Cytoplasmic Myosin from *Drosophila melanogaster*

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Abstract. Myosin is identified and purified from three different established *Drosophila melanogaster* cell lines (Schneider's lines 2 and 3 and Kc). Purification entails lysis in a low salt, sucrose buffer that contains ATP, chromatography on DEAE-cellulose, precipitation with actin in the absence of ATP, gel filtration in a discontinuous KI-KCl buffer system, and hydroxylapatite chromatography. Yield of pure cytoplasmic myosin is 5–10%.

This protein is identified as myosin by its cross-reactivity with two monoclonal antibodies against human platelet myosin, the molecular weight of its heavy chain, its two light chains, its behavior on gel filtration, its ATP-dependent affinity for actin, its characteristic ATPase activity, its molecular morphology as demonstrated by platinum shadowing, and its ability to form bipolar filaments.

The molecular weight of the cytoplasmic myosin's light chains and peptide mapping and immunochemical analysis of its heavy chains demonstrate that this myo-

sin, purified from *Drosophila* cell lines, is distinct from *Drosophila* muscle myosin. Two-dimensional thin layer maps of complete proteolytic digests of iodinated muscle and cytoplasmic myosin heavy chains demonstrate that, while the two myosins have some tryptic and alpha-chymotryptic peptides in common, most peptides migrate with unique mobility. One-dimensional peptide maps of SDS PAGE purified myosin heavy chain confirm these structural data. Polyclonal antiserum raised and reacted against *Drosophila* myosin isolated from cell lines cross-reacts only weakly with *Drosophila* muscle myosin isolated from the thoraces of adult *Drosophila*. Polyclonal antiserum raised against *Drosophila* muscle myosin behaves in a reciprocal fashion. Taken together our data suggest that the myosin purified from *Drosophila* cell lines is a bona fide cytoplasmic myosin and is very likely the product of a different myosin gene than the muscle myosin heavy chain gene that has been previously identified and characterized.

MYOSIN, a hexameric protein that usually consists of two heavy and four light polypeptide chains, is the mechanochemical force transducer that interacts with actin to convert energy from the hydrolysis of ATP into force for muscular contraction (reviewed in Harrington and Rodgers, 1984). In higher eukaryotes, cytoplasmic isoforms of myosin can be identified and presumably generate such diverse cellular motilities as platelet-mediated clot retraction, intracellular vesicle movement, cytoplasmic streaming, cell locomotion, and cytokinesis (Pollard, 1981). Indeed, a functional role for cytoplasmic myosin in cytokinesis has been demonstrated best in antibody injection studies: anti–cytoplasmic myosin specifically inhibits actin-activated myosin ATPase activity in vitro and, when microinjected into living cells, inhibits the function of the contractile ring during cleavage in a specific and dose-dependent fashion (Mabuchi and Okuno, 1977; Kiehart et al., 1982).

In avian and mammalian species, distinct isoforms of both myosin heavy and light chains are responsible for contractility in skeletal, cardiac, smooth muscle, and nonmuscle tissues (reviewed in Nadal-Ginard et al., 1982; Whalen et al., 1982; and Buckingham and Minty, 1983). Further diversity is common even within a particular tissue: fast and slow iso-

forms of skeletal muscle, distinct isoforms of atrial and ventricular cardiac muscle (reviewed in Nadal-Ginard et al., 1982; Whalen et al., 1982; and Buckingham and Minty, 1983), and distinct isoforms of cytoplasmic myosin (Burridge and Bray, 1975; Wong et al., 1985) have been identified. While the origin of this diversity is not uniformly clear, some of it is specified at the DNA level (Nadal-Ginard et al., 1982; Buckingham and Minty, 1983; Robbins et al., 1986). In avian and mammalian species, the myosin heavy chain gene family apparently consists of ~10 distinct but closely related members. In avian species there are as many as 20 more distantly related forms. The heterogeneity in myosin heavy chain genes may reflect differences in sequence that alter regulation of the gene's expression, allowing coordinate expression with different sets of genes in different tissues or cell types at various times during development. Alternately, genes that differ in the sequence of their protein coding regions may result in appropriate expression of specific isoforms that are custom-tailored for their particular mechanochemical application.

In contrast to the mammalian and avian systems described above, only a single myosin heavy chain gene has been identified in *Drosophila* (Bernstein et al., 1983; Rozek and

Davidson, 1983). This suggests three interesting possibilities: That no diversity in myosin isoforms is necessary in *Drosophila*, that necessary diversity can be accommodated by posttranscriptional or posttranslational modifications of the products of a single myosin gene, or that additional, more distantly related, and therefore non-cross-hybridizing myosin heavy chain genes exist. Indeed, alternate splicing of the primary transcripts of both myosin heavy and light chain genes suggest that posttranscriptional modifications are responsible for at least some, albeit minor diversity in myosin heavy and light chain isoforms at the polypeptide level (Bernstein et al., 1986; Rozek and Davidson, 1986; Falkenthal et al., 1985).

Here we purify a protein from *Drosophila* cell lines, identify it as myosin by five structural and three functional criteria, and establish on the polypeptide level that its heavy chains are extensively different from muscle myosin heavy chain isoforms. The substantial differences between muscle and cytoplasmic isoforms of *Drosophila* myosin heavy chain support the existence of a distinct myosin gene responsible for encoding this cytoplasmic polypeptide.

Preliminary accounts of this work were presented at meetings of the American Society for Cell Biology and the Biophysical Society (Kiehart and Feghali, 1985, 1986).

Materials and Methods

Cell Culture, Harvest, and Homogenization

Drosophila Kc and Schneider's 2 and 3 cells are distinct, established cell lines that are derived from embryonic tissue explants and do not display characteristics of myogenic cell lines (Schneider and Blumenthal, 1978). They were grown by standard methods in spinner culture (Schneider and Blumenthal, 1978). Approximately 15–30 g (wet weight) of cells grown to a density of $0.5–2 \times 10^7$ cells/ml were harvested by centrifugation and washed three times by gentle sedimentation and resuspension in ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaPO$_4$, pH 7.2). All subsequent steps were performed at 0–4°C unless otherwise specified. Washed cells were resuspended in 10 vol of PBS, incubated in diisopropylfluorophosphate (5.7 mM final concentration) for 5 min, washed in fresh PBS, then resuspended in 4–6 vol of ice-cold homogenization buffer (0.34 M sucrose, 20 mM imidazole-Cl, pH 7.0, 5 mM EGTA, 5 mM ATP, 5 mM dithiothreitol [DTT],[1] 5 mM phenylmethylsulfonyl fluoride, 3 mM NaN$_3$, 22 μM Pepstatin A, 0.25 mg/ml soybean trypsin inhibitor). Cells were lysed by sonication with a Branson sonifier set at maximum for a microtip (40 W). Cell homogenates were fractionated by centrifugation at 43,000 rpm in a Sorvall T647.5 rotor (137,600 g_{av}).

