Low Ionic Strength Solubility of Myosin in Sea Urchin Egg Extracts Is Mediated by a Myosin-binding Protein

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Abstract. We identify a novel myosin-binding protein, designated 53K, which appears to mediate the low ionic strength solubility of myosin in extracts of unfertilized sea urchin eggs. The protein possesses a subunit molecular mass on SDS-PAGE of 53 kD, an S value of 7, may be organized into disulfide-linked oligomers, and is associated with myosin in egg extracts. Both myosin and 53K co-precipitate from extract upon the addition of nucleoside triphosphates and co-sediment with an S value of 24 by sedimentation velocity centrifugation. Myosin in extracts not associated with 53K has an S value of 10. Further, myosin can be immunoprecipitated from extract with antibody to 53K and the 53K in extracts binds to a myosin affinity column. When extract is depleted of 53K, a majority of the myosin precipitates out of extract in a nucleotide-independent manner. Whereas purified myosin precipitates in the absence of nucleotide when recombined with dialysis buffer or myosin-depleted extract, reconstituting 53K and myosin before addition to buffer or myosin-depleted extract partially restores the low ionic strength solubility demonstrated by myosin in fresh egg extracts. The 53-kD protein may represent a new class of authentic myosin-binding proteins that may regulate the supramolecular organization of myosin in nonmuscle cells.

ACTIN and myosin have been shown to be involved in many forms of motility in nonmuscle cells. Regulation of motility depends in part on the state of polymerization of both actin and myosin. Actin can exist in many forms: as F-actin, as G-actin, as oligomers, and in complexed forms with various actin-binding proteins (for review see Pollard and Cooper, 1986). The regulation of actin polymerization and organization depends on actin's interactions with actin-binding proteins and the proclivity of actin to polymerize into filaments under physiological conditions. Heretofore, there has been limited progress in the analysis of factors responsible for the assembly and distribution of myosin in nonmuscle cells.

Myosin, in vitro, also exists in either polymerized or monomeric forms depending on ionic strength (Harrington and Rodgers, 1984). However, the extent of polymerized myosin appears much less than what would be predicted given intracellular ionic conditions. Some of the best evidence for this conclusion is that myosin remains soluble in high speed isosmotic extracts of several cell types (Pollard, 1976; Stossel and Hartwig, 1976; Condeelis and Taylor, 1977; Kane, 1983). Once purified, however, nonmuscle myosins exhibit typical low solubility at physiological ionic strength (Clarke and Spudich, 1977). The dynamic cytoskeletal reorganizations necessary for processes such as cytokinesis, ameboid movement, or neural tube formation suggest that myosin's polymerization state in the nonmuscle cell may be flexible. This idea is supported by immunofluorescence studies on *Dictyostelium* amebas demonstrating that myosin is capable of reorganization in response to cAMP stimulation (Yumura and Fukui, 1985).

Furthermore, a complex relationship exists between myosin assembly and enzymatic activity in vitro. Myosin ATPase activity and assembly state in many nonmuscle systems is regulated by either light chain or heavy chain phosphorylation (for review see Kuznicki, 1986). In general, light chain phosphorylation promotes bipolar filament assembly and enhances myosin's actin-activated ATPase activity, whereas heavy chain phosphorylation inhibits bipolar filament assembly. Phosphorylation is also partially responsible for regulating the extent of myosin's interaction with actin. Unlike actin, there have been to date relatively few reports of myosin-binding proteins. Titin, nebulin, myomesin, C-protein, and an 86-kD protein have all been found in skeletal, and in some cases, cardiac muscle where they appear to bind to myosin thick filaments and conceivably play a role in thick filament organization and alignment (Moos and Feng, 1980; Eppenberger et al., 1981; Moos, 1981; Wang, 1985; Horowits et al., 1986). Finally, myosin light chain and heavy chain kinases and their respective phosphatases must be myosin-binding proteins, even if transient compared to the structural proteins of the skeletal sarcomere.

Unfertilized sea urchin eggs contain the following: soluble actin and myosin, both in an unpolymerized form; profilin;

a 220-kD protein; α-actinin; fascin; spectrin; and tropomyosin. Extracts prepared from such eggs contain most of these proteins (Kane, 1976; Ishimoda-Takagi, 1979; Mabuchi and Hosoya, 1982; Kane, 1983; Mabuchi et al., 1985; Kuramochi et al., 1986). Interestingly, myosin remains soluble in low ionic strength extracts, conditions under which it would be expected to form insoluble bipolar filaments in vitro. Soluble myosin can then be converted into a form, presumably bipolar filaments, capable of generating force in reconstituted contractile gels upon the addition of ATP (Kane, 1980).

The present study centers on the nature of myosin's solubility properties in extracts of unfertilized sea urchin eggs. We find a protein of subunit molecular mass 53 kD present in extracts that appears to confer solubility on myosin in a low ionic strength environment. This 53-kD polypeptide, designated 53K, likely in the form of a high molecular mass disulfide-linked oligomer, appears to be an authentic nonmuscle myosin-binding protein that regulates the solubility state of myosin.

Materials and Methods

Preparation of Extracts

Extracts of unfertilized sea urchin eggs (*Lytechinus variegatus*) were prepared according to Kane (1975). Briefly, after dejellying, eggs were homogenized in 0.9 M glycerol, 0.1 M Pipes, 5 mM EGTA, 1 mM dithiothreitol (DTT), pH 7.0 (1 vol packed eggs/1.5 vol homogenization solution). The egg homogenate was centrifuged at 100,000 *g*, at 4°C for 1 h, the soluble supernatant was dialyzed overnight against extract dialysis buffer (5 mM Pipes, 0.1 mM EGTA, 0.1 mM ATP, pH 7.0), and then clarified at 25,000 *g* for 20 min before use. To determine the effect of pH, the extract was directly dialyzed into buffer at the appropriate pH. Protease inhibitors were included in egg homogenization buffer and extract dialysis buffer as follows: 0.3 μg/ml aprotinin, 25 μg/ml soybean trypsin inhibitor, and 1 μg/ml leupeptin.

