were solubilized with 50 μl of 2 N NaOH, the solution was neutralized with 50 μl of 2 N HCl, and 95 μl of the mixture was counted in 8 ml of Beckman EP Ready Solv scintillation cocktail in a liquid scintillation spectrophotometer.

Nonspecific binding was determined in three ways that yielded essentially identical values. Labeled, nonspecific, control antibody was incubated in wells

[1] *Abbreviations used in this paper:* STTAB, 150 mM NaCl, 10 mM Tris-Cl, pH 7.7, 3 mM NaN_3, 0.1% Triton X-100, 0.1% bovine serum albumin; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

containing myosin-II; specific, labeled antimyosin-II was incubated in wells containing an antigen unrelated to myosin-II; or (for ^{125}I-labeled antibodies) labeled, specific antibody was incubated in the presence of 1–10 × 10^3-fold excess of cold, homologous antibody. Backgrounds determined in these three ways were identical in almost all cases tested and were usually 0.5–1% of the total counts of specific antibody bound in the absence of competing antibody. In each experiment raw counts were adjusted by subtracting the background determined in one of these three ways.

RESULTS

Hybridomas Produce Antibodies Against Myosin-I and -II

We have produced and partially characterized monoclonal antibodies against myosin-I and -II. Initially, we identified hybridomas secreting antibodies directed against cytoplasmic myosins from *Acanthamoeba* with a solid-phase binding assay using native myosin-I or -II as test antigens. We selected and cloned 23 hybridomas producing antimyosin-II and eight producing antimyosin-I (see appendix). Each antibody producing cell line was named according to the eliciting antigen ("M1." for myosin-I and "M2." for myosin-II) followed by a unique integer suffix to designate individual clones. Thus antibodies M2.1 through M2.27 are antibodies to myosin-II secreted by distinct clones. The production, purity, and a number of the physical properties of each antibody are described in the appendix and are summarized in the appendix in Table III. Knowledge of these properties is essential for subsequent design of experiments and interpretation of the binding and inhibition studies described in later papers (23, 25, 26).

Specificity of Monoclonal Antibody Binding to Acanthamoeba Polypeptides

The monoclonal antibodies against myosin-I and -II react specifically and exclusively with myosin heavy chains among all of the *Acanthamoeba* polypeptides resolved by gel electrophoresis in SDS (Fig. 1). The M2 antibodies bind only to the 175,000-mol-wt myosin heavy chain. All M1 antibodies bind to the 125,000–130,000-mol-wt myosin-I heavy chain. The polyacrylamide gels used for these experiments do not resolve the myosin-IA and -IB heavy chains, so we do not know whether our antibodies bind to one or both myosin-I isozymes. The myosin-I antibodies occasionally stained a low molecular weight species (<11,200) that co-migrated with the dye front, well ahead of the myosin light chains. Unlike antibody staining of the myosin heavy chains, staining of the material at the dye front was not reproducible and may have been due to proteolytic fragments of myosin generated during sample preparation. Some of the myosin-I antibodies cross react with myosin-II heavy chain (Fig. 1 *b*). Similar results are obtained when purified myosin-I and -II are used instead of whole amoeba extract. These experiments demonstrate the specificity of the monoclonal antibodies to the two myosin isozymes and that the two myosins have at least one common antigenic site that is considered in detail below.

Monoclonal Antibody Binding to Myosin-II Peptides

Two monoclonal antibodies, M2.17 and M2.18, bind unambiguously to the myosin-II head because each reacts with

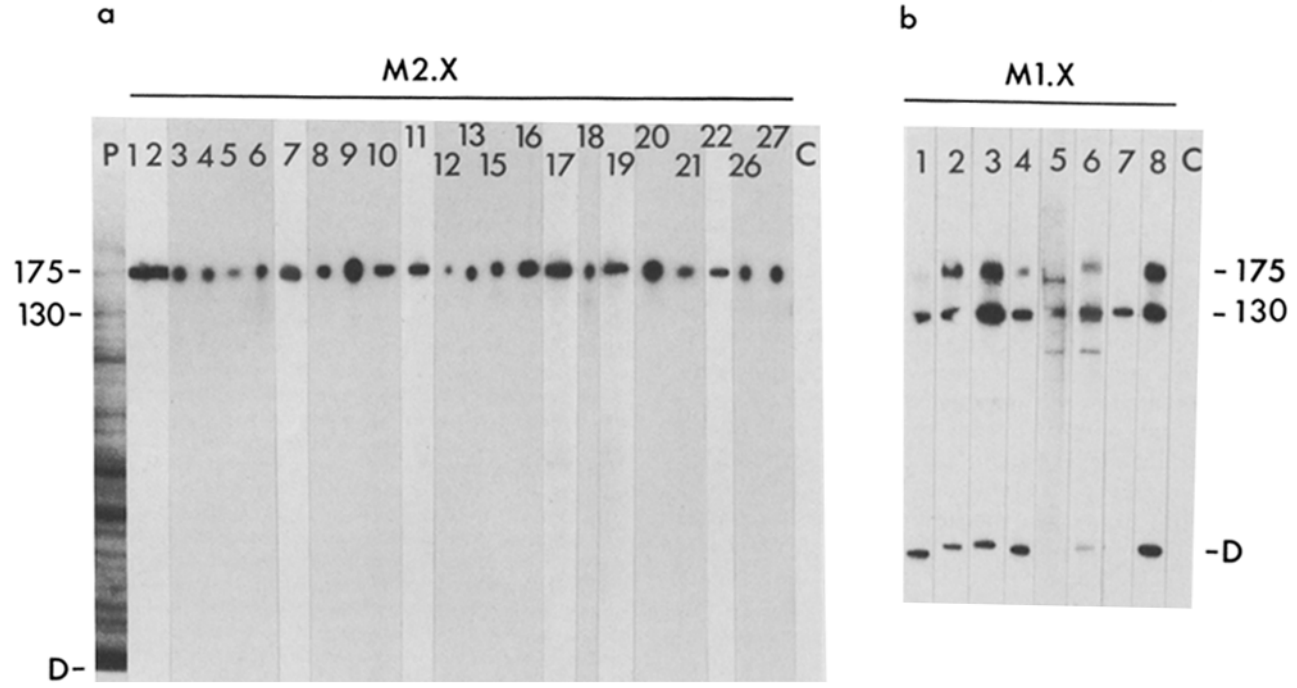

FIGURE 1 Antibody staining of whole *Acanthamoeba castellanii* polypeptides separated by PAGE in SDS and transferred to nitrocellulose paper. (Strip *P*) Whole amoeba polypeptides stained with amido black. (Numbered strips) (*a*) Autoradiograms depicting the staining of *Acanthamoeba* myosin heavy chains by antibodies to myosin-II (M2.x). (*b*) A different experiment shows staining of whole amoeba proteins by myosin-I antibodies (M1.x). (Strips C) Staining by a control antibody directed against chicken pectoralis myosin subfragment-1. All blot strips were stained with antibody in culture supernatant to which Triton X-100 had been added to a final concentration of 0.1%. Indistinguishable results were obtained when blots were stained with purified antibody in STTAB buffer. Migration of myosin heavy chains are marked with their respective molecular weights (175,000 for myosin-II, 130,000 for myosin-I). *D* marks the position of the dye front. Antibody binding at the dye front was not reproducible like the staining of the 125,000–130,000- and 175,000-mol-wt peptides (see text).

a 70,000-mol-wt tryptic peptide previously established to comprise the bulk of the head and contain the amino terminus of the myosin-II heavy chain (5). This 70,000-mol-wt fragment also contains the ATP and actin-binding sites. We observed identical peptide binding results when myosin-II was cleaved into 70,000- and 105,000-mol-wt peptides during a dephosphorylation experiment (Fig. 2). All the antibodies bind to the intact heavy chains, also shown on these blots.

The remaining 21 antibodies, including the two M1 antibodies that cross-react with myosin-II, bind to the 105,000-mol-wt tryptic fragment that includes the carboxy terminus of the heavy chain and all of the myosin-II tail. Assuming that the myosin tail has the same mass per unit length (885 mol wt/nm) as tropomyosin, another alpha-helical coiled coil, we estimate that 80,000 mol wt of heavy chain is required for the 90-nm tail of myosin-II. Thus, the N-terminal 25,000 mol wt of the 105,000-mol-wt tryptic fragment is part of the myosin-II head and antibodies that bind the 105,000-mol-wt fragment may bind either the head or the tail.