Purification of Myosin from the High Speed Supernatant

The supernatant was chromatographed on DEAE-cellulose (DE-52, Whatman Inc., Clifton, NJ; prepared as described by Pollard, 1982) that was equilibrated with homogenization buffer in the absence of proteolytic inhibitors. Approximately 100 ml of high speed supernatant was applied to a 2.5 × 35-cm column. The column was washed with 3–5 vol of homogenization buffer, then eluted with 0.8–1 liter of a linear, 0–0.5 M KCl gradient in homogenization buffer. Fractions that contained myosin were pooled.

Subsequent purification relied on the high affinity of myosin for actin in the absence of ATP. Endogenous *Drosophila* actin in the high speed supernatant or in selected fractions from DEAE-chromatography was polymerized by warming the sample to 25°C, adjusting the KCl concentration to 50–150 mM (fractions from DEAE were already ∼150 mM in KCl), then adding MgCl$_2$ to a final concentration of 2 mM. To maximize recovery of myosin in subsequent steps, additional actin was required. Chicken F-actin

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1. *Abbreviation used in this paper*: DTT, dithiothreitol.

(in 50 mM KCl, 2 mM MgCl$_2$, 2 mM Tris-Cl, pH 8.0, 0.75 mM NaN$_3$, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM CaCl$_2$), purified by established protocols (Spudich and Watt, 1971; Pardee and Spudich, 1982), was added to a final concentration of 0.25 mg/ml. Exogenous chicken actin did not contain any contaminating polypeptides that co-migrate with the 205-kD band as shown by SDS PAGE of heavily overloaded samples (data not shown). In addition, polyclonal antibodies made against the purified *Drosophila* cytoplasmic myosin (see below) did not cross-react with purified chicken skeletal muscle myosin on Western blots (data not shown).

ATP was removed from the mixture of pooled DEAE fractions and exogenous actin after adding glucose (50 mM) and hexokinase (1 U/ml). After incubation for 30 min at 25°C and then for 8–12 h at 0°C, the preparation was layered over a 30% sucrose cushion containing 50 mM KCl, 2 mM MgCl$_2$, and 10 mM imidazole-Cl, pH 7.0, and filamentous actin. Any actin binding proteins were precipitated by centrifugation at 30,000–43,000 rpm in a Sorvall T1270 rotor (61,300–126,000 g_{av}) for 3 h. Without exogenous actin, more than half of the 205-kD polypeptide remained in the supernatant (even when the high speed supernatant of cell homogenate was not subjected to DEAE chromatography first; Fig. 1 *B*). With added chicken actin, nearly all of the 205-kD polypeptide that otherwise remained in the supernatant was sedimented (Fig. 1 *C*).

The actomyosin pellet was dissolved in 4.3 ml of KI buffer (0.72 M KI, 20 mM imidazole-Cl, pH 7.0, 1 mM EGTA, 5 mM DTT, 5 mM ATP, 3 mM NaN$_3$, 10% glycerol), homogenized with a tight-fitting glass pestle Dounce, then clarified by centrifugation for 30 min at 30,000 rpm in a Sorvall T1270 (63,400 g_{av}). The clarified supernatant was gel filtered on a A15-m (Bio-Rad Laboratories, Richmond, CA) column (1.5 × 85 cm) equilibrated with KCl buffer (0.6 M KCl, 10 mM imidazole-Cl, pH 7.0, 1 mM DTT, 1 mM EGTA, 3 mM NaN$_3$, 10% glycerol) that was loaded with 12–19 ml of KI buffer just before addition of the protein sample.

Further purification of myosin was achieved by repetition of the actin affinity and gel filtration steps and/or by chromatography on hydroxylapatite (Bio-Rad Laboratories) equilibrated with 0.5 M KCl, 50 mM imidazole-Cl, pH 7.0, 1 mM EGTA, 0.5 mM DTT, 3 mM NaN$_3$, 10% glycerol. Pooled fractions from gel filtration were added to the hydroxylapatite in batch (12–20 ml/ml bed) and tumbled slowly for ∼12 h. The hydroxylapatite with absorbed protein was poured into a small column and washed with 5–10 vol of equilibration buffer. Absorbed proteins were eluted with a linear 0–300 mM KPO$_4$ gradient in equilibration buffer.

Drosophila Thoracic Muscle Myosin

Myofibrils from *Drosophila* thoraces were isolated as detailed by Mogami et al. (1982), then dissolved in KI buffer (see above). Subsequent purification of *Drosophila* muscle myosin was by gel filtration on an agarose A15-m column and by hydroxylapatite chromatography as described for the myosin purified from *Drosophila* cell lines.

SDS PAGE

PAGE was performed using standard methods (Laemmli, 1971). To prepare samples that contained low concentrations of protein for SDS PAGE, aliquots were mixed with exogenous tRNA (from *E. coli*; Boehringer Mannheim Biochemicals, Indianapolis, IN), precipitated with 10% TCA as described by Cabib and Polacheck (1984), then resuspended in SDS sample buffer (Laemmli, 1971) with bromophenol blue as an indicator dye. The pH was adjusted to near neutrality by the vapor phase addition of NH$_4$OH until the indicator turned blue. Polyacrylamide gels were stained with Coomassie Brilliant Blue as described elsewhere (Fairbanks et al., 1971).

Western Blotting

Polypeptides were electrophoretically blotted from 5% polyacrylamide gels by a modification of the procedure devised by Towbin et al. (1979; Kiehart et al. 1984*a*; Kiehart et al. 1986). Nitrocellulose blots were incubated briefly in STTMB (150 mM NaCl, 10 mM Tris-Cl, pH 7.7, 0.1% Triton X-100, 0.1% bovine serum albumin, 0.01% thimerosal) or Blotto (150 mM NaCl, 10 mM NaPO$_4$, pH 7.4, 5% non-fat dry milk, 0.05% antifoam A, and 0.01% thimerosal; Johnson and Elder, 1983), then overlaid with antibody in the same blocking buffer. Localization of antibody that bound to the blots was accomplished with ^{125}I-labeled second antibody (Kiehart et al., 1984*a*) or with peroxidase-labeled second antibody (HyClone Laboratories, Hogan, UT) diluted in STTMB or Blotto by 500–2,000-fold, followed by autoradiography or development for peroxidase reaction product with 4-chloro-1-napthol and hydrogen peroxide as substrates (Hawkes et al., 1982).

Concentration of Myosin in Drosophila Cells in Culture

The concentration of myosin in *Drosophila* Schneider's 3 cells was estimated with an antibody binding assay similar to the one used by Kiehart and Pollard (1984) to estimate the concentration of myosin-II in *Acanthamoeba* extracts. Dilutions of hydroxylapatite-purified *Drosophila* cell myosin were used as standards, a 1,000-fold dilution of anti-*Drosophila* cytoplasmic myosin was used to probe a blot that contained both standards and whole cell samples, and peroxidase-labeled second antibody was used to estimate first antibody binding.

Analytical Gel Filtration

The Stokes radius of *Drosophila* cytoplasmic myosin was calculated from partition coefficients obtained by gel filtration of 0.5-ml samples containing 80 µg/ml myosin on a 1.5 × 85-cm agarose A15-m column equilibrated with KCl buffer by the method of Ackers (1967) as described by Pollard et al. (1978).