Precipitation

Nucleoside triphosphates, at a final concentration of 2 mM unless otherwise specified, were added to extracts on ice along with indicated chelators or accessory ions for 3 h during which time microscopic fibrillar aggregates formed. EGTA or EDTA were both used at a final concentration of 10 mM; Mg^{++} or Ca^{++} at 1 mM; $NaPP_i$, ADP, and AMP at 2 mM. Precipitates were centrifuged at 7,000 *g* for 20 min, washed twice in 10 mM Pipes, pH 7.0, and repelleted before further analysis.

High/Low Salt Cycling

ATP-generated precipitate was resolubilized in a small volume of 0.5 M KCl, 10 mM imidazole, 1 mM EDTA, pH 7.2, and then cycled into low salt by dialysis into 100 mM KCl, 5 mM Pipes, 0.1 mM EGTA, pH 7.2, until a precipitate was observed. Samples were centrifuged in an airfuge (Beckman Instruments, Inc., Palo Alto, CA) at 22 psi for 20 min. The pellet was resolubilized and the cycle repeated. Samples were taken from all pellet and supernatant fractions for PAGE.

Myosin Purification

The ATP-generated precipitate was solubilized in a minimal volume of 0.4 M KCl, 50 mM $NaPP_i$, 50 mM NaF, 20 mM Pipes, 1 mM EDTA, 1 mM DTT, 1 mM ATP, pH 7.5. The sample was then run on either a Sephacryl S-300 or an S-1000 column (1.0 × 60 cm, Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with the same buffer. Fractions containing myosin, determined by SDS-PAGE, were pooled and dialyzed against 10 mM KCl, 10 mM Pipes, 1 mM EDTA, 1 mM DTT, pH 7.2, for ~4 h. After centrifugation at 25,000 *g* for 30 min, the pellet was washed once in 10 mM Pipes, pH 7.0, and then resolubilized in 0.5 M KCl, 10 mM imidazole, 1 mM EDTA, pH 7.2. Myosin obtained through this protocol was judged to be >95% pure as judged by SDS-PAGE.

Sedimentation Velocity Centrifugation

An aliquot of extract, to which ATP had been added, was loaded onto a 10–50% linear sucrose gradient prepared in extract dialysis buffer with a 2-ml cushion of saturated sucrose, and centrifuged for 12 h at 52,000 *g*, 4°C in a SW 28 rotor. Fractions of 1 ml were collected from the bottom of the tube and their OD_{280} measured. Separate parallel gradients with 3 mg of rabbit skeletal muscle aldolase or equine splenic apoferritin were run as standards for sedimentation coefficient values. Purified filamentous egg myosin was also run on a separate parallel gradient. The polypeptide composition of peak fractions was determined by SDS-PAGE.

Antibody Production and Immunoprecipitation

Polyclonal antibodies were generated in New Zealand White rabbits. Rabbits were injected with 300–400 μg of purified myosin or an enriched fraction containing 53K. Booster injections were given 3 wk after the initial injection and subsequently at 2-wk intervals. Rabbits were bled 4 d before receiving a boost. The sera was then fractionated by NH_4SO_4 precipitation and ion exchange chromatography to isolate the IgG fraction which was used in all experiments. Specificity of antibody reactivity was assessed by immunoblotting against both purified proteins and extract. Preimmune sera was routinely used as an experimental control to determine specificity of antibody binding in experiments. Immunoprecipitation was carried out by incubating the sample with antibody for 1 h at 4°C. An aliquot of protein A-Sepharose beads (Pharmacia Fine Chemicals), previously equilibrated and washed in extract dialysis buffer, was added and the sample incubated for another hour. Beads were pelleted by brief centrifugation in a Beckman microfuge at 10,000 *g* and washed five times in buffer containing 0.1% SDS before bound proteins were solubilized by boiling in SDS-PAGE sample buffer.

Immunoblotting

Proteins were transferred onto nitrocellulose from microgels at 500 mA for 35 min in transfer buffer containing 192 mM glycine, 25 mM Tris-HCl, 0.1% SDS, 20% methanol, pH 8.3. Efficiency of transfer was determined by Amido black staining of the nitrocellulose. Blots were blocked in TB (500 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.1% Tween-20. Antibody incubations were carried out in TB containing 1% gelatin for 2 h after which the blots were washed three times in TB containing 0.05% NP-40. Visualization of immunoreactive bands was accomplished by either using ^{125}I- or horseradish peroxidase–coupled goat anti-rabbit antibody. Iodinated antibody was prepared using the Iodogen method or purchased (2–10 μCi/μg; New England Nuclear, Boston, MA) and used at a concentration of 0.5 μCi/ml. When horseradish peroxidase–coupled secondary antibody was used, blots were developed in 0.5 mg/ml 4-chloro-1-naphthol, 0.015% H_2O_2 in TB.

Dot Blots

Samples in TB were dotted onto nitrocellulose using a microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA) and allowed to incubate at room temperature for 30 min before vacuum filtration. The nitrocellulose was removed and processed as described above for immunoblots with slight modification. Blocking buffer contained TB plus 20 mg/ml BSA and 0.1% Tween-20, and the antibody incubation buffer contained TB plus 0.5 mg/ml BSA. ^{125}I-labeled goat anti-rabbit antibody incubation was followed by extensive washing after which the nitrocellulose sheet was allowed to air dry for 10 min. Individual dotted samples were excised with a razor blade and counted on a Beckman 5500 gamma counter. Each assay included a standard curve of the appropriate protein ranging from 10 ng to 1.5 μg for myosin or from 10 to 750 ng for 53K. The amount of 53K dotted onto nitrocellulose for each standard curve was determined by combining a protein assay with scanning densitometry of a Coomassie Blue–stained microgel lane of the enriched 53K fraction. Background cpm, subtracted from all experimental samples, were obtained by inclusion of control wells dotted with 0.5, 1.0, and 5.0 μg of ovalbumin on each blot. In addition, all samples were dotted at a minimum of three dilutions so that final values represent an average of at least three wells.