Reaction of each antibody with the myosin-II peptides produced by more extensive hydrolysis by four other proteolytic agents establishes that the library of monoclonal antibodies recognizes a wide variety of antigenic determinants on myosin-II. Chemical cleavage (NH_2OH or CNBr) or partial proteolysis (V-8 protease or alpha chymotrypsin) generated numerous peptides that we separated by gel electrophoresis and stained with each antibody to evaluate the relationship among the antigenic sites. Fig. 3 and Table I illustrate the results for the hydroxylamine peptides. Table IV, in the appendix, summarizes the data for the other cleavages.) Because the digests are incomplete, the antigenic determinants usually appear on a variety of peptides that range in size up to the 175,000-mol-wt heavy chain. For unknown reasons, no antibodies reproducibly stain peptides with molecular weights <11,000. Using peptides generated by all five of the hydrolytic agents, several autoradiogram exposures of each antibody stained map, and both peptide mobility and staining intensity, we verified the similarities or differences in the antibody staining patterns in order to determine the relationship between the antigenic sites recognized by each of the antibodies (summarized in Tables I and II and in the appendix in Table IV). As described below, we grouped the antibodies into families delineated by the proximity of their binding sites on the myosin-II molecule.

For each type of digest, several antibodies bind to unique sets of peptides. For example, antibodies M2.1, -5, -13, -15, -16, -19, and -20 each bind to a unique constellation of NH_2OH peptides (Fig. 3 and Table I). The antigenic sites that these antibodies recognize must be separated from each other by at least one NH_2OH cleavage site.

Alternately, more than one antibody may bind to an identical set of peptides. These antibodies bind to antigenic sites that are close to or identical with one another, so we refer to these antibodies as members of a family. Between the two extremes are antibodies that bind to antigenic sites found on common high molecular weight peptides, but different small peptides. The antigenic sites that these antibodies recognize are probably close to one another, but separated by cleavage sites.

The largest and most clear cut family of antibodies includes M2.4, -6, -7, and -26 and provides an excellent example of how the antibody families are delineated by the peptide mapping studies. The antigenic sites to which these antibodies bind must be very close together, because none of the five hydrolytic agents (Fig. 3, Tables I, II, and, in the appendix, Table IV) used resolves them onto different peptides. These antibodies are also indistinguishable by competitive binding assays (see below). Because of the close relationship between their antigenic sites (they may be identical), we refer to these antibodies as members of a "core" family. Additional family

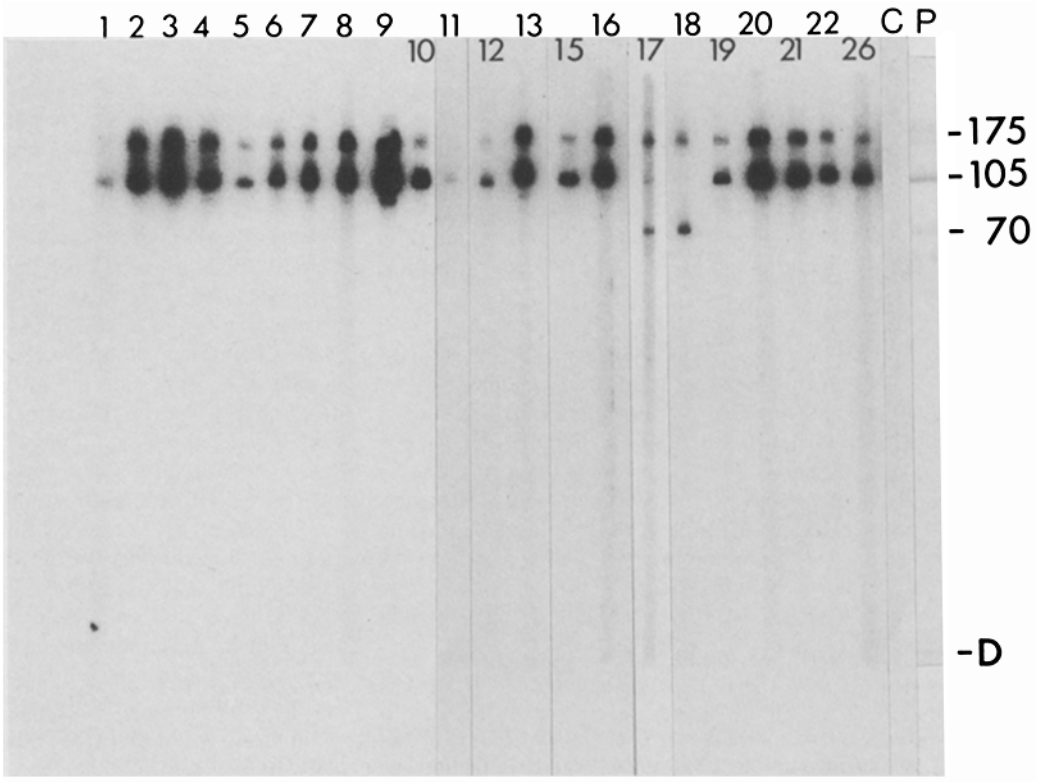

FIGURE 2 Antibody staining of 105,000 and 70,000-mol-wt fragments of myosin-II separated by PAGE in SDS and transferred to nitrocellulose paper. (Strip *P*) Amido black stained peptides. (Numbered strips) Autoradiograms show staining of intact myosin-II heavy chain and the 105,000- and the 75,000-mol-wt fragments of myosin-II by antibodies to myosin-II (M2.x) and myosin-I (M1.x). (Strip C) Staining by a control antibody directed against chicken pectoralis myosin subfragment-1.

TABLE I

Antibody Staining of NH_2OH Peptide Maps

a. Antibody staining of NH_2OH peptides

Antibody	Antibodies with identical staining	Antibodies with related staining (differences)
M2.-		
1	Unique	
2	4, 6, 7, 10, 21, 26, 27	22 (<32,000 mol wt) 27 (<29,000 mol wt)
3		9 (<32,000 mol wt)
4	4, 6, 7, 10, 21, 26	2, 27 (<29,000 mol wt) 22 (<32,000 mol wt)
5	Unique	
6	4, 7, 10, 21, 26	2, 27 (<29,000 mol wt) 22 (<32,000 mol wt)
7	4, 6, 10, 21, 26	2, 27 (<29,000 mol wt) 22 (<32,000 mol wt)
8	Unique	
9		3 (<32,000 ml wt)
10	4, 6, 7, 21, 26	2, 27 (<29,000 mol wt) 22 (<32,000 mol wt)
11	*	
12	*	
13	Unique	
15	Unique	
16	Unique	
17		18 (< 32,000 mol wt)
18		17 (<32,000 mol wt)
19	Unique	
20	Unique	
21	4, 6, 7, 10, 26	2, 27 (<29,000 mol wt) 22 (<32,000 mol wt)
22		2, 4, 6, 7, 10, 21, 26, 27 (<32,000 mol wt)
26	4, 6, 7, 10, 21, 26	2, 27 (<29,000 mol wt) 22 (< 32,000 mol wt)
27		2, 4, 6, 7, 10, 21, 26 (<29,000 mol wt) 22 (<32,000 mol wt)

b. NH_2OH families and their members

1	
4, 6, 7, 1, 21, 26	2, 22, and 27 differ slightly.
3, 9	
5	
8	
16	
17, 18	
19	
13, 15, 20	Could be seen, but little staining <60,000.
*11, 12	

* Staining pattern was nonexistent or too restricted to be properly interpreted.

members include antibodies M2.2, -10, -21, -22, and -27 that bind to a constellation of peptides that is similar, but not identical to the constellation stained by the "core" family. For example, antibody M2.27 stains the same alpha-chymotryptic and V-8 protease peptides as the core family (data not shown), but on NH_2OH (Fig. 3, Table I) and CNBr maps (data not shown) M2.27 stains an additional 26,000-mol-wt peptide and an 11,000-mol-wt peptide, respectively, that are not recognized by the core family. Antibody M2.27 stained peptides >29,000 mol wt on the NH_2OH map and >18,000 mol wt on the CNBr map in a manner identical to staining by core family members, so one interpretation is that M2.27 recognizes an antigenic site on a 26,000-mol-wt NH_2OH peptide that is generated from a 29,000-mol-wt peptide by NH_2OH hydrolysis ~3,000 mol wt from one of its ends. Similarly, M2.27 recognizes an 11,000-mol-wt CNBr peptide that could result from cleavage at a methionine ~7,000 mol wt from the end of an 18,000-mol-wt fragment. Antibodies of the core family recognize no fragments smaller than the 29,000- and 18,000-mol-wt fragments, respectively.

Competitive Binding of Antibodies to Myosin-II

Competitive binding experiments between each ^{125}I-labeled monoclonal antibody and a large excess of each unlabeled monoclonal antibody provide independent evidence at higher resolution than peptide mapping for the relative positions of the monoclonal antibody binding sites on myosin-II summarized in (Fig. 4).

Of the 21 antibodies tested, 12 bind to unique sites on myosin-II; only an excess of homologous unlabeled antibody blocks the binding of labeled antibodies M2.1, -2, -5, -9, -12, -15, -16, -17, -18, -19, -22, and -27. The binding of the remaining nine antibodies is blocked by one or more unlabeled heterologous antibodies. In all five cases tested, Scatchard plots show that the unlabeled heterologous antibodies reduce the apparent affinity of the labeled antibody without influencing the stoichiometry of binding (see Fig. 7). Thus they are competitive inhibitors of antibody binding.