Two-dimensional Peptide Maps

Purified myosins were resolved on SDS PAGE, heavy chains were excised from the gel, iodinated, cleaved by trypsin (13 µg/ml) or alpha-chymotrypsin (200 µg/ml) at 37°C for 8–12 h, then mapped on cellulose thin layer plates as detailed by Elder et al. (1977). In each experiment, duplicate gel slices for each myosin were processed and revealed precise reproducibility. Tryptic maps were performed on three different preparations of cytoplasmic myosin and on two preparations of muscle myosin. Alpha-chymotryptic maps were performed on two samples each of *Drosophila* cytoplasmic and muscle myosin. In all cases, reproducibility between identically treated samples was excellent. To compare reliably the mobility of peptides from the two *Drosophila* myosin heavy chain isoforms, two plates were each spotted with peptides from either the cytoplasmic or the muscle isoforms, and a third composite plate was spotted with equal amounts of both. The presence of weak spots was verified by inspection of longer autoradiographic exposures and the resolution of closely spaced, strong spots was evaluated on shorter exposures. All 47 of the prominent cytoplasmic peptides and all 43 of the prominent muscle peptides were identified on the composite map. Thus the cytoplasmic and muscle isoform peptides could be compared directly, with confidence, despite subtle differences in peptide migration from one thin-layer plate to the next. To more quantitatively analyze the data each peptide on the cytoplasmic and the muscle myosin map was assigned a number and was located on the combined map. Peptides that overlapped were considered identical. This is clearly a simplification that tends to overestimate the degree to which the myosins are homologous.

Platinum Shadowing

Samples of *Drosophila* myosins in KCl column buffer were mixed with glycerol to a final concentration of 70% or were dialyzed into a mixture of 70% glycerol and 30% 0.6 M ammonium formate, pH 7.5 and rotary shadowed with platinum as described previously (Kiehart et al., 1984b; Tyler and Branton, 1980). Electron micrographs were taken on a Philips 301 at magnifications of 25,000.

ATPase Activity

ATP hydrolysis by K^+-EDTA-, Ca^{++}-, Mg^{++}-, and actin-activated Mg^{++}-ATPases were measured by ^{32}P-gamma-ATP as described previously (Kiehart and Pollard, 1984).

Actin–Myosin Binding Assay

Actin (5.3 µM in 2 mM Tris-Cl, pH 8.0, 0.75 mM NaN_3, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM $CaCl_2$) was polymerized by the addition of salts (0.6 M KCl, 20 mM imidazole-Cl, pH 7.0, 4 mM $MgCl_2$, 3 mM NaN_3, 0.1 mM DTT) and incubation at 37°C for 30 min. Parallel samples containing actin alone were treated identically. All concentrations shown are for the final concentration in the assay. Phalloidin (10 µM) and glucose (50 mM) were added, then samples were either supplemented with 2 mM ATP or depleted of ATP by the addition of hexokinase (12 U/ml) and incubated for 5 min at 37°C. Each preparation was chilled to 0°C, mixed with myosin (42 nM, assuming a molecular mass of 480 kD), allowed to incubate for 10 min at 0°C, then layered onto a sucrose cushion (30% sucrose, 0.6 M KCl, 20 mM

imidazole-Cl, 4 mM $MgCl_2$, 3 mM NaN_3, 0.1 mM DTT) with or without 2 mM ATP and sedimented for 40 min at 24 psi in a Beckman airfuge (Beckman Instruments, Inc., Palo Alto, CA). The polypeptide composition of the supernatants and pellets was analyzed by SDS PAGE.

Polyclonal Antibodies against Drosophila Myosins

Polyclonal antibodies were prepared in rabbits by popliteal lymph node injection (Sigel et al., 1983) of 3–10 µg of purified *Drosophila* cytoplasmic or muscle myosin in 0.2 ml of 400 mM NaCl, 5 mM imidazole-Cl, pH 7.0 mixed with an equal volume of Freund's complete adjuvant. After 1 mo rabbits were boosted with 10–20 µg of purified protein in the same solution mixed with Freund's incomplete adjuvant by injection into several sites intradermally. The antiserum raised against *Drosophila* cytoplasmic myosin reacts with a single, 205-kD polypeptide on immunoblots of both whole Schneider's 3 cell homogenates and of purified myosin from *Drosophila* cell lines (see Results). Preimmune serum showed no reaction against either fraction.

Other Proteins

Two monoclonal antibodies (PM 1 and PM 4) against human platelet myosin were prepared in collaboration with Drs. Albert Wong and Thomas D. Pollard. They react specifically with myosin from a variety of sources (manuscript in preparation; and Wong et al., 1985). Chicken myosin and actin were purified from chicken muscle and muscle acetone powder by standard methods (Kielly and Harrington, 1960; Spudich and Watt, 1971; Pardee and Spudich, 1982).

Protein Concentration

Protein concentration was estimated with an Amido black dye binding assay as described by Nakamura and co-workers (1985), with chicken skeletal muscle myosin as a standard.
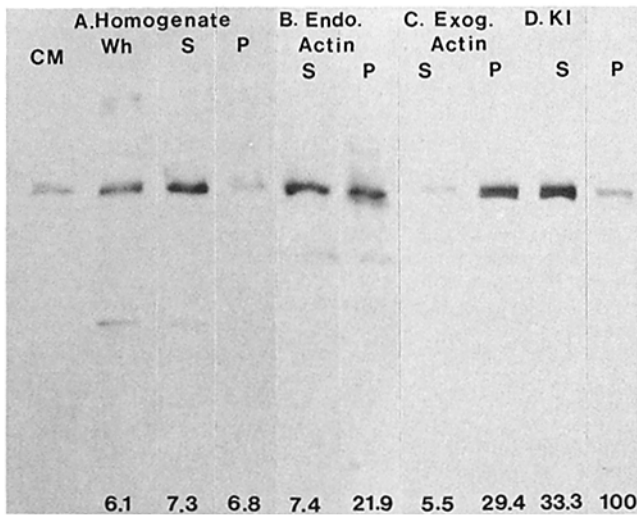
Reagents

All salts and pH buffers were reagent grade and deionized water (Millipore Corp., Bedford, MA) was used throughout these experiments. Suppliers of chromatographic resins are given in the text. ATP, diisopropylfluorophosphate, EGTA, hexokinase, phenylmethylsulfonyl fluoride, pepstatin A, and soybean trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO, and DTT was from Sigma Chemical Co. or Boehringer Mannheim Biochemicals. SDS was from Bethesda Research Laboratories (Gaithersburg, MD) and other reagents for SDS PAGE were from Bio-Rad.

Results

Identification, Purification, and Partial Characterization of Myosin from Drosophila Cells in Culture

Identification of Myosin in Drosophila Cell Lines. A 205-kD polypeptide in three *Drosophila* cell lines, Kc and Schneider's 2 and 3, was tentatively identified as myosin by establishing its cross-reactivity with two monoclonal antibodies directed against human cytoplasmic myosin heavy chain (Fig. 1). SDS PAGE and Western blots overlaid with these antibodies were used to follow the distribution of the 205-kD polypeptide during cell fractionation and subsequent purification from Schneider's 3 cells. The majority of the 205-kD polypeptide was extracted into the high speed supernatant of cell homogenate as revealed by monoclonal anti–platelet myosin overlay of Western blots of pellet and supernatant fractions (Fig. 1). Further extraction of the pellet with 10 vol of homogenization buffer supplemented with 0.5 M KCl, 40 mM sodium pyrophosphate, or 1% Triton-X-100 did not significantly reduce the amount of 205-kD polypeptide in the high speed pellet (data not shown).