Affinity Column Chromatography

Affinity columns were made by coupling either purified soluble egg myosin or antibodies to Affigel 10 (Bio-Rad Laboratories) at 5 mg/ml in 0.1 M mor-

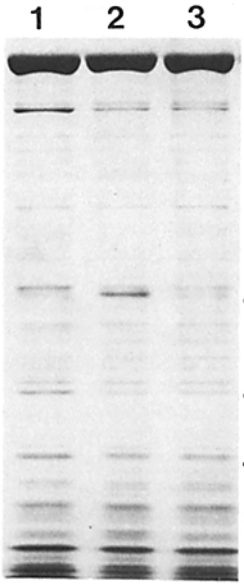

Figure 1. Effect of nucleoside triphosphates on precipitation of myosin from egg extract. Coomassie Blue–stained SDS-PAGE of protein precipitated from extract in the presence of 2 mM ATP (lane *1*), 2 mM ITP (lane *2*), and 2 mM γ-S-ATP (lane *3*). *M*, myosin heavy chain. The molecular mass standards (phosphorylase B [97 kD], BSA [66 kD], ovalbumin [45 kD], and carbonic anhydrase [29 kD]) are indicated on this and other figures.

pholinepropanesulfonic acid (MOPS), 0.35 M KCl, pH 7.0, or PBS, pH 7.5, respectively. Columns were washed extensively and equilibrated in appropriate loading buffer before use.

SDS-PAGE

Electrophoresis was performed according to Laemmli (1970) on either 0.75-mm thick 5–20% polyacrylamide gradient gels or 0.50-mm thick 12% microgels (Matsudaira and Burgess, 1978). Gels were stained with Coomassie Brilliant Blue R-250. Densitometry (E-C Apparatus Corp., St. Petersburg, FL) was performed on stained gels both across lanes equally loaded (micrograms of protein) and down lanes. In this manner an estimate was made of both the relative ratio of myosin (for example) contained in each lane with respect to total myosin and the ratio of myosin to total protein in each lane.

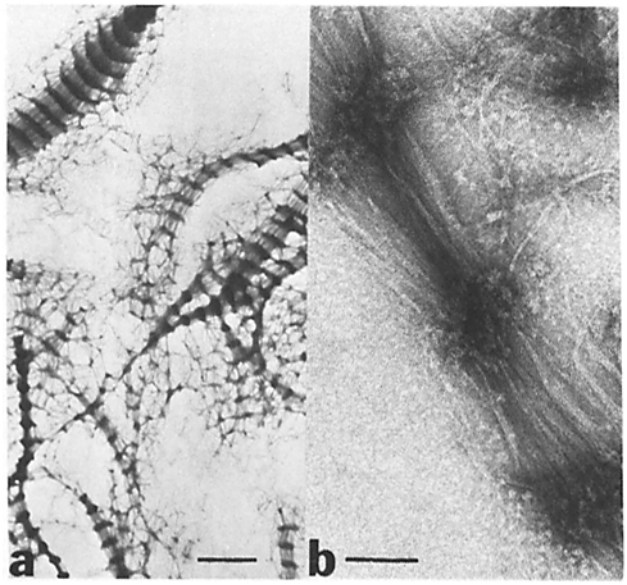

Figure 2. Negative stain EM of purified filamentous egg myosin. (*a*) Low magnification view of a field of myosin bipolar filaments demonstrating extensive arrays arranged in head-to-head pattern. (*b*) Higher magnification of egg myosin bipolar filaments showing more detailed filament structure. The bipolar filaments appear to be composed of a small number of individual myosin molecules. Bars: (*a*) 1.0 μm; (*b*) 0.1 μm.

Two-dimensional Gel Electrophoresis and Analysis of Myosin Light Chain Phosphorylation

Electrophoresis in two dimensions was performed according to O'Farrell (1975) with some modification. IEF was carried out essentially according to the method of Broschat et al. (1983). The second dimension was performed according to Laemmli (1970) on 15% SDS-PAGE. Purified chicken gizzard myosin was a gift of K.O. Broschat. Myosin was precipitated from extracts as described above using 2 mM ATP plus 100 μCi γ-^{32}P-ATP (1,000–3,000 Ci/mmol). The pelleted, washed precipitate protein (~80% myosin) was analyzed by two-dimensional gel electrophoresis. The stained gels were prepared for fluorography with DMSO and 2,5-diphenyloxazole or with Enlightening (New England Nuclear), dried, and then exposed at −70°C using Kodak X-OMAT AR film. Quantitation of light chain phosphorylation was performed on stained gels using densitometry as described by Broschat et al. (1983).

Negative Stain Electron Microscopy

An aliquot of purified extract myosin in extract dialysis buffer was reserved from the sample subjected to sedimentation velocity centrifugation. Samples were stained with 1% uranyl acetate on Formvar- and carbon-coated grids and viewed with a JEOL 100CXII at 80 kV.

Protein Determinations

The protein concentration of samples was assayed by the Bradford method (Bio-Rad Laboratories) using ovalbumin as the protein standard.

Results

Conditions of Myosin Precipitation

The major determinant of myosin precipitation from sea urchin egg extracts is added nucleotide. Precipitates form on the addition of ATP, ITP, or γ-S-ATP (Fig. 1) and are composed of essentially the same components: approximately 80% myosin and various other minor polypeptides as seen on SDS-PAGE analysis. To visualize the structure of the ATP-generated precipitates, negative-stain EM was carried out. Despite repeated attempts only large amorphous aggregates could be observed. No evidence of filamentous material within or extending from the aggregates was noted. In contrast, negative stain EM of purified myosin that had been dialyzed into extract dialysis buffer revealed bipolar filaments, often in head-to-head association, typical of myosin filaments purified from various nonmuscle sources (Fig. 2). In addition, extensive sheets of myosin bipolar filaments forming a regular three-dimensional array arranged in a head-to-head pattern were readily seen (Fig. 2). Therefore, it appears that the ATP-generated precipitate from extract, while containing myosin, was not organized into classical myosin bipolar filaments whereas purified myosin was fully competent to assemble.