There are three families of antibodies by competitive binding criteria: M2.3 and -8; M2.4, -6, -7, and -26; and M2.13 and -20. The binding site of M2.10, while close to the binding sites of M2.4, -6, -7, and -26 by antibody binding of peptide maps, must be slightly different, because it does not inhibit binding of the other members of its family as well as the members of the core family. This is confirmed by the reactions of the antibodies with alpha-chymotryptic peptides: M2.10 binds to a slightly different constellation of peptides than the antibodies in the core family (data not shown).

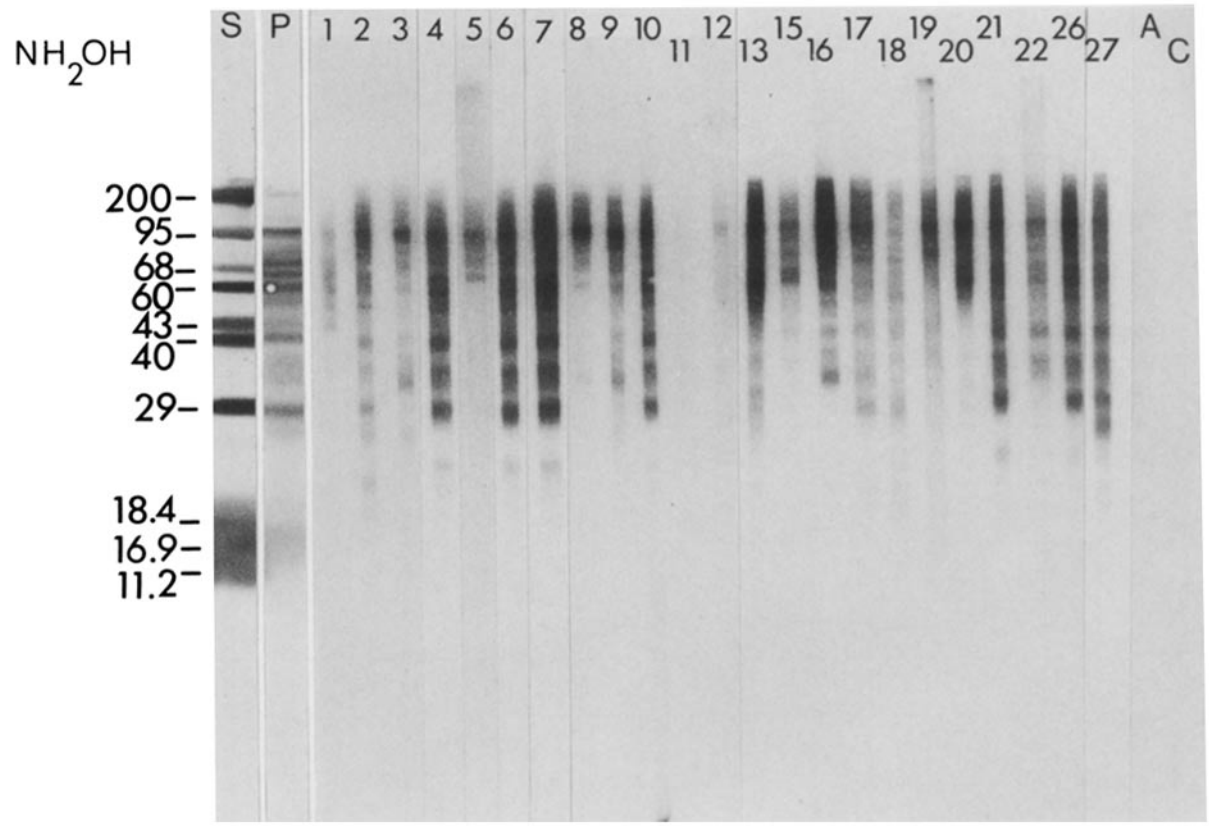

FIGURE 3 Antibody staining of myosin-II peptides produced by cleavage with hydroxylamine, separated by PAGE in SDS and transferred to nitrocellulose paper. (Strip *S*) Molecular weight standards (X 10^{-3}, left). (Strip *P*) Peptides stained with amido black. (Strips *1–27*) Incubated with specific monoclonal antibodies M2.1-M2.27 and bound antibodies localized by reaction with ^{125}I-labeled goat antimouse F(ab')$_2$ and autoradiography.

TABLE II

Summary of Families of Antigenic Sites by Competitive Binding and Peptide Staining

Family	Adjacent sites by competitive binding	Additional adjacent sites by peptide staining
A		1, 11
B	4, 6, 7, 10, 26	21, 27 > 22 > 2
C	3, 8	9, 12
D		5, 16, 19
E	13, 20	15
F		17, 18

Two of the antibody families delineated by these competitive binding studies are subsets of those revealed by the peptide map staining studies. The competitive binding studies demonstrate a close relationship between antibody M2.3 and -8 that was not observed by peptide mapping. This may be because M2.8 stained the NH_2OH generated peptide maps only poorly and failed to stain the CNBr, alpha-chymotryptic and V-8 protease maps in an interpretable fashion (see Table IV). In general, the competitive binding approach distinguishes differences in antibody binding sites with greater resolution than the one-dimensional peptide maps.

A number of unlabeled antibodies appear to enhance the binding of the ^{125}I-labeled antibodies. For example, M2.3 more than doubles the amount of ^{125}I-labeled M2.12 bound to the solid-phase assay. These two antibodies bind close to one another on the tail of myosin-II (23). In addition, most of the members of the family that includes M2.2, -4, -6, -7, etc., enhance the binding of M2.5. These observations suggest that the mechanism of enhanced binding of labeled antibody may be caused by specific changes in the structure of the myosin molecule induced by the binding of unlabeled antibody. A less likely explanation is that increased binding may result from potentiated binding of myosin antigen to the solid-phase substrate throughout the incubations and washes of the binding assay.

Myosin-I and -II Share Antigenic Determinants

Certain myosin-I antibodies bind both to myosin-I and myosin-II heavy chain (Fig. 1). To examine this cross reactivity more closely, we stained myosin-II peptide maps with myosin-I antibodies and evaluated the ability of high concentrations of unlabeled myosin-I antibodies to block the binding of ^{125}I-labeled M2 antibodies to intact myosin-II. Antibodies M1.3 and M1.4 stain myosin-II peptides generated by four of the five hydrolytic methods described above (Fig. 5 shows the data for the V-8 protease peptide maps). On each map these M1 antibodies stained a constellation of peptides identical to those that the major family of M2 antibodies recognized. In addition M1.4 inhibited the binding of ^{125}I-labeled M2 antibodies from core family that includes M2.4, -6, -7, and -26 (Fig. 4). Therefore M1.3 and M1.4 bind to sites on myosin-II that are close to one another and that must be structurally related to antigenic determinants found on myosin-I. Subsequent electron microscope localization studies show that this major family of M2 antibodies binds to a domain on the