Purification of Myosin from Drosophila Cell Lines. Be-

| CM	A.Homogenate			B. Endo. Actin		C. Exog. Actin		D. KI	
	Wh	S	P	S	P	S	P	S	P
	6.1	7.3	6.8	7.4	21.9	5.5	29.4	33.3	100

Figure 1. The *Drosophila* 205-kD polypeptide cross-reacts with antibody against human cytoplasmic myosin and reveals the distribution of 205-kD heavy chain in fractions obtained during the purification of *Drosophila* cytoplasmic myosin. Western blots were overlaid with PM-4, a monoclonal anti–human platelet myosin antibody. Antibody binding was localized with peroxidase-labeled second antibody. The amount of each fraction that was loaded is indicated in parentheses and is expressed in thousandths of a percent of the total protein (or volume) present in the fraction. Comparison of the relative intensity of peroxidase label at 205 kD in the supernatant and pellet fractions in *B* and *C* reveal that most of the 205-kD polypeptide is not recovered in the actomyosin pellet unless exogenous actin is included. Chicken skeletal muscle myosin, also stained by this antibody, is in the lane labeled *CM*. Samples were obtained from four steps: (*A*) The homogenate, including the whole cell homogenate (*Wh*), the high speed supernatant of cell homogenate (*S*), and the high speed, cell debris pellet (*P*). (*B*) The endogenous actin pelleting steps (*Endo. Actin*; steps not usually included during standard purification but shown here to document the efficacy of adding exogenous actin), including the supernatant (*S*) that followed centrifugation of the endogenous actin–myosin complex and the actomyosin pellet (*P*). (*C*) The exogenous actin pelleting steps (*Exog. Actin*) including the supernatant (*S*) following centrifugation of the supernatant from *B* after the addition of exogenous chicken skeletal muscle actin and the exogenous actin–myosin complex pellet (*P*). (*D*) The KI-solubilized actomyosin pellet after centrifugation that resulted in a clarified supernatant (*S*) and the KI-insoluble pellet (*P*).

havior of the 205-kD polypeptide during purification, in part, helps to identify the polypeptide as a bona fide myosin.

The high speed supernatant was chromatographed on DEAE cellulose to remove contaminating nucleic acids and enrich for the 205-kD polypeptide. Fractions eluting between 0.13 and 0.2 M KCl contained an immunoreactive 205-kD polypeptide and a prominent 205-kD polypeptide on Coomassie Blue–stained gels. These fractions were pooled.

Like other myosins, more of the protein that contains 205-kD polypeptide binds to F-actin in the absence of ATP than in its presence as shown in co-sedimentation experiments (Fig. 2). To take advantage of this property during purification of the *Drosophila* 205-kD polypeptide, exogenous F-actin (see Materials and Methods) was added to pooled fractions from DEAE, ATP was removed by addition of hexokinase and glucose, then the actin and actin-binding proteins were precipitated by high speed centrifugation
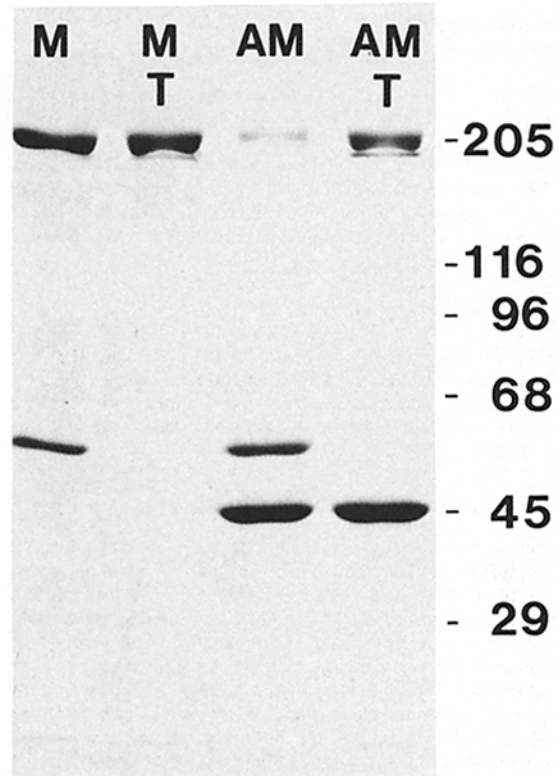

Figure 2. ATP inhibits the binding of 205-kD polypeptide to actin filaments. Coomassie Blue–stained SDS PAGE was used to analyze the polypeptide composition of supernatants from an actin–myosin co-pelleting experiment. Myosin quantitatively remained in the supernatant in the absence or presence of 2 mM ATP (*M* and *MT*, respectively). In contrast, sedimentation of myosin in the presence of actin is profoundly inhibited by the presence of ATP. Nearly all of the myosin co-sediments with actin in the absence of ATP (*AM*), but most remains in the supernatant in the presence of 2 mM ATP (*AMT*). The 58-kD band in lanes *M* and *AM* is hexokinase, used to deplete the samples of ATP. SDS PAGE of the pellets confirms these results (data not shown).

through a 30% sucrose cushion. Pellets were dissociated in potassium iodide solution and fractionated by gel filtration (Fig. 3).

Hydroxylapatite chromatography of fractions pooled from the gel filtration column allowed further purification of myosin, which eluted as a peak at 210 mM KPO_4 (Fig. 3 *b*). SDS PAGE of peak fractions revealed at least two light chains migrating at 16 and 18 kD.