Analysis of Precipitate Components

To investigate whether a component(s) of the precipitate might be involved in myosin's initial solubility in extracts, an ATP-generated precipitate was rapidly cycled twice through high and low salt buffers. SDS-PAGE analysis of the proteins that cycle with myosin indicated a large number of polypeptide bands with a broad range of molecular masses. However, one polypeptide band at an apparent molecular mass of 53 kD that co-pelleted with myosin through two polymerization cycles was enriched (data not shown).

Sedimentation velocity centrifugation of extract incubated

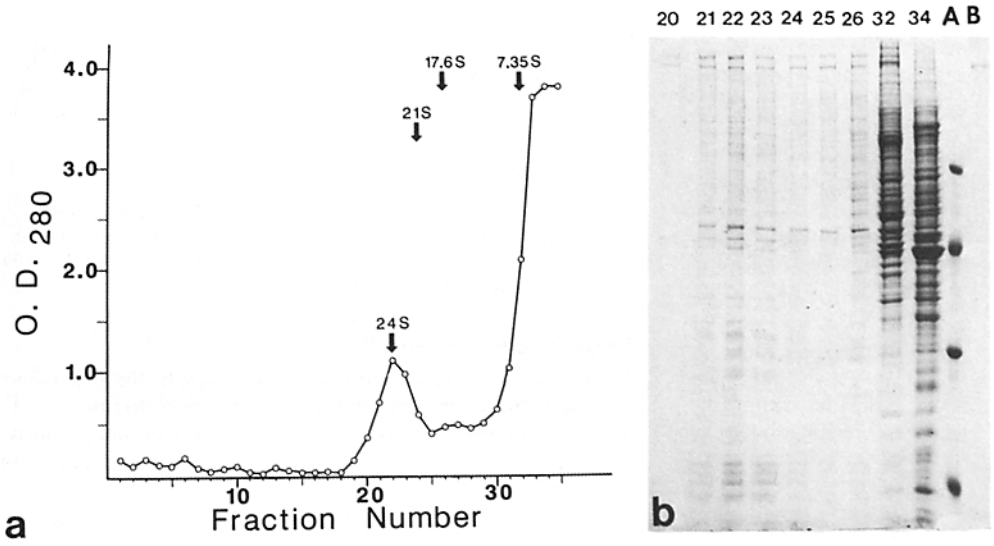

Figure 3. Sedimentation velocity centrifugation of egg extract in the presence of ATP. Extract was incubated in 2 mM ATP for 3 h before loading onto a 10–50% sucrose gradient (prepared in extract dialysis buffer) followed by centrifugation at 52,000 *g* for 12 h. (*a*) The figure shows OD_{280} versus fraction number with the top of the gradient at the extreme right. An arrow indicates the 24 S peak fraction. Arrows at the top of the figure indicate the sedimentation coefficients determined for skeletal muscle aldolase (*7.3 S*), splenic apoferritin (*17.6 S*), and purified filamentous egg myosin (*21 S*). (*b*) A Coomassie Blue–stained gel of gradient fractions indicated at the top of each lane. (Lane *A*) Molecular mass markers: BSA (66 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), and β-lactoglobulin (18 kD). (Lane *B*) Egg myosin standard.

with ATP at low ionic strength demonstrated that the ATP-generated precipitate had a sedimentation coefficient of 24 S (Fig. 3). Purified filamentous myosin run on a parallel gradient system sedimented at a value of 21 S. SDS-PAGE performed on individual 24 S peak fractions demonstrated an enrichment of myosin (myosin heavy chain at 200 kD), a 53-kD polypeptide, and three other peptides with apparent molecular masses of 220, 48, and 43 kD, along with other minor polypeptide bands (Fig. 3).

Efforts to purify the 53-kD polypeptide were undertaken to determine if this component interacted with myosin. When an ATP-generated precipitate (Fig. 4, lane *1*) was fractionated on Sephacryl S-1000, 53K and myosin eluted in consecutive but partially overlapping fractions. When the column peaks containing myosin and 53K were dialyzed against very low salt buffer in the absence of ATP, myosin assembled into bipolar filaments that were easily pelleted by centrifugation (Fig. 4, lane *2*) while 53K remained in the supernatant (Fig. 4, lane *3*). This difference in solubility allowed the isolation of a highly enriched 53-kD fraction used in the generation of antibodies and in further experiments. This dialysate supernatant was estimated to be enriched ~20-fold in 53K over the precipitate starting material and also contained minor polypeptides at ~130 and 37 kD as seen on SDS-PAGE (Fig. 4, lane *3*). Evidence that 53K exists as an oligomer in its native form was provided by gel electrophoresis under nonreducing conditions. 53K showed a dramatic retardation in its mobility; it migrated as a polypeptide of much higher molecular mass that barely entered the running gel (Fig. 4, lane *4*). No such high molecular mass band was seen in the enriched fraction of 53K run on SDS-PAGE under reducing conditions.

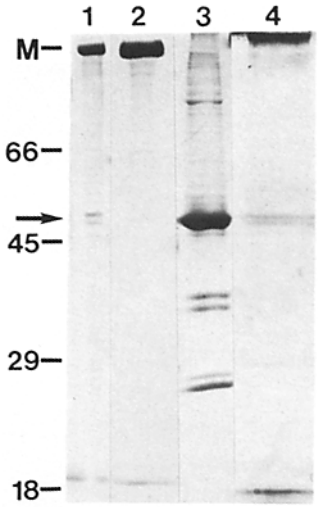

Figure 4. Fractionation of ATP-generated precipitate by gel filtration and differential solubility properties. SDS-PAGE of ATP-generated precipitate starting material (lane *1*), purified egg myosin from S-1000 column after fractionation and pelleting of bipolar filaments as shown in Fig. 2 (lane *2*), enriched 53K fraction (lane *3*), and enriched 53K fraction run under nonreducing conditions demonstrating retarded mobility on SDS-PAGE (lane *4*). The high molecular mass band indicated in lane *4* barely enters the running gel and is not present when 53K is run on SDS-PAGE under reducing conditions. *Arrow*, 53K. *M*, myosin.