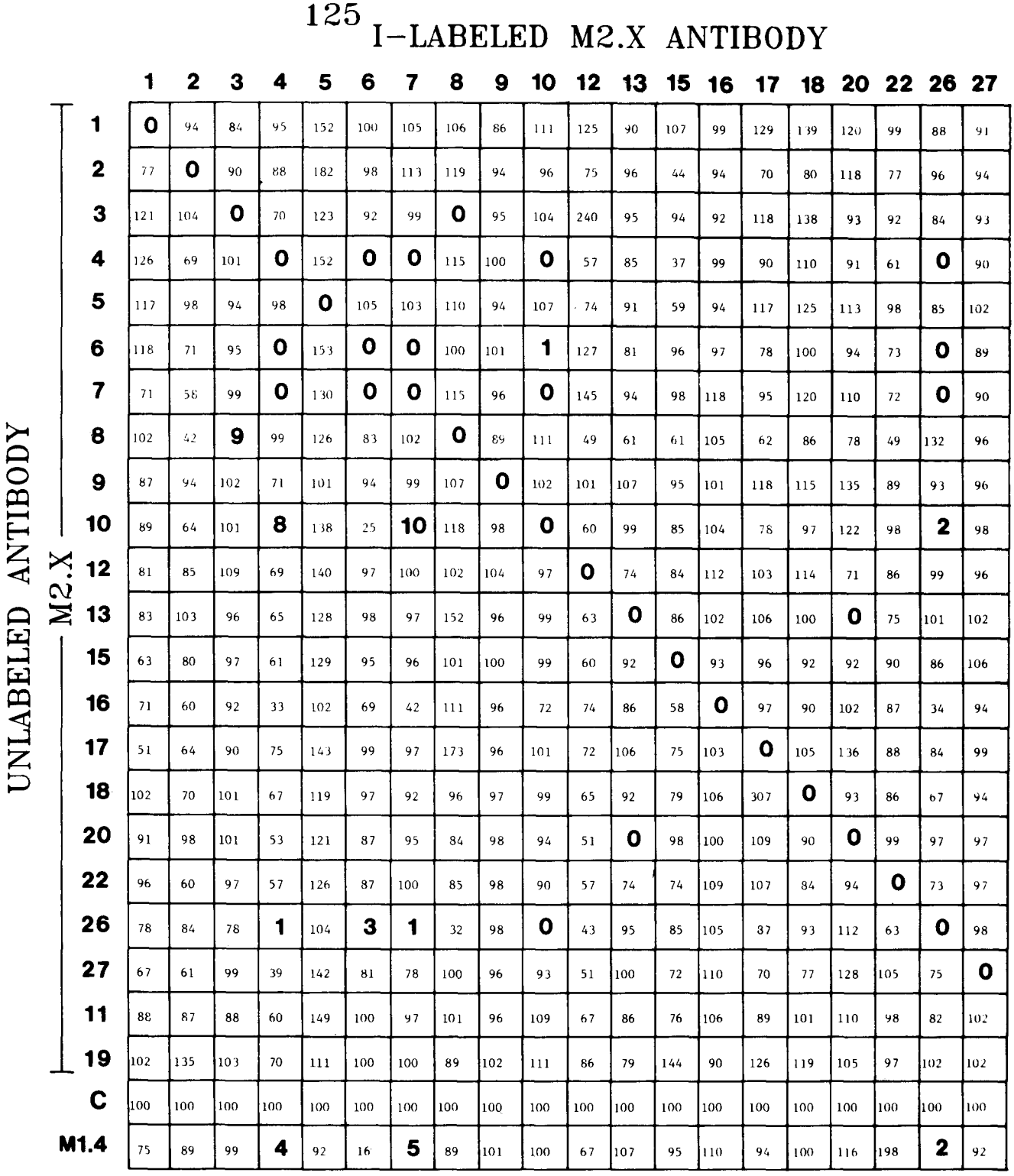

FIGURE 4 Summary of competitive binding experiments. ^{125}I-labeled antibodies are numbered across the top. Unlabeled, competing antibodies are numbered on the left side. The values in the matrix are the percent of control binding by ^{125}I-labeled antibody in the presence of 10^3–10^4 excess of unlabeled homologous (on the diagonal) or heterologous antibodies. Control binding was established by incubating labeled antibody in the absence of unlabeled antibody. Bold face numbers highlight the cases where binding of labeled antibodies was inhibited by 90% or more.

myosin-II tail near its junction with the heads. These experiments and functional studies show that these antibodies are potent inhibitors of myosin ATPase activity and can block contraction of gelled extracts of *Acanthamoeba* cytoplasm. Together these studies demonstrate that myosin-I shares some limited structural homology with a domain in the myosin-II tail that is essential for actin-activated ATPase activity and gel contraction.

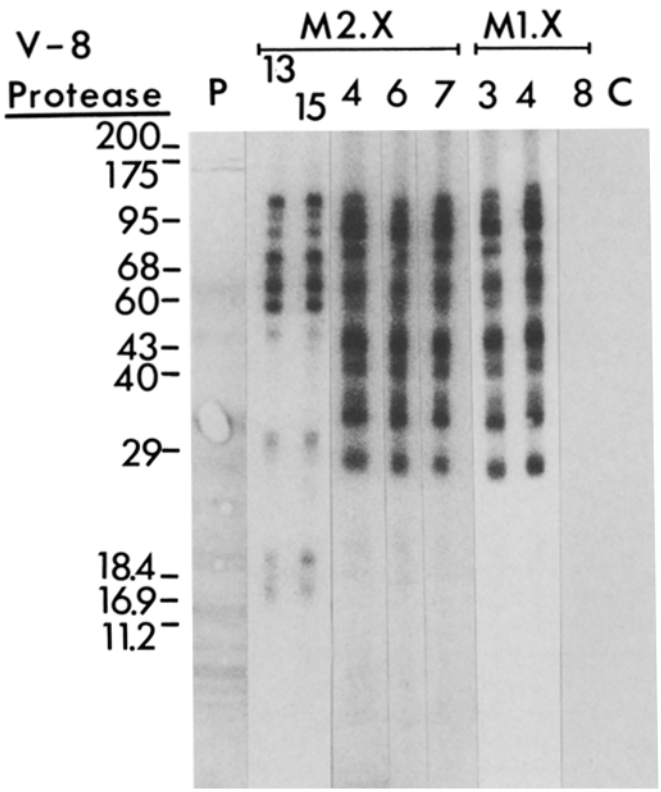

FIGURE 5 Monoclonal antibody staining of myosin-II peptides generated by digestion with V-8 protease (*S. aureus*) and separated by gel electrophoresis demonstrates immunological cross reactivity between myosin-I and -II. Molecular weight standards (X 10^{-3}) are on the left. (Lane *P*) Amido black stained peptides. (Numbered lanes) Autoradiograms of one dimensional peptide maps stained with antibodies to myosin-II (M2.x) and myosin-I (M1.x) (Lane *C*) Control monoclonal antibody to chicken pectoralis myosin subfragment-1. Two myosin-I antibodies (M1.3 and M1.4) react with the same myosin-II peptides as the major core family of myosin-II antibody that includes M2.4, -6, -7, and -26.

It is not clear why several M1 antibodies that bound intact myosin-II heavy chain in whole cell extracts (Fig. 1) failed to recognize myosin-II peptides generated by various hydrolytic agents. We speculate that these antibodies may have had lower apparent affinities to binding sites on myosin-II or that such sites were destroyed during proteolysis.

Comparative Studies with Rabbit Antibodies to Acanthamoeba Myosins

Antibodies produced by rabbits immunized with electrophoretically purified myosin-I heavy chains, myosin-II light chains or myosin-II heavy chains confirm and extend the conclusions drawn from the experiments with monoclonal antibodies. Solid-phase antibody binding assays with purified myosins (see Table V) and antibody staining of myosin polypeptides separated by gel electrophoresis (Fig. 6) establish the specificity of the polyclonal antibodies. Antibodies to the myosin-II heavy chain or light chains react exclusively with myosin-II heavy or light chain, respectively, whether purified myosin-II or whole cell extract is used as test antigen in gel overlay experiments. Both rabbits immunized with myosin-I heavy chains produced antibodies that react with the heavy chains of myosin-I and one of the antimyosin-I antibodies also cross-reacts with myosin-II heavy chain. Rabbit myosin-II light chain antibodies react exclusively with myosin-II light chains: no cross-reactivity with either myosin heavy chain or myosin-I light chains was observed. The rabbit antibodies to the myosin-II heavy chains compete poorly with most of the ^{125}I-labeled monoclonal antibodies to myosin-II for binding to myosin-II. The exception is that sera from one of the rabbits immunized with myosin-II heavy chain (AM-2) strongly inhibits the binding of M2.8 to myosin-II (data not shown).

DISCUSSION

Myosin-I and -II Are Different Gene Products

Myosin-I and -II are likely the products of distinct genes. All of the antibodies against myosin-II and some of the antibodies against myosin-I recognize antigenic determinants found specifically on the myosin isozyme against which they were raised. Therefore, each myosin has major domains that are antigenically and, therefore, structurally unique. None of the antibodies recognize polypeptides with higher molecular weight than the myosin-II heavy chain. Thus, putative higher

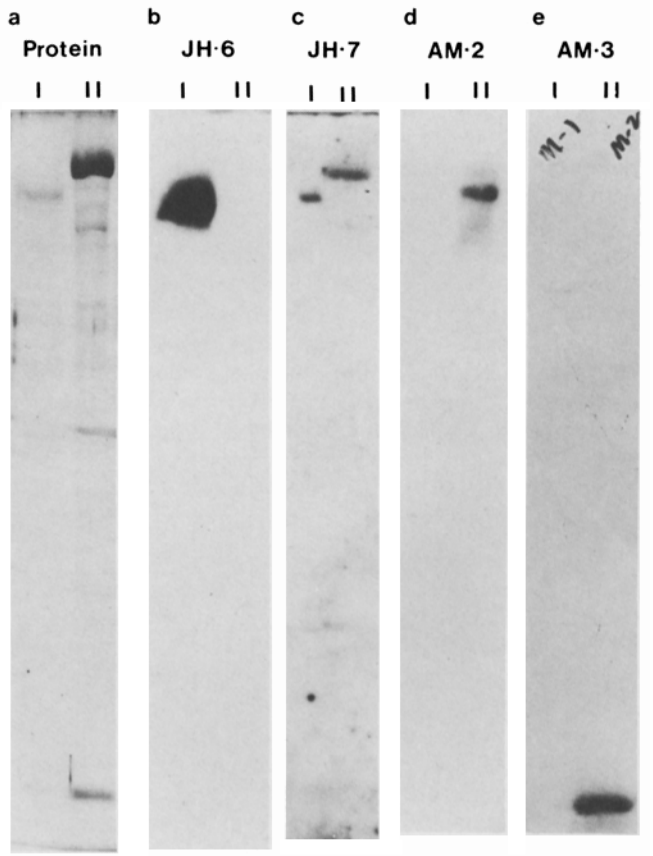

FIGURE 6 Polyclonal antibody overlay of myosin-I and -II peptides separated by gel electrophoresis. (*a*) Coomassie Blue-stained gels indicated polypeptide composition of purified myosin-I (I) and -II (II) (*b-e*) Autoradiograms of gels incubated with different antibodies and bound antibodies localized with ^{125}I protein A. (*b*) JH-6, an immune serum directed against myosin-I, stains myosin-I heavy chain and not myosin-II heavy chain. (*c*) JH-7, a sera from a rabbit also immunized with myosin-I heavy chain, stains both myosin-I and -II heavy chains. (*d* and *e*) AM-2 and AM-3, immune sera from rabbits immunized with electrophoretically purified myosin-II heavy chains and light chains respectively. They stain only their respective antigens.