Typically 300–500 µg of hydroxylapatite-purified myosin was obtained from 20–25 g (wet weight) of *Drosophila* Schneider's 3 cells. The concentration of myosin in whole cells was estimated by comparing antibody binding on Western blots of whole cells and of purified cellular myosin (Fig. 4). The polyclonal, rabbit antibody that was used for these experiments was raised against purified myosin from *Drosophila* cells and is specific for myosin as demonstrated by antibody overlay of nitrocellulose blots of SDS PAGE resolved purified myosin or of whole cell sample (Fig. 4). Gel lanes that had an equivalent of 53 nl of whole cells contained ∼10–15 ng of myosin. Thus, the concentration of myosin in these cells is ∼0.5 µM, and the yield of myosin in the most highly purified fractions is 5–10%.
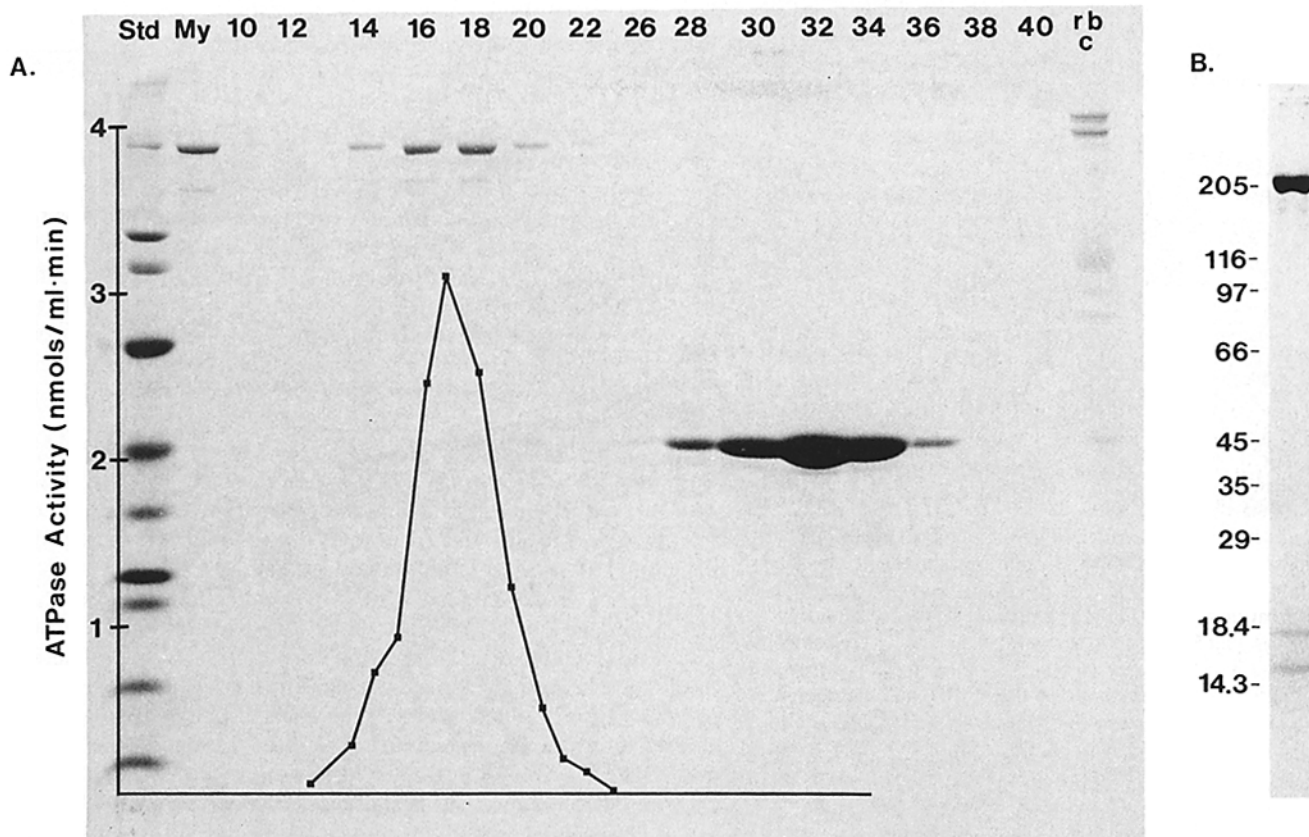
Figure 3. ATPase activity and polypeptide subunit composition of purified *Drosophila* cytoplasmic myosin. (*A*) K⁺-EDTA ATPase activity coelutes with 205-kD heavy chain when cytoplasmic myosin is gel filtered on agarose A15-m. Activity is plotted with respect to the axis shown on the left and corresponds to uniform specific activity of ∼550 nmol/mg·min across the peak. Lanes include molecular weight standards (*Std*, with molecular weights as in *B*), chicken myosin (*My*), numbered fractions from agarose A15-m gel filtration column (*10–40*, after a second cycle of purification by actin affinity and gel filtration; see Materials and Methods), and red blood cell ghosts (*rbc*). (*B*) SDS PAGE of ∼7.5 µg of *Drosophila* cytoplasmic myosin purified by hydroxylapatite chromatography reveals two light chains migrating at 16 and 18 kD.

Partial Characterization of Myosin from Drosophila Cell Lines. The 205-kD polypeptide and the K⁺-EDTA ATPase activity, a functional signature of myosin, coeluted precisely on gel filtration: specific activity was essentially constant across the peak (∼550 nmol Pi/mg·min; Fig. 3 *a*). Further analysis of the ATPase activities of the fractions that contained 205-kD polypeptide showed other activities indicative of myosin. They had comparable activities in 10 mM CaCl₂, 0.5 M KCl, or 0.05 M KCl and little activity in the presence of 5 mM MgCl₂ in 0.01 M KCl. Gel filtration revealed that the 205-kD polypeptide elutes with a partition coefficient (K_d) of 0.136, which corresponds to a Stokes radius of 17 nm.

Electron microscopy of rotary-shadowed specimens dried from glycerol (Fig. 5) revealed that the predominant molecular species in the fractions that contain 205-kD polypeptide and K⁺-EDTA ATPase activity was myosin: The two-headed molecules have a long tail (132 nm average) and aggregate in 180 mM ammonium formate to form bipolar filaments that have a bare zone averaging 204 nm (± 19 nm, $n = 5$) with projecting heads at each end (14.9 ± 3 heads per end). This suggests that each filament consists of 16 myosin molecules with antiparallel tails that overlap >60 nm or almost half of their length.

Drosophila Cytoplasmic and Muscle Myosin Isoforms Can Be Distinguished Structurally and Immunochemically

In addition to differences between the molecular weight of this myosin's and *Drosophila* muscle myosin's light chains, structural and immunological analyses of its heavy chains suggest that this myosin is a true cytoplasmic isoform of myosin, distinct from the muscle isoforms previously isolated from *Drosophila* thorax.

Two-dimensional Peptide Maps Reveal Significant Differences between Drosophila Cytoplasmic and Thoracic Muscle Myosin Heavy Chains. The structural relationship between these two *Drosophila* myosins was revealed in two-dimensional peptide mapping experiments (Fig. 6). Even cursory inspection of the maps revealed significant differences in the structure of these two related proteins: most of the cytoplasmic and muscle myosin peptides appeared to migrate with unique mobility as determined by comparing the position, shape, and intensity of the various spots (several uniquely migrating spots are highlighted with arrows in Fig. 6). When the maps were compared semiquantitatively (see Materials and Methods), approximately half of the tryptic peptides from *Drosophila* cytoplasmic (23 of 47) and muscle (22 of 43) myosins were observed to mi-