Generation of Antibodies

Polyclonal antibodies against both myosin and 53K were generated in rabbits (Fig. 5). The myosin antibody recognized myosin heavy chain in whole extract and precipitates and demonstrated cross-reactivity against both heavy and light chains of purified myosin (Fig. 5 *a*). No cross-reactivity was detected against 53K or any other polypeptide. Antibodies generated to 53K were depleted of any potential cross-reactive myosin antibodies by repeated passage over a myosin affinity column. The efficacy of this procedure was tested

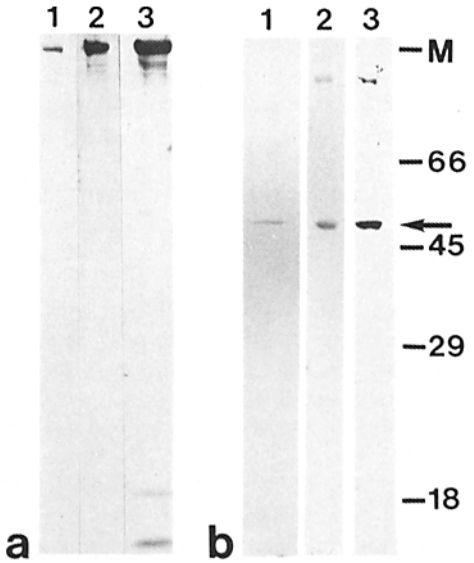

Figure 5. Characterization of antibodies generated to myosin and 53K and distribution of these antigens in extract and precipitate. (*a*) Immunoblots of egg extract (lane *1*), ATP-generated precipitate (lane 2), and purified myosin with antibody to myosin. (*b*) Immunoblots of egg extract (lane *1*), ATP-generated precipitate (lane 2), and the enriched 53K fraction (lane *3*) with antibody to 53K. *Arrow*, 53 kD. *M*, myosin.

by immunoblots of the eluent material against multiple extract fractions showing, in all cases, that antibody reactivity is primarily to a 53-kD polypeptide band with minor additional reactivity to a 130-kD band (Fig. 5 *b*). There was no antibody reactivity to myosin heavy chain or to myosin light chains. Immunoblots performed with anti-53K on egg extract, ATP-generated precipitate, and the 53K-enriched fraction (Fig. 5 *b*) all exhibited staining of a polypeptide band at 53 kD; the strongest reaction, as expected, with the latter sample. There is only light staining of 53K in egg extract indicating the small amount of 53K relative to other extract proteins.

Further Characterization of Extract

The yield of precipitate under varying conditions ranged from 0.30 to 8% of total extract protein (Table I). The antibodies against myosin and 53K were used to quantitate the amounts of each protein in extract and precipitate and to further analyze the precipitate. To make a depleted extract, ATP-generated precipitate was removed from extract by centrifugation and the remaining extract was dialyzed in extract buffer to remove excess nucleotides and chelators. Using this quantitation procedure the concentration of myosin in extract and in the remaining depleted extract in three different preparations averaged 8.2 and 1.9 μg/mg extract protein, respectively. The amount of myosin in different extract preparations varied significantly. Myosin remaining in depleted extracts was not precipitable by further addition of nucleotide. The concentration of 53K in extract and in depleted extract was 0.7 and 0.3 ug/mg, respectively. As with myosin, no further 53K could be precipitated by addition of more ATP. This method was also used to quantitate the amount of myosin and 53K precipitated by nucleotides and chelators (Table I). These results indicate that ATP and γ-S-ATP precipitate much more myosin than other nucleotides or any other factors. It further appears that approximately half the 53-kD protein contained in extract is precipitated by nucleotides, but that little is precipitated by chelators or by the inclusion of calcium with ATP.

Incubation of extracts with EDTA or EGTA in the absence of nucleotide precipitated many other extract proteins but myosin and 53K were specifically precipitated only in the presence of nucleotide. Varying the concentrations of KCl in the presence of ATP had little effect on myosin or 53K precipitation. While ATP with millimolar Ca^{++} yielded more precipitated protein, only a minor fraction of that precipitated was myosin and no 53K was detected in the precipitate. With $NaPP_i$, AMP, or ADP the yield of precipitate was 0.03–0.07% of total protein, of which essentially none was myosin or 53K. Although the intracellular pH rises from 6.8 to 7.2 after fertilization (Shen and Steinhardt, 1978), the yield of myosin precipitated with ATP was independent of pH between 6.8 and 7.5 (data not shown).

Table I. Myosin and 53K Distribution and Myosin LC_{19} Phosphorylation Levels in Precipitates

Nucleotide	Additional treatment	Precipitated protein	Precipitated myosin	Precipitated 53K	% myosin LC_{19} phosphorylation
		μg/mg extract protein	*μg/mg extract protein*	*μg/mg extract protein*	
ATP	–	4.3	3.8	0.4	36
γ-S-ATP	–	7.2	7.1	0.3	57
ITP	–	3.7	3.0	0.3	5
AMP-PNP	–	3.1	1.2	0.2	4
ADP	–	0.4	0.2	ND	–
$NaPP_i$	–	0.4	ND	ND	–
EGTA	–	16.6	0.7	ND	–
EDTA	–	6.9	1.1	ND	–
ATP	150 mM KCl	2.2	1.8	0.2	–
ATP	1 mM Ca^{2+}	79.9	1.0	ND	–

Determination of the total protein, myosin, and 53K induced to precipitate from extract in the presence of nucleotides, chelators, pyrophosphate, salt, and calcium. All values shown represent the average of at least three independent experiments. The precipitated protein from extract incubated for 3 h on ice under each condition was pelleted at 7,000 *g* for 20 min at 4°C, washed twice in 10 mM Pipes by resuspension, and the concentration of precipitated protein determined. Quantitation of myosin and 53K was determined by dot blotting and developing with antibodies to myosin and 53K. Myosin light chain phosphorylation was quantitated by scanning stained two-dimensional gels of precipitate. ND, not detectable under conditions of assay.