molecular weight precursors of either myosin isozyme are present only briefly or in very low concentration. Taken together, these observations provide strong evidence that the two classes of myosin isozymes from *Acanthamoeba* are the products of distinct genes. Our observations with monoclonal antibodies therefore confirm the polyclonal immunochemical studies (12, 33) and peptide mapping experiments (13) on the two classes of myosin (12, 33). Further, they concur with the observations that myosin-I is not generated from myosin-II during purification.

Myosin-I Shares an Antibody Binding Site Located on the Myosin-II Tail

Although myosin-I and -II are clearly distinct they have some structural homology. Two monoclonal antibodies (M1.3 and M1.4) and one polyclonal (JH-7) serum bind tightly to antigenic determinants on the heavy chains of both myosin-I and -II. By both competitive binding and peptide mapping the two cross-reactive M1 antibodies bind to the same region of the myosin-II heavy chain as M2.4, -6, -7, and -26. This family of M2 antibodies binds to sites on the myosin-II tail near its junction with the heads (23). Thus, these common antigenic sites are unlikely to be either the actin-binding sites or the catalytic sites of the two myosins. This is the first evidence that myosin-I may have a short tail. This is an unexpected finding, because the tail of myosin-II is presumably a helical coil composed of two parallel polypeptide chains, making it unclear how myosin-I with a single heavy chain could form such a structure.

This structurally homologous region of the myosin isozymes appears to be important for myosin function in vitro, and probably in vivo, because the M2 antibodies that bind to this region inhibit both the actomyosin-II ATPase activity and the contraction of gelled extracts of amoeba cytoplasm (25). The fact that myosin-I shares a homologous region suggests that this region may be fundamentally important for the function of both myosin isozymes.

Characteristics of the Antigenic Sites

All of the antimyosin monoclonal antibodies and at least some of the antibodies in the polyclonal sera bind to myosin in solid-phase binding assays and to myosin heavy chains that were SDS denatured and transferred to nitrocellulose paper. In the solid-phase binding assay, the myosin probably retains at least part of its native structure, but it is unlikely that significant renaturation of the SDS denatured myosin takes place on the nitrocellulose. It is therefore possible that the antibodies recognize the primary sequence at the antigenic site in both its native and denatured form. We conclude that the three-dimensional structure of the antigenic determinant is probably not important for antibody binding, but we have not critically compared the relative apparent affinities of the antibodies for native versus denatured myosin.

The 23 monoclonal antibodies directed against myosin-II recognize at least 15 unique antigenic determinants on the myosin-II heavy chain. They can be grouped into core families of antibodies whose members competitively inhibit each other's binding to myosin-II. Associated with these core families are other family members that stain one-dimensional maps of the myosin-II heavy chain in a pattern similar or identical to that of the core family (Table II). In a following paper (23), we use electron microscopy in conjunction with the relationships established here to localize directly all but six of the antibody binding sites on the myosin-II molecule.

Origin of Cells That Secrete Antibodies to Common Antigenic Sites

The relationship between antibodies within core families is fascinating. It is likely that the antibodies we have characterized are the result of unique fusion events because each secreting cell line comes from a different culture well plated with hybridoma fusion products and, in addition, the hybridoma colonies are physically separated into individual culture plates during cloning. However, it is possible that more than one fusion might have occurred between myeloma cells and B-lymphocytes that came from the same clone in the immunized mouse. Consider the core family that includes M2.4, -6, -7, and -26. Three of the antibodies are of the IgG_{2B} isotype. M2.26 is an IgG_1. Antibodies M2.4, -6, and -7 may be secreted by monoclonal hybridomas that were the result of fusions between parent myeloma cells and identical but distinct B-lymphocytes that were clonally related to one another. Such B-lymphocytes could have been sister progeny derived during maturation of the immune response to a single antigen recognition event in the mouse. Because immunoglobulin class switching during maturation of the immune response can give rise to closely related B-lymphocytes secreting antibodies of different immunoglobulin isotypes directed against identical antigenic sites (42), it is even possible that M2.26 share some common lineage with other members of the core family.

Alternatively, the members of the core family may recognize unique antigenic determinants that are so close to one another that the differences between them remain unresolved by our techniques. Under such circumstances, each antibody would be the result of a unique antigen recognition event in the mouse. Subtle differences in the functional effects of these antibodies on actomyosin-II ATPase activity (25) suggest that this interpretation may be correct. The extent of inhibition by antibodies M2.4, -6, -7, and -26 are all subtly different.

APPENDIX

This appendix is divided into two parts. First, we detail antibody production and characterization. Second, we supply supporting data that provides guidelines for reproducing these experiments and supplies important but not essential data for interpreting the experiments discussed in the text.

Antibody Production and Characterization

IMMUNIZATION: 5–10-wk-old female C57B6 mice, obtained from Jackson Labs (Bar Harbor, ME), were immunized and boosted as described by Strand (41) with purified native proteins (~20 μg of myosin-I and ~200 μg of myosin-II) in 0.15 M NaCl buffered with 10 mM sodium phosphate (pH 7.5) or 10 mM imidazole-Cl (pH 7.0). Protein solutions were mixed with an equal volume of Freund's complete adjuvant for the primary immunization and with Freund's incomplete adjuvant for all but the last boost. Between days 24 and 40, blood was obtained from the tail vein and tested for antibodies against myosin by the solid-phase binding assay described below. The assay was considered positive when dilutions of one part in 10^4 to 10^5 were higher than background binding. Positive mice were given a final, intraperitoneal boost with antigen in buffered saline (no adjuvant).

FUSION AND HYBRIDOMA CULTURE: Mice were cervically dislocated 3–4 d following the final boost, their spleens were removed surgically, rinsed three times in Dulbecco's modified Eagle's medium (DME), then dissociated mechanically into DME by maceration through a stainless steel screen with a rubber policeman. Subsequent steps were a modification of a fusion protocol described previously (19, 27). Erythrocytes were removed by lysis in ice cold

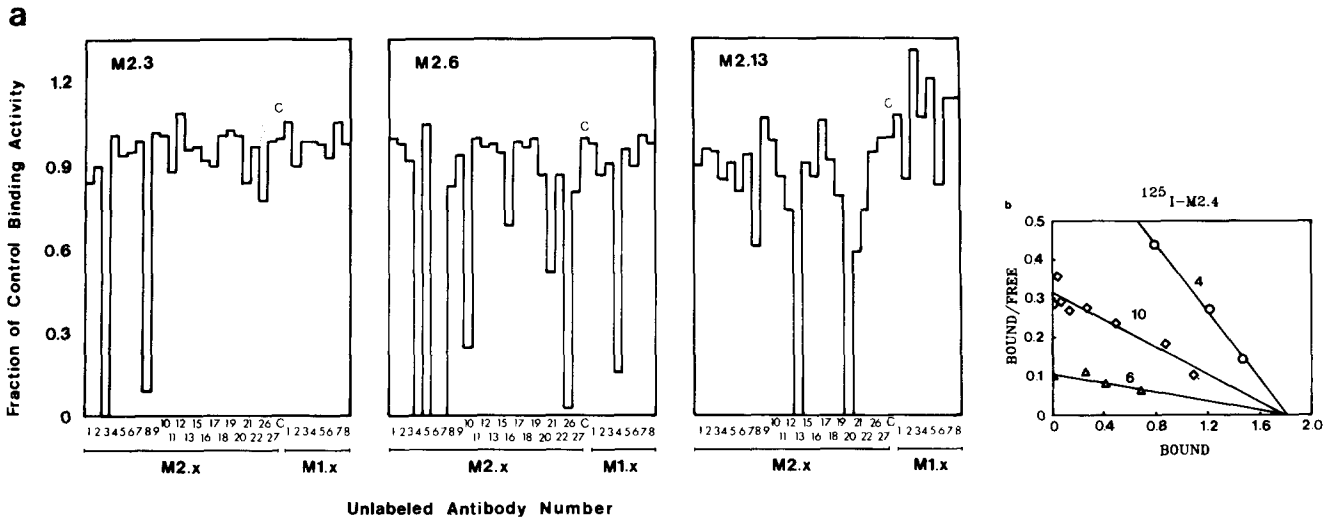

FIGURE 7 Competition between ^{125}I-labeled monoclonal antibodies and unlabeled antibodies for binding to myosin-II. (*a*) Competition between M2.3, M2.6, and M2.13 and a panel of monoclonal antibodies to myosin-II (M2.x) and myosin-I (M1.x). Binding is expressed as a fraction of ^{125}I-labeled antibody binding in the absence of unlabeled antibody. Homologous antibody plus one or more heterologous antibodies compete with each antibody. (*b*) Scatchard plots of ^{125}I-labeled antibody binding in the presence of approximately equivalent amounts of unlabeled antibody demonstrate competitive inhibition of binding. ^{125}I-labeled antibody M2.4 alone (○); binding of ^{125}I-labeled M2.4 in the presence of approximately equimolar concentrations of unlabeled M2.10 (◇) or M2.6 (△). The differences in the slopes show that M2.10 does not inhibit binding of M2.4 as well as M2.6.