A. Drosophila Cyto. Myo. (ng) **B. Wh. Cell (nl)**
90 60 30 25 20 15 10 5   79 53 26
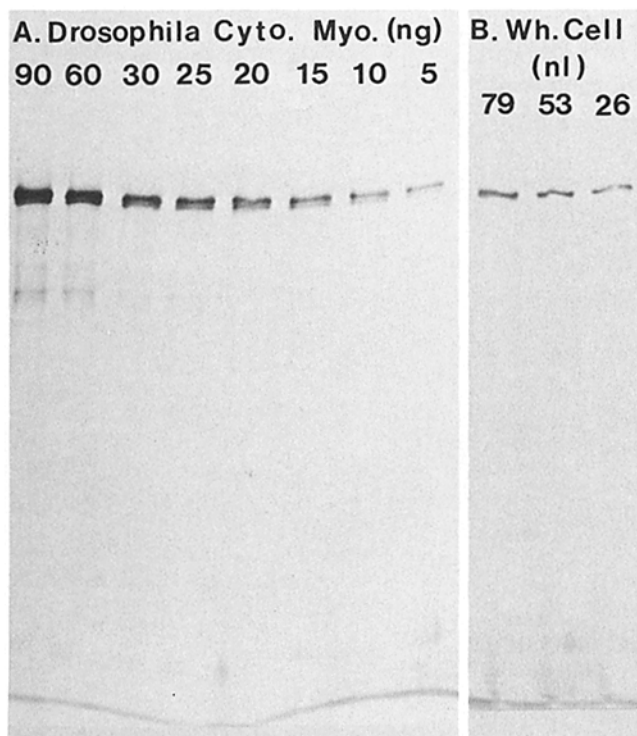
Figure 4. The specificity of anti-*Drosophila* cytoplasmic myosin antibody and the concentration of myosin in *Drosophila* cells in culture. Anti-*Drosophila* cytoplasmic myosin was used to overlay a Western blot of a dilution series of purified cytoplasmic myosin and whole cell sample. Antibody was localized with peroxidase-labeled goat anti-rabbit antibody. The dilution series of purified *Drosophila* cell myosin (*A*) verifies that the antibody reacts with myosin and provides a standard for estimating the concentration of myosin in the dilution series of whole cell sample (*B*). The amount (in nanograms) of cellular myosin standard is given at the top of each lane. The relative volume of whole cell sample loaded is given in nanoliters, and reflects the extent to which cells were diluted during sample preparation. Each lane of both sample and standard was actually loaded with 20 μl of sample. The lane containing 53 nl of sample contained 10–15 ng of total protein.

grate with unique mobility on the two-dimensional maps. The remaining peptides overlapped on the composite map. Some of these overlapping peptides probably precisely comigrated with one another while others were close to one another but likely to be distinct. Thus, the resolution of the semi-quantitative analysis used was insufficient to document the relationship between these polypeptides unambiguously and tended to overestimate the degree to which the myosins are homologous.

Overall, the data suggest that, while the two types of myosin isolated from *Drosophila* share some homology, significant differences distinguish *Drosophila* cytoplasmic from muscle myosin. Two-dimensional maps of alpha-chymotryptic peptides of the two *Drosophila* myosin isoforms and one-dimensional maps of peptides generated by V8 protease verify the significant differences in the primary sequences of the two polypeptides (data not shown).

Drosophila Cytoplasmic and Muscle Myosin Heavy Chains Can Be Distinguished Immunologically. Polyclonal antisera raised against either purified *Drosophila* cytoplasmic myosin or purified *Drosophila* muscle myosin were used to overlay Western blots of *Drosophila* cytoplas-

mic and muscle myosins (Fig. 7). These experiments show that the two myosin heavy chain isoforms are significantly different immunologically.

Anti-cytoplasmic myosin antibodies distinguished easily between the cytoplasmic and muscle isoforms of *Drosophila* myosin heavy chain (Fig. 7). At a 200-fold dilution of anti-cytoplasmic myosin, 1 ng of the cytoplasmic myosin isoform was detectable. 100 ng of the muscle myosin isoform was required to produce a peroxidase reaction product of only slightly greater intensity. At a 2,000-fold dilution of anti-cytoplasmic myosin antiserum, reaction products with 10 ng of cytoplasmic myosin and 1,000 ng of muscle myosin were comparable. Thus the anti-cytoplasmic myosin bound selectively to cytoplasmic myosin by approximately two orders of magnitude.

The immunological differences between the cytoplasmic and muscle isoforms of *Drosophila* myosin were more striking when the Western blots were probed with the anti-muscle myosin antiserum. While as little as 1 ng of muscle myosin was detected by 200-fold dilutions of the anti-muscle antiserum, 1,000 ng of cytoplasmic antigen was necessary to produce a detectable reaction product. At anti-muscle myosin dilutions of 2,000-fold the reaction product with 1 ng of muscle myosin appeared approximately equivalent to the reaction product observed with 1,000 ng of cytoplasmic myosin. Thus the anti-muscle antiserum was even more selective than the anti-cytoplasmic antiserum.

The immunological differences between these isoforms of *Drosophila* myosin have recently been confirmed with antisera from two mice immunized with *Drosophila* muscle myosin and two mice immunized with *Drosophila* cytoplasmic myosin. Like the rabbit polyclonal antisera described above, each of the four antisera displays marked specificity for the isoform against which it was made (Lutz, D. A., and D. P. Kiehart, unpublished observations).

Discussion

Five structural criteria, i.e., cross-reaction with monoclonal antibodies directed against human platelet myosin, molecular weight of myosin heavy chains, existence of two heterologous light chains, behavior on gel filtration, molecular architecture as revealed by electron microscopy of platinum-shadowed specimens, and three functional criteria, i.e., ATP-dependent association with actin, high K⁺-EDTA, and Ca⁺⁺-ATPase activities, and ability to form bipolar filaments, demonstrate that this protein, purified from *Drosophila* cells in culture, is a bona fide myosin.