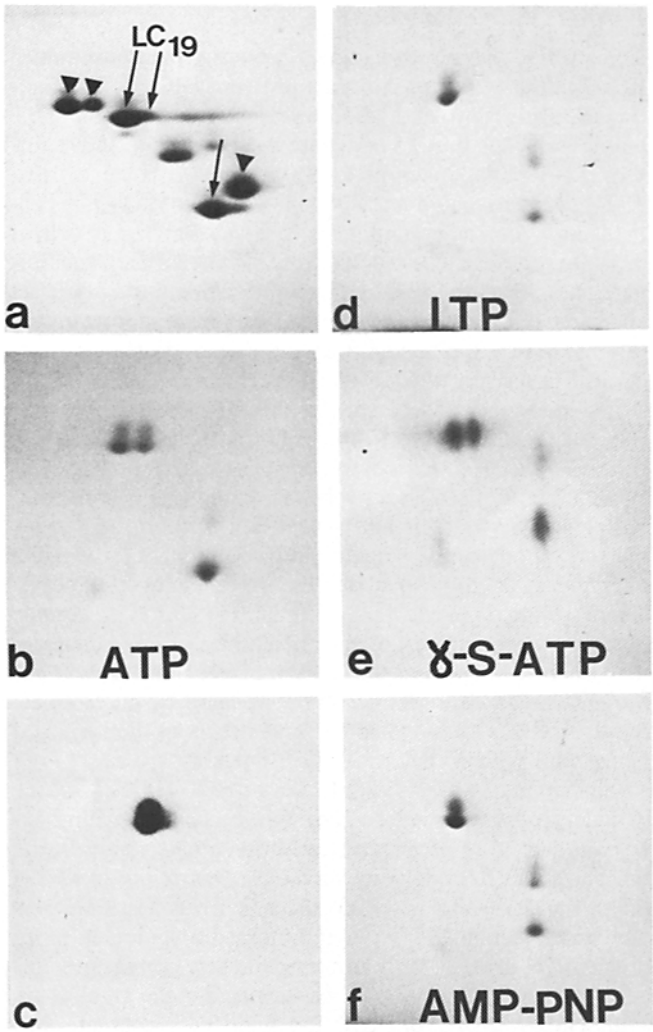

Figure 6. Two-dimensional gel electrophoresis of precipitate proteins. Only that portion of the gel containing myosin's light chains is shown. The more acidic portion of each gel is to the right. (*a*) Coelectrophoresis of gizzard myosin and egg myosin. The gizzard (*arrowheads*) myosin 20-kD light chain and the egg (*arrows*) myosin 19-kD light chain separate into two polypeptide spots. Gizzard myosin 16-kD light chain has a more acidic pI than the myosin 15-kD light chain from egg extract. (*b*) Precipitate generated by incubation of extract with 2 mM ATP and 100 μCi γ-^{32}P-ATP. There is clear separation of two myosin 19-kD light chain polypeptides. (*c*) Autoradiograph of gel in *b* showing one radioactive peptide corresponding to the more acidic myosin 19-kD light chain polypeptide. (*d*) Precipitate generated by incubation of extract with 2 mM ITP. (*e*) Precipitate generated by incubation of extract with 2 mM γ-S-ATP. (*f*) Precipitate generated by incubation of extract with 2 mM AMP-PNP. The 19-kD light chain polypeptide spots of myosin precipitated in the presence of ITP or AMP-PNP do not exhibit a shift to a more acidic pI, evidence of the lack of light chain phosphorylation in these samples.

The fact that ATP, ITP, or γ-S-ATP but not $NaPP_i$, ADP, or AMP caused precipitation of myosin suggested that the change in solubility of myosin might be due to phosphorylation of the myosin 19-kD light chain. Precipitates generated by these nucleotides were analyzed by two-dimensional gel electrophoresis to determine the state of myosin light chain phosphorylation. Precipitate formed with γ-^{32}P-ATP resulted in only one phosphorylated polypeptide corresponding to the more acidic polypeptide of the 19-kD myosin light chain as detected by two-dimensional gels (Fig. 6). However, analysis of myosin precipitated by various nucleotides showed that precipitation is independent of light chain phosphorylation (Fig. 6 and Table I).

An effort was made to determine the association state of myosin and 53K in isolated precipitate and in ATP-treated extract (Fig. 7). Analysis of fractions of ATP-treated extract from sedimentation velocity centrifugation revealed two peaks of both myosin and 53K (Fig. 7 *a*). Both myosin and 53K were found in significant quantity in the peak detected at 24 S by OD_{280} as shown in Fig. 3. In addition, a second prominent peak of myosin in ATP-treated extract was detected at an S value of 10. As with myosin, a second peak of 53K was detected which had an S value of 7. A separately run fraction highly enriched in 53K and lacking myosin exhibited an S value of 7 by sucrose gradient sedimentation (data not shown). Analyzing the eluant fractions of ATP-generated precipitate from gel filtration chromatography by immunoblotting showed a broad myosin peak; a major portion of the myosin peak lacking 53K eluted before a shoulder which also contained the major fraction of 53K (Fig. 7 *b*). A minor amount of the 130-kD polypeptide eluted with the tail end of the 53-kD peak in fractions lacking myosin.

Further evidence that 53K and myosin are associated in extracts comes from immunoprecipitation and affinity chromatography. Immunoprecipitation using antibody to 53K precipitated myosin from extracts (Fig. 8). In addition, anti-53K also precipitated myosin from the 24 S peak fraction resolved upon sedimentation velocity centrifugation (Fig. 8). Immunoprecipitation of extract or the 24 S peak with preimmune serum showed no myosin co-precipitation. Unfortunately, the antibody heavy chain bands on SDS-PAGE would obscure a 53-kD band so that the complementary immunoprecipitation experiment with anti-myosin was not performed since interpretable results could not be expected.