TABLE III

Physical Properties of Monoclonal Antibodies to Acanthamoeba Myosins

Antibody	Isotype	KCl	pI*	Apparent K_D
		mM		*nM*
M2.x				
1	IgG_{2A}	52	6.0	10–15
2	IgG_1	86	6.0	30–50
3	IgG_{2B}	63	6.0	2–3
4	IgG_{2B}	42	7.2	10
5	IgG_{2A}	62	5.9	0.1
6	IgG_{2B}	36	7.2	3
7	IgG_{2B}	—	—	—
8	IgM	—	—	4
9	IgG_1	33	7.2	16
10	IgG_1	28	7.0	4
11	IgG_1	52	6.4	5
12	$IgGD_{2B}$	66	6.2	38
13	IgG_1	38	7.1	4
15	IgG_1	49	6.5	3
16	IgM	—	—	6
17	IgG_1	73	6.6	7
18	IgG_{2B}	38	7.0	26
19	IgG_1	32	7.3	12
20	IgG_1	36	7.4	10
21	IgG_{2A}	—	—	—
22	IgG_1	42	6.6	13
26	IgG_1	33	7.4	1
27	IgG_{2B}	48	7.2	5
M1.x				
1	IgG_{2B}	56	6.4	—
2	IgM	—	—	—
3	IgM	—	—	—
4	IgM	—	—	—
5	IgM	—	—	—
6	IgM	—	—	—
7	IgG_{2B}	52	6.7	—
8	IgG_1	48	6.8	—

* pI: Isoelectric focusing of these proteins resulted in four to five closely spaced Coomassie Blue-stained bands, a pattern typical for purified monoclonal antibodies. The pI tabulated was the average pI for the bands.

TABLE IV

Families Defined by Antibody Staining of Peptide Maps

Alpha-chymotryptic families	V-8 Protease "families"	CNBr Families
1, 11	1	3, 9
2	2, 4, 6, 7, 10, 21, 26, 27*	4, 6, 7, 10, 21, 22, 26, 27
3	9	5, 19
4, 6, 7, 21, 22, 26, 27‡	13, 15, 20	17
9, 12	17	18
13, 15§	18	
16, 19		
17		
18		
5, 8‖	3, 5, 8, 10, 12, 16, 19‖	1, 2, 8, 11, 12, 13, 15, 16, 20‖

* M2.22 stains myosin-II peptides >40,000 mol wt like the members of this family.
‡ M2.10 stains myosin-II peptides >24,000 mol wt like the members of this family.
§ M2.20 stains myosin-II peptides >29,000 mol wt like the members of this family.
‖ Staining pattern was too restricted to be interpreted.

0.83% NH_4Cl. The remaining cells were pelleted through ice cold 100% fetal bovine serum (FBS), and washed once in DME. Cells of the parent myeloma line (P3-X63-Ag8.653 [see reference 17]) were grown at low density (5–50 × 10^4 cells/ml) for 4 d before fusion in 20% FBS, 80% DME containing 130 μM 8-azaguanine and then washed three times in DME to completely remove serum, which inhibits cell fusion. Cells were mixed in a ratio of 2:1 to 10:1 spleen to myeloma cells and co-pelleted in a 50 ml conical centrifuge tube. Supernatant was removed and the cells were gently dispersed by tapping the tube. Fusagen (1 ml of 25% polyethylene glycol [PEG 1000], 15% dimethyl sulfoxide in DME adjusted to pH 7.4 with NaOH) was added dropwise over the course of 1 min. After 30–60 s, 6 ml of DME were added dropwise over the course of 6 min. Slow addition of fusagen and diluents is required, probably to minimize osmotic shock. The cell suspension was mixed by intermittent, gentle swirling to maintain homogeneity throughout the procedure. After a 3-min incubation, the fusion reaction was inhibited by slow addition (10 ml in 2 min) of 20% FBS, 80% DME. Cells were pelleted, and the supernatant was carefully removed. Cells were resuspended in HAT-hybridoma growth medium consisting of 70% DME, 20% FBS, 10% Medium NCTC 135 (a growth

TABLE V

Solid Phase Binding Assays Reveal the Cross-reactivity of Polyclonal Rabbit Antibodies

	Antigen applied to well			
	0.2 μg Myosin-I		0.2 μg Myosin-II	
Antibody	^{125}I-Protein A specific cpm bound*	(Serum dilution)	^{125}I-Protein A specific cpm bound*	(Serum dilution)
Antimyosin-I heavy chain				
Rabbit JH-6	11,600	(10^{-2})	20	(10^{-2})
Rabbit JH-7	5,100	(10^{-2})	4,100	(10^{-2})
Antimyosin-II heavy chain				
Rabbit AM-1	460	(10^{-2})	299,000	(10^{-3})
Rabbit AM-2	230	(10^{-2})	352,000	(10^{-3})
Antimyosin-II light chains				
Rabbit AM-3	580	(10^{-2})	158,000	(10^{-3})
Rabbit AM-4	730	(10^{-2})	103,000	(10^{-3})

* Specific cpm, cpm with immune serum minus cpm with preimmune serum at the same dilution from the same rabbit. The preimmune cpm were in the range 500 to 2,000 cpm.

supplement), to which glutamine (2 mM), insulin (0.25 U/ml), pyruvate (450 μm), oxalacetic acid (1.1 mM), and HAT (hypoxanthine, 100 μM, aminopterin, 0.4 μM, and thymidine, 20 μM) were added. Sometimes penicillin (100 U/ml) and streptomycin (100 μg/ml) were included, but fungicides were avoided. The postfusion cells were immediately plated into 24 well plates (3.5-ml wells) at a density of 1-10 × 10^6 spleen cells/well in 1 ml of growth medium. The wells were preplated with 0.5 ml of hybridoma growth medium containing ~10–100 × 10^3 mouse peritoneal macrophages per well (9). Plates were incubated at 37°C in humidified air containing 5–8% CO_2. Cells were fed every 3–5 d by replacing 0.5 ml of culture supernatant in each well with fresh HAT hybridoma growth medium. After 2–3 wk cells were weaned from HAT in an identical growth medium from which aminopterin was omitted (HT-hybridoma growth medium). In 2–3 wk, or as soon as growth was sufficient to turn one or more of the culture wells orange-yellow, cultures were assayed for antibody production with the solid-phase binding assay described below. Assays were repeated every 3–4 d for ~10 d after new wells failed to appear with growth. Wells were considered positive if counts were five times background. Cells from positive wells were grown in HT-hybridoma growth medium in flasks for freezing and cloning as soon as possible.

CLONING AND FREEZING: Cells from positive cultures were cloned in 0.33% agar (Bacto Agar, Difco Laboratories, Detroit, MI) in HT supplemented hybridoma growth medium over a basal layer of 0.5% agar (29). After growth in flasks, all of the lines producing antibodies to myosin-II and some producing antibodies to myosin-I were recloned, then frozen, and stored in liquid nitrogen.

Hybridomas (1–4 × 10^6/ml) were frozen in 40% FBS, 50% DME, and 10% DMSO, or 90% FBS and 10% DMSO using 1 ml storage vials (Nunc, InterMed, Denmark). An immersion cooler (Cryocool; Neslab, Portsmouth, NH) controlled the rate of the temperature change: −3°C/min from 37°C to −10°C, then −1°C/min to −70°C. Frozen cells were stored in liquid nitrogen.

ANTIBODY PRODUCTION BY ASCITES TUMORS IN MICE: For large scale production of antibodies, cloned, antibody producing cells were grown as ascites tumors in pristane primed C57B6-BALBC F1 mice (16). After 2–15 wk ascites fluid was collected by peritoneal tap. After removal of cells by centrifugation, antibodies were precipitated with an equal volume of saturated ammonium sulfate (pH 7.0) containing 10 mM K^+-EDTA and stored as a pellet at 4°C.