Electron microscopy of platinum-shadowed specimens and gel filtration of native cytoplasmic myosin demonstrate that this myosin is similar in size and shape to other myosins and as a consequence likely consists of two heavy chains and four light chains. By SDS PAGE, the molecular mass of the *Drosophila* cytoplasmic myosin heavy chains is 205 kD. Two bands, migrating on SDS PAGE at 16 and 18 kD, co-purify with the 205-kD heavy chain and are likely myosin light chains. The molecular mass of the light chains of *Drosophila* muscle myosin are 34 or 31 kD (LC 1), 30 kD (LC 2), and 20 or 18 kD (LC 3), respectively (Takano-Ohmuro et al., 1983; Toffenetti, J., personal communication). Thus at least one of these light chains is probably unique to the cytoplasmic myosin isoform.
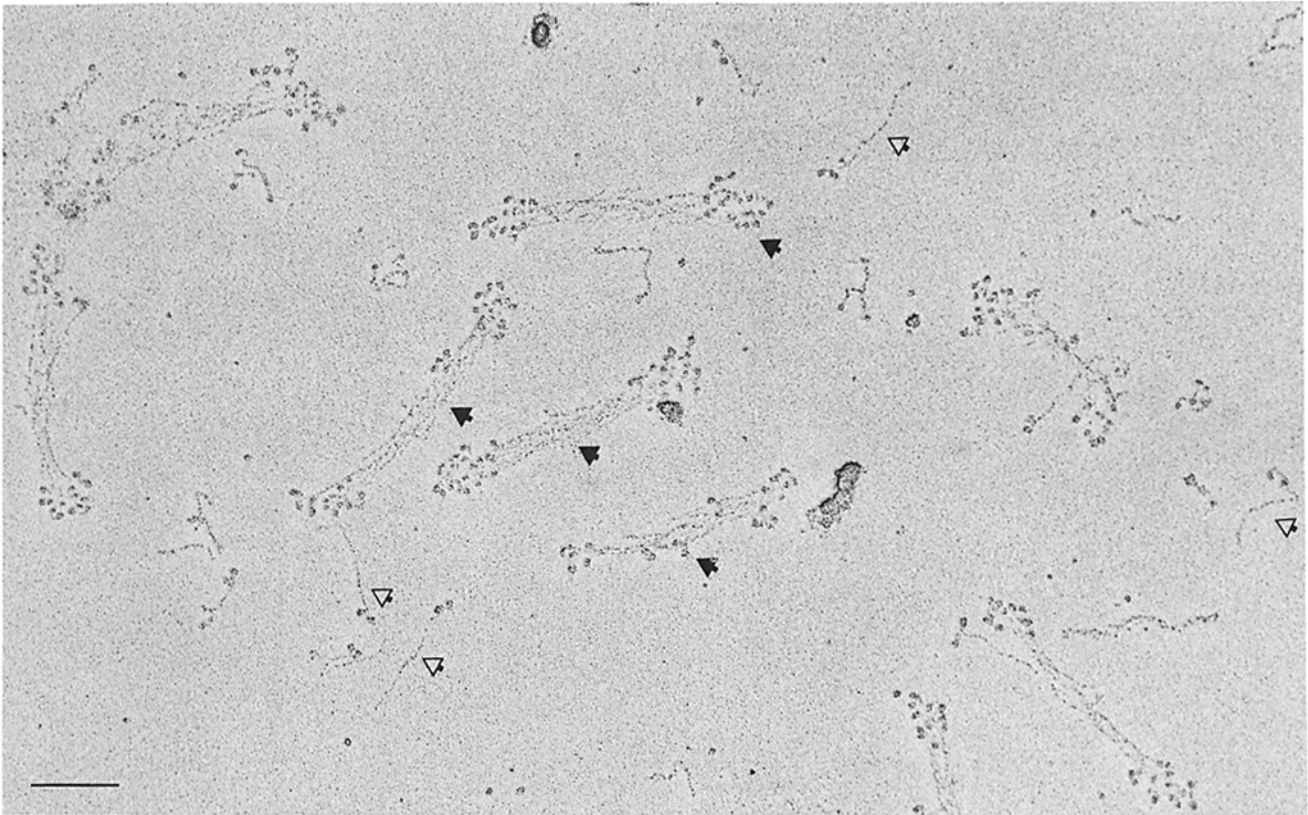
Figure 5. Drosophila cytoplasmic myosin has a long tail, two heads (*open arrows*), and assembles into bipolar filaments (*solid arrows*) with a bare zone. Specimens of *Drosophila* cytoplasmic myosins purified by two cycles of precipitation with actin and gel filtration were rotary platinum shadowed and photographed at 25,000×. Bar, 100 nm.

In preliminary experiments, actin activation of the Mg^{++}-ATPase activity of *Drosophila* cytoplasmic myosin has not been demonstrated. This is not surprising and is probably due to our failure to identify appropriate conditions for activ-

ity, which may require variation in solution conditions or in the state of phosphorylation of the heavy or light chains.

In addition to differences between the molecular weight of *Drosophila* cytoplasmic and muscle myosin light chain poly-
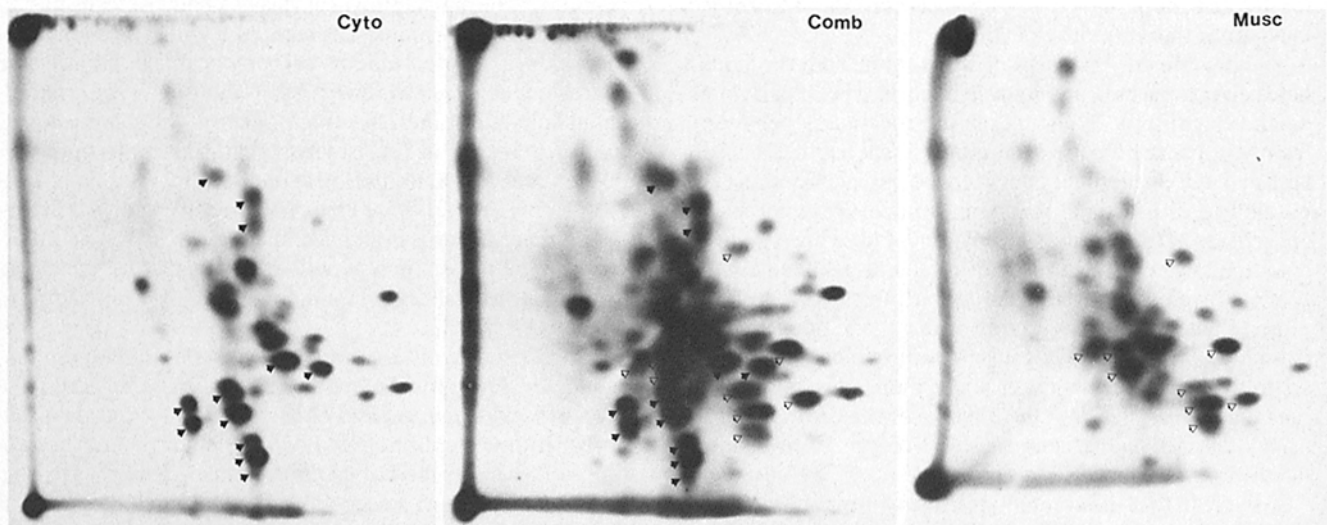