Extract passed over a myosin affinity column resulted in binding of 53K which was highly enriched among the polypeptides released in the presence of high salt (Fig. 9). Sequential elution with ATP, 0.15 M KCl, then 2 M KCl resulted in elution of various polypeptides with a broad range of molecular masses. 53K was eluted with high specificity only in the presence of 2 M KCl. Dot blotting with anti-53K to quantitate release showed that ~60% of total extract 53K loaded onto the column was eluted by the 2 M KCl wash. We were unable to determine the disposition of the balance of 53K due to its dilution in the eluant fractions. Finally, ATP and high salt together did not result in further protein elution.

Role of Precipitate or Extract Components

To separate the solubility properties of myosin in the ATP precipitate from those of pure myosin, myosin was column purified and reconstitution experiments were performed (Table II). Myosin redissolved in a small volume of high salt buffer was added to either depleted extract (described above) or to dialysis buffer. In both cases, myosin precipitated in a nucleotide-independent manner. In dialysis buffer 99% of the myosin precipitated after a short incubation on ice without the addition of nucleoside triphosphate. However, there

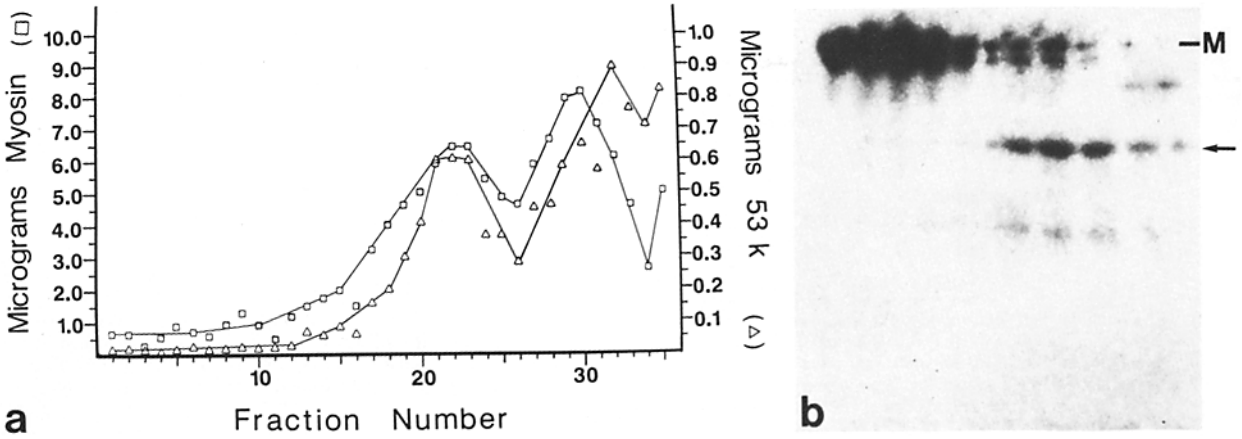

Figure 7. (*a*) Aliquots of each fraction from a sedimentation velocity centrifugation run of ATP-treated extract analyzed for quantity of myosin and 53K by dot blots using antibodies against 53K and myosin. Myosin was detected in two peaks having S values of 24S and 10S whereas 53K was also found in the 24S peak but was also present as a 7S peak. The fractions analyzed are aliquots of the same fractions shown in Fig. 3. (*b*) Immunoblot of Sephacryl S1000 sequential fractions with antibodies against myosin and 53K with the earlier eluting fractions on the left showing myosin and elution of 53K as a tail on the major portion of the myosin peak. *Arrow*, 53 kD; *M*, myosin.

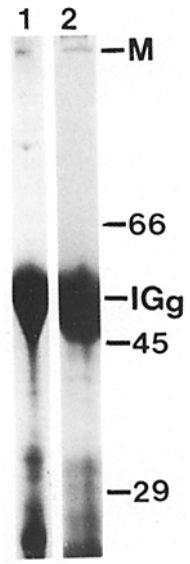

Figure 8. SDS-PAGE showing immunoprecipitates of egg extract (lane *1*) and 24S peak fraction No. 22 (lane *2*) from sucrose density gradient (see Fig. 6) with anti-53K demonstrating precipitation of myosin heavy chain (200 kD). Controls performed with preimmune serum did not demonstrate immunoprecipitation of any polypeptide bands. *M*, myosin heavy chain.

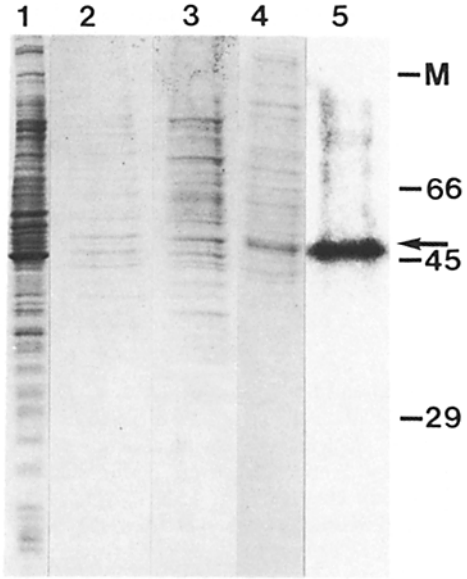

Figure 9. Myosin affinity column characterization of extract components. Purified egg myosin coupled to Affigel 10 was washed extensively and equilibrated in extract dialysis buffer. Repeated passage of an aliquot of egg extract (lane *1*) over the column was followed by washing with 200 column volumes (100 ml) of buffer. SDS-PAGE of proteins eluted with 20 column volumes of buffer supplemented with 5 mM ATP (lane *2*), 0.15 M KCl (lane *3*), and 2.0 M KCl (lane *4*). Lane *5* shows an immunoblot with anti-53K of the 2.0 M KCl eluant confirming that the 53-kD polypeptide detected by Coomassie staining is 53K. *Arrow*, 53K.

was slight restoration of myosin solubility in depleted extract as 81% of the pure myosin precipitated independently of nucleotide. The state of myosin light chain phosphorylation did not play an important role in restoring solubility in reconstitution experiments since myosin purified from extract with either ATP- or ITP-generated precipitates demonstrated identical solubility properties when added to either depleted extract or dialysis buffer (data not shown).