EFFICACY OF MONOCLONAL ANTIBODY TECHNIQUES: Using these methods for producing monoclonal antibodies, we found that myosin-II proved an excellent antigen: two fusions yielded cells secreting antibody to myosin-II in 200 out of 200 wells each seeded with 10^6 spleen cells. Three myosin-I fusions yielded 29 positive wells out of 300 wells seeded. This lower success rate may be related to the smaller amounts of myosin-I used for immunization. Cells from 23 of the wells producing antimyosin-II and from eight wells producing antimyosin-I were successfully cloned in soft agar. All antimyosin-II producing clones were cloned a second time and subclones produced antimyosin-II that was of the same, single Ig isotype as the parent clone, so we conclude that the second cloning step was probably unnecessary. We verified that our antibodies were monoclonal by analyzing antibody labeled biosynthetically in culture and antibody purified from ascites fluid by SDS gel electrophoresis and by isoelectric focusing.

Supplemental Details for Experimental Protocols

DEAE PURIFICATION OF MONOCLONAL ANTIBODIES: Antibodies were purified from the 50% ammonium sulfate pellets by DEAE chromatography by standard techniques except the ion exchange column was equilibrated with 20% sucrose. The sucrose kept IgGs in solution at concentrations in excess of 20 mg/ml even under low salt (5 mM Tris-Cl, pH 8.0) conditions. Partial purification of specific monoclonal Igs from other serum proteins and from contaminating endogenous mouse Igs was greatly facilitated because the proteins were actually chromatographed, rather than pooled in the flow through from the ion exchange column. Ammonium sulfate precipitates of ascites fluid were washed one to three times by pelleting and resuspension in 50% ammonium sulfate. Washed pellets were resuspended in a minimal volume of column buffer, then dialyzed for at least 18 h against three changes of 500 vol of column buffer. Samples of 1–10 ml contained 5–50 mg/ml protein and were clarified by centrifugation for 15 min at 18,000 *g* then chromatographed on the DEAE. After a two column volume buffer wash, a 200-ml linear gradient of 0–300 mM KCl in column buffer was applied to elute the antibodies. The antimyosin activity eluted with the major peak of protein at a salt concentration characteristic of the particular antibody. IgMs were purified by two to three cycles of precipitation in 5 mM Tris-Cl, pH 8.0, and then dissolved in 150 mM NaCl, 5 mM Tris-Cl, pH 8.0. SDS gel profiles of the chromatographed antibodies establish their purity and are shown in Fig. 8. The molecular weights of the heavy and light chains of the IgGs vary somewhat but average ~50,000 and 25,000 respectively. The two IgMs (M2.8 and 16) have 70,000-mol-wt heavy chains and 25,000-mol-wt light chains. Interestingly, antibody M2.5 is an IgG_{2A} by double diffusion analysis, and is soluble in low salt, but the heavy chains are about the same size as IgM on SDS gels.

ISOELECTRIC FOCUSING: Small samples of DEAE-purified antibody, ^{125}I-antibody, or ^{35}S-antibody in culture supernatant were isoelectric focused in 12 × 22 × 0.1 cm 1% agarose (Isogel, FMC Marine Colloids, Rockland, ME) gels cast on Gel Bond (FMC Marine Colloids) using a flat bed apparatus (LKB, Multiphor) by the protocol described by the manufacturer. Ampholines (Pharmacia Fine Chemicals, LKB Stockholm, Sweden; or Separation Sciences, Attleboro, MA) ranged from pH 6.5 to pH 9.0 or from pH 3 to 10.

IODINATION OF SPECIFIC MONOCLONAL ANTIBODIES, GOAT ANTIMOUSE ANTIBODIES, AND PROTEIN A: DEAE-purified monoclonal antibodies, affinity-purified goat anti-mouse $F('ab)_2$ (Cappel Laboratories, Cochranville, PA), and Protein A (Pharmacia Fine Chemicals) were iodinated with $Na^{125}I$ (~50 μCi/μl diluted from ~500 μCi/μl stock with deionized water just before use) using chloramine T as an oxidant (15). When iodinated proteins that had been chromatographed on G-25 were precipitated with 10% ice cold trichloroacetic acid, all of the iodine precipitated with the protein. Specific activity varied from 5–50 × 10^{17} cpm/mol and the molar ratio of iodine to protein varied from 0.5–2 mol/mol. Antibodies labeled in this fashion displayed little nonspecific binding in the solid-phase binding assay described below: if wells containing myosin-II were incubated with ^{125}I-control antibody, ~0.5–1% of the total counts were bound. Similarly, if wells plated with skeletal muscle myosin were incubated with ^{125}I-myosin-II antibodies, only 0.5–1% of the counts bound. In contrast, 15–50% of all the counts bound to myosin-II coated wells provided that antibody was not saturating the antigen present. Two of 22 specific antibodies labeled had insufficient specific activity for the competitive binding assays described in the methods (M2.11 and M2.19).

SOLID-PHASE BINDING ASSAY: Antibody concentrations in serum, culture medium, ascites fluid, and purified antibody preparations were measured semi-quantitatively by a modified solid-phase binding assay (44). Native antigen (10–1,000 ng) in 50 μl of 10 mM imidazole-Cl, pH 7.0, was dried onto the bottom of polystyrene wells (~380 μl, ~0.32 cm^2 bottom surface area) using a hair dryer. Temperatures in the bottom of the well reached ~45–50°C. Alternately 50 μl of antigen was placed in the wells, incubated ~15 min, then removed without drying. Wells stored at 4°C for up to 30 wk displayed sufficient binding activity for these solid-phase binding assays. Wells coated with myosin were rinsed once with STTAB wash solution (see Materials and Methods) to minimize nonspecific binding of antibodies and protein A. Test samples of 50 to 250 μl containing antibodies (culture medium supernatant or dilutions of antibody in STTAB) were next plated onto each well and incubated at room temperature for 1–4 h. For each assay, controls included incubation with hybridoma growth medium, STTAB alone, or purified monoclonal antibody that was previously shown not to bind to myosin-I or -II. The control antibody used most frequently was a mouse monoclonal antibody to chicken skeletal muscle myosin subfragment-1 (24). Test solutions were removed, and the plates were rinsed rapidly with three changes of 350 μl STTAB. Next, the plates were incubated with 50 μl of STTAB containing ~50,000 cpm of ^{125}I-labeled goat antibody to mouse $F(ab')_2$ antibody fragments. Alternately, we incubated the

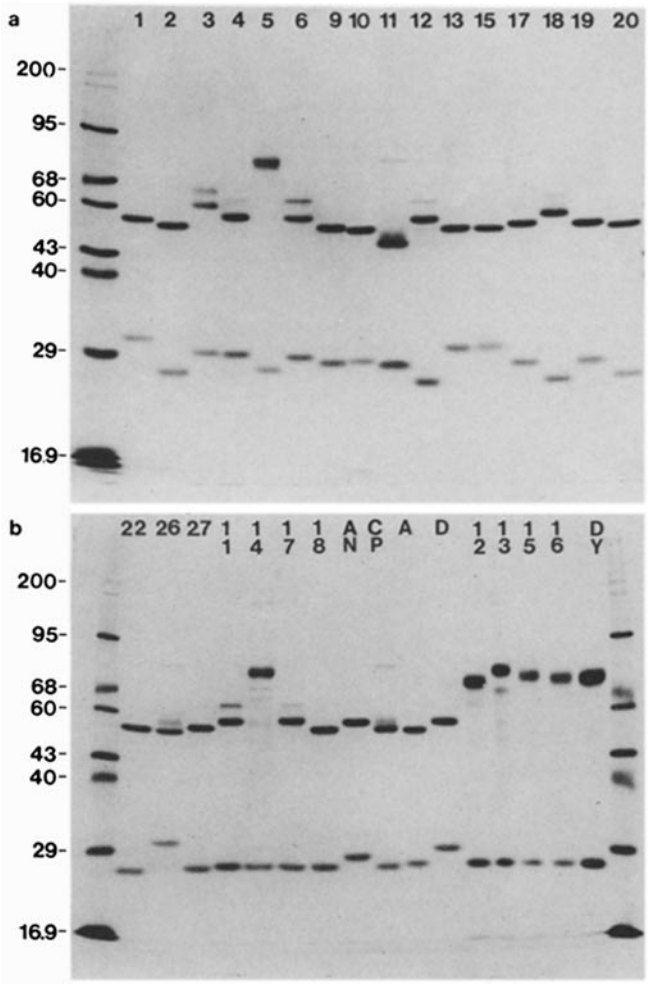

FIGURE 8 SDS gel electrophoresis of monoclonal antibodies purified from ascites fluid by DEAE chromatography. (*Upper panel* and lanes *22–27* in *lower panel*) Monoclonal antibodies against myosin-II (M2.x). (*Lower panel*) Lanes *1/1, 1/4, 1/7, 1/8, 1/2, 1/3, 1/5,* and *1/6* are monoclonal antibodies directed against myosin-I. Other monoclonals are directed against: *AN,* an unknown antigen; *CP, Acanthamoeba* capping protein produced by John Cooper, Don Kaiser, and Dan Kiehart; and *D,* chicken pectoralis myosin subfragment-1; *DY,* sea urchin egg cytoplasmic dynein. Molecular weight markers migrate as shown in lanes marked *S.* Molecular weight (X 10^{-3}) is shown to the left of each gel.

plates with unlabeled goat antimouse antibody (Cappel Laboratories), washed three times with STTAB, and incubated with ~50,000 cpm of ^{125}I-protein A in 50 μl of STTAB. After 1-h incubation, the plates were washed three times with STTAB to remove unbound, radiolabeled antibody or protein A. Wells were removed and counts bound were quantified in a gamma counter. The assay was reliable as a semi-quantitative measure of antibody concentration even if the time or temperature of incubation was varied, providing that all samples compared were treated in an identical fashion.