Figure 6. Two-dimensional peptide maps of ^{125}I-labeled myosin heavy chains reveal numerous distinct differences between cytoplasmic and muscle isoforms of *Drosophila* myosin heavy chain. Autoradiographs of two-dimensional peptide maps are oriented with electrophoretic and chromatographic dimensions horizontally (anode toward the right) and vertically, respectively. The end panels are labeled to indicate maps of cytoplasmic (*Cyto*) and muscle (*Musc*) isoforms. The center panel (*Comb*) was spotted with equal amounts of cytoplasmic and muscle myosin peptides and serves to compare peptides despite small anomalies in the migration of identical peptides on individual plates. Several of the peptides unique to the cytoplasmic and muscle isoforms are indicated with solid and open arrows, respectively.
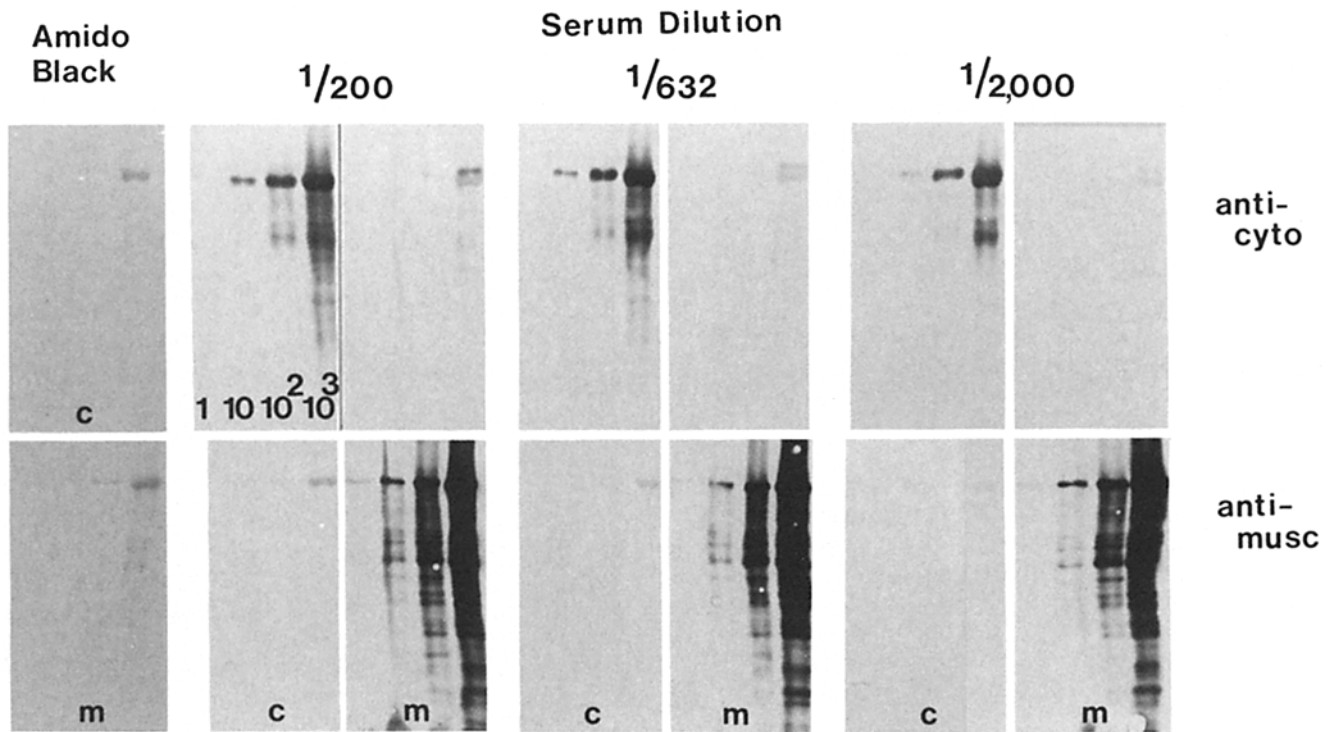
Figure 7. Cytoplasmic and muscle myosin heavy chain isoforms can be distinguished immunologically. Antibody overlay of Western blots demonstrates that polyclonal antisera raised against *Drosophila* cytoplasmic and muscle myosin isoforms react selectively with the heavy chain of the isoform against which they were made. Shown are 14 nitrocellulose blots, each containing a dilution series (1, 10, 100, or 1,000 ng, labeled *1, 10, 10², 10³* in one of the panels) of hydroxylapatite-purified *Drosophila* cytoplasmic (*c*) or muscle (*m*) myosin. Two blots at the left were stained with Amido black. The lanes containing 1,000 ng of protein are readily visible, the lanes with 100 ng are less so. 1 and 10 ng of protein are not detected by the Amido black. The 12 remaining blots (six cytoplasmic, *c*, and six muscle, *m*) were stained with three different dilutions (200-, 632-, or 2,000-fold dilutions in STTMB) of anti-*Drosophila* cytoplasmic (*top, anti-cyto*) or anti-*Drosophila* muscle myosin (*bottom, anti-musc*) as shown. Peroxidase-labeled second antibody (diluted 1,000-fold) was used to localize antibody binding to the blots.

peptides, two independent peptide mapping protocols and six independent polyclonal antisera establish that the cytoplasmic isoform of *Drosophila* myosin heavy chain is structurally and immunologically distinct from the isoform isolated from thoraces of adult flies.

The two-dimensional peptide maps reveal some homology between cytoplasmic and muscle isoforms, but clearly demonstrate significant differences in the constituent peptides of the myosin heavy chains. One-dimensional peptide maps establish that the differences between the polypeptides revealed by the two-dimensional mapping protocol were not simply an artifact of differential iodination of tyrosine, cysteine, histidine, or tryptophan residues on the respective peptides.

Six polyclonal antisera, one rabbit, and two mouse sera directed against the cytoplasmic isoform, and one rabbit and two mouse sera directed against the muscle isoform, underscore the differences between these molecules. These findings suggest that the two *Drosophila* isoforms are as different as cytoplasmic and striated muscle isoforms of myosin isolated from vertebrate sources.

It is clear that differential splicing of myosin heavy and light chain genes gives rise to heterogeneity in the mature messenger RNAs that encode for various isoforms of *Drosophila* muscle myosin heavy and light chains (Bernstein et al., 1983; Rozek and Davidson, 1983; 1986; Bernstein et al., 1986; Falkenthal et al., 1985). However, differential splicing results in significant changes in the sequence of the 3' un-

translated region of both myosin heavy and light chain mRNAs and can account for only minor changes in the amino acid sequence near the carboxy-termini of these polypeptides. One muscle myosin heavy chain isoform has 27 unique carboxy-terminal amino acids, while the other isoform has a single, unique carboxy-terminal amino acid (Bernstein et al., 1986; Rozek and Davidson, 1986). Differential splicing of the *Drosophila* alkali light chain primary transcript results in two isoforms that differ only in the sequence of the 14 amino acids at their carboxy-terminal ends (Falkenthal et al., 1985). Thus, no currently known differential splicing patterns could account for the extensive differences in the myosin polypeptide isoforms that we document through structural and immunological analyses of the purified proteins.

In concert, the observations provide good, albeit circumstantial evidence for the existence of at least one additional myosin heavy chain gene in *Drosophila*. Since our data suggest that the cytoplasmic and muscle isoforms are substantially different at the polypeptide level, it is not surprising that the existence of a second myosin heavy chain gene was missed when muscle myosin DNA probes were used to search for additional myosin genes in *Drosophila* (Bernstein et al., 1983; Rozek and Davidson, 1983). Supporting this conclusion is the observation that, although hybridization between nematode muscle and *Acanthamoeba* myosin genes shows some detectable homology between muscle and cyto-

plasmic myosin genes, in chickens and in humans the muscle myosin DNA probes have failed to identify unambiguously the gene(s) for cytoplasmic myosin (Leinwand, L., personal communication). Unequivocal demonstration of the existence of a second myosin heavy chain gene in *Drosophila* that encodes the cytoplasmic myosin isoform will require identification and cloning of the cytoplasmic gene and subsequently its detailed comparison with the gene for the muscle isoform that has already been identified and cloned.

The study of the myosin polypeptides and their genes in *Drosophila* may be particularly rewarding because, in contrast to the vertebrate systems, the apparently small number of myosin genes (perhaps as few as two) should allow a more complete description of the relationships between myosin genes, the regulation of their expression, and the functional importance of the diversity between the polypeptide isoforms that they encode. In addition, classical genetics and modern molecular biology, including mutant analysis, reverse genetics, production of anti–sense message, and germ and somatic cell line transformation can be brought to bear on the mechanism of myosin function and the role this protein plays in cellular homeostasis and movements during early embryogenesis.

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