IG ISOTYPING: The isotype of each monoclonal antibody was determined by Ouchterlony double diffusion analysis (11) against rabbit Ig typing sera for mouse IgG_1, IgG_{2A}, IgG_{2B}, IgG_3, IgM, and whole mouse Ig (Miles Laboratories, Elkhart, IN). The test monoclonal antibodies were concentrated approximately 20-fold from culture medium by precipitation with 50% ammonium sulfate followed by resuspension in, and dialysis against, 0.1 M sodium phosphate buffer, pH 8.0.

BIOSYNTHETIC LABELING OF SPECIFIC MONOCLONAL ANTIBODIES WITH [^{35}S]METHIONINE: Antibodies were labeled biosynthetically by growing 10^7 cells overnight in 5–10 ml of culture media consisting of RPMI 1640 without methionine, 10% NCTC (which contributes 3 μM cold methionine, ~3% of its level in normal RPMI growth mediums), 50 μCi (3.5–7 μM) of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) and 10% FBS dialyzed against 150 mM NaCl, glutamine, HT, and IPO (see above, under fusions). Specific activities of labeled proteins ranged from 10^{15} to 10^{16} cpm/mol Ig. Three of the 23 monoclonals (M2.1, -21, and -27) did not label well enough for use in competitive binding assays.

DETERMINATION OF APPARENT BINDING AFFINITY: ^{125}I-labeled and ^{35}S-labeled antibodies were used in a solid-phase binding assay to evaluate the apparent K_D for each antibody binding to myosin-II. Wells containing ~200 ng (0.5 pmol) of bound myosin-II were incubated with various concentrations of labeled antibody and washed as described above. Bound antibody was measured by counting the ^{125}I or ^{35}S. The concentration of ^{35}S-antibodies in culture medium was estimated with a solid-phase binding assay using myosin-II–containing wells, DEAE-purified, homologous antibody as the standard, and ^{125}I-anti mouse F(ab')$_2$ antibody to detect both the standard and the unknown concentration of ^{35}S-labeled antibody. The ^{35}S was not detected by the gamma counter. Backgrounds were determined as described in the competitive binding assays section. The K_D of each antibody was determined from a Scatchard plot.

The K_D, as determined by a solid-phase binding assay, is an apparent value that is theoretically related to the amount of myosin-II that is bound to the solid-phase support (18). The apparent K_D that we measured experimentally varied by a factor of 2 as the amount of antigen plated in the polystyrene wells varied from 0.2 to 3.2 μg (data not shown). The apparent K_D's tabulated are probably a measure of the apparent affinity of the antibodies within an order of magnitude for three reasons. First, apparent dissociation constants determined with ^{125}I-labeled antibodies were all within an order of magnitude of those determined with ^{35}S-labeled antibodies and second, the dissociation constant for antibody M2.2, determined by two other methods (competitive binding and kinetic methods, data not shown, see reference 45 for methods) resulted in values within a factor of two of those tabulated. Finally, Kennel (18) characterized the apparent K_D of a monoclonal antibody against human fibrinogen fragment D under solution and solid-phase conditions. The values he obtained agreed within an order of magnitude.

The wells contained ~0.5 pmol of myosin-II (estimated with ^{125}I-labeled myosin-II, data not shown) and the abscissal intercept of all the Scatchard plots showed that 0.3 to 0.6 pmol of antibody bound at saturation. Thus the ratio of antibody to myosin was about 1:1 and the antigenic sites on myosin-II were equally available.

PREPARATION OF MYOSIN-II PEPTIDES BY PROTEOLYTIC AND CHEMICAL CLEAVAGE: Fragments of myosin-II were generated by three proteolytic and two chemical cleavage agents and then separated by gel electrophoresis before transfer to nitrocellulose paper and reaction with the monoclonal antibodies.

TRYPSIN: Native myosin-II (0.47 mg in 0.4 ml) in 30% sucrose, 10 mM imidazole-Cl, pH 7.0 was digested for 1 min at 0°C with 4 μl of 0.04 mg/ml of TPCK trypsin (Worthington Biochemical Corp., Freehold, NJ). The reaction was quenched by the addition of 7.5 μl of 0.1 mg/ml of soybean trypsin inhibitor (Worthington Biochemical Corp.). Gel samples were prepared immediately by adding an equal volume of boiling SDS gel two times sample buffer (0.3 M Tris-Cl, pH 6.8, 30% sucrose, 4 mM EDTA, 0.02% bromophenol blue, 4% SDS, 10% beta-mercaptoethanol), and boiling 3 min. Peptides similar to those generated by tryptic digestion were once formed during dephosphorylation of myosin-II (method described in reference 25). The results of antibody staining of the 105,000- and 70,000-mol-wt peptides are shown in Fig. 2.

HYDROXYLAMINE: Myosin-II was reduced and alkylated by the method described by Craven et al. (8), then cleaved with NH_2OH (2). The sample was chromatographed on Sephadex G-25-150 equilibrated with 70% formic acid. Fractions in the void volume were pooled and lyophilized. Gel samples were prepared by boiling in 1× sample buffer. Results are shown in text Fig. 3.

ALPHA-CHYMOTRYPSIN: Myosin-II (0.5 ml of a 0.5 mg/ml solution) was dialyzed into 25 mM Tris-Cl, pH 6.8, 2 mM K^+-EDTA, then denatured by adding SDS to 0.1% and boiling immediately for 3 min. The sample was brought to 25°C and hydrolyzed with 4 μl of 0.5 mg/ml alpha-chymotrypsin (Worthington Biochemical Corp.) at 25°C for 3 min. The reaction was terminated by boiling in an equal volume of two times sample buffer.

V-8 PROTEASE: SDS-denatured myosin-II was hydrolyzed with 21 μl of a 1 mg/ml solution of V-8 protease (Worthington Biochemical Corp.) by the protocol described for alpha-chymotrypsin.

CYANOGEN BROMIDE: Myosin-II was reduced and alkylated as described by Craven et al. (8), dialyzed into 0.1 M NH_4CO_3, and lyophilized. The myosin (1 mg) was resuspended in 1 ml of 70% formic acid, 25.2 μl of 10% CNBr (wt/vol) in 70% formic acid was added and the mixture was incubated for 24 h at 25°C. The solution was frozen and lyophilized. The peptides were dissolved in boiling gel sample buffer.

Materials

CHEMICALS: Reagent grade chemicals were obtained from the following sources. Aldrich Chemical Co., Milwaukee, WI: oxalacetic acid and Pristane (2,6,10,17-tetramethylpentadecane). J. T. Baker Chemical Company, Phillips-

burg, NJ: formic acid, glacial acetic acid; hydrochloric acid, lithium hydroxide, methanol, potassium hydroxide, sodium chloride, sodium hydroxide, sodium dibasic phosphate, ammonium carbonate, sucrose and trichloroacetic acid. British Drug House, Poole, England: SDS. Eastman Kodak Co., Rochester, NY: acrylamide, *bis*-acrylamide, chloramine T, and DATD. Fisher Scientific Company, Pittsburgh, PA: ammonium chloride, bromophenol blue, cyanogen bromide, DMSO, and polyethylene glycol 1000. Grand Island Biological Co., Grand Island, NY: DME, Freund's complete and incomplete adjuvant, glutamine, Medium NCTC 135, penicillin, RPMI 1640 without methionine, sodium pyruvate, and streptomycin. Merck and Co., Inc., Rahway, NJ: sulfasalicylic acid. Sigma Chemical Co., St. Louis, MO: aminopterine, 8-azoguanine, Coomassie Brilliant Blue R, EDTA, glutamic acid, hypoxanthine, hydroxylamine, imidazole, insulin, mercaptoethanol, ovalbumin (chicken egg), Sephadex G-25-150, sodium azide, sodium barbitol, thymidine, trizma base, and tyrosine. Schwarz-Mann, Orangeburg, NY: ammonium sulfate, guanidine. Sterile Systems, Inc., Logan, UT: HyClone fetal bovine serum.

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