The reconstitution experiments indicate that, on one hand, purified egg myosin exhibits solubility properties at low ionic strength comparable to other purified nonmuscle myosins. On the other hand, a component(s) present in both the myosin-depleted extract and the crude precipitate is necessary for myosin's nucleotide-dependent precipitation and low ionic strength solubility, respectively.

Perturbation of Myosin and 53K Interaction

We investigated whether perturbing the apparent interaction between myosin and 53K would affect the solubility properties of myosin in extracts. Extract was repeatedly passed over an anti-53K column and the flow through designated as 53K-depleted extract (Fig. 10 *a*). Immunoblots with the same dilution of anti-53K which yielded a positive reaction against

Table II. The Effect of 53K on Myosin Solubility

Initial protein concentrations			Myosin				53K			
Myosin	53K	Incubation conditions	Pelleted		Soluble		Pelleted		Soluble	
μg/ml	μg/ml		μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%
77	–	Dialysis buffer	76	99	0.1	0	–	–	–	–
77	–	Depleted extract	62	81	12	16	–	–	–	–
84	6	Dialysis buffer	34	40	48	57	1	17	4	67
84	4	Depleted extract	57	68	21	25	2	50	1	25

Purified myosin, with or without 53K, in a volume of 0.5 ml of dialysis buffer or depleted extract made 0.5 M KCl was rapidly dialyzed against sequential changes of 250 ml of dialysis buffer containing 0.4, 0.3, 0.2, 0.1, and 0.05 M KCl, respectively, and then against extract dialysis buffer. Samples were incubated on ice for 1 h, protein was pelleted by centrifugation at 10,000 *g* for 15 min, and pellets were solubilized in 0.5 M KCl, 10 mM imidazole, 1 mM EDTA, pH 7.2, before protein determination. Dot blots of pelleted myosin and 53K and that remaining in the supernatant were quantitated with antibodies to myosin and 53K. The values represent the average of at least two determinations from separate preparations.

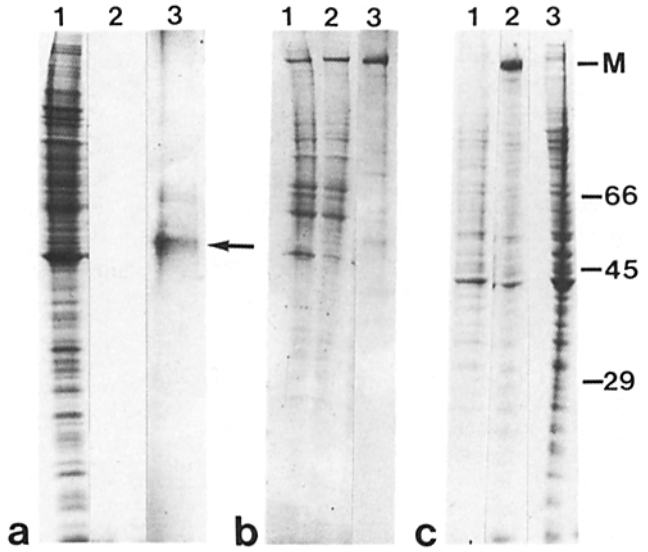

Figure 10. Myosin precipitation from extracts depleted of 53K. (*a*) Lane *1*, Coomassie Blue–stained gel lane of 53K-depleted extract. Lane *2*, immunoblot of 53K-depleted extract with antibody to 53K revealing no cross-reactivity. Immunoblots were performed with the same dilution of antibody used in Fig. 5. Lane *3*, SDS-PAGE of protein eluted from anti-53K affinity column as detected by silver staining. Note the highly enriched elution of only one polypeptide with an apparent molecular mass of 53 kD. (*b*) SDS-PAGE of proteins sequentially pelleted from extract passed over an anti-53K or (*c*) a denatured anti-53K affinity column at 7,000 *g* after a 1-h incubation at 0°C with no further treatment (lanes *1*), at 7,000 *g* after an additional 1-h incubation in the presence of 2 mM ATP (lanes *2*), and at 7,000 *g* after overnight incubation with no further treatment (*b*, lane *3*); (*c*, lane *3*), final supernatant. The percent of myosin pelleted at each step was quantitated by densitometry of myosin heavy chain in stained gels which were loaded with equivalent amounts of pelleted protein. *Arrow*, 53K; *M*, myosin.

egg extract showed no reactivity to the 53K-depleted extract (Fig. 10 *a*). Elution of the anti-53K antibody column yielded only the 53-kD polypeptide but not myosin nor a 130-kD polypeptide (Fig. 10 *a*).

Depletion of 53K from egg extracts radically changed the solubility properties of myosin. Earlier experiments demonstrated that myosin and 53K only precipitate from extract in the presence of nucleoside triphosphates. In fact, 95% of precipitable myosin precipitated within 1 h after ATP addition in normal extracts. In contrast, depletion of 53K from extract resulted in 20% of the myosin precipitating in the absence of ATP within 1 h of 53K depletion (Fig. 10 *b*). After adding ATP, an additional 26% of the myosin precipitated within 1 h and the remaining 46% required an additional 18 h before precipitating. Control experiments were carried out by passing extract over a denatured anti-53K column and proceeding as above (Fig. 10 *c*). In contrast to the experimental results, more than 90% of the myosin precipitated 1 h after the addition of ATP to the control but no myosin precipitated before ATP addition. Finally, the absence of further precipitation of myosin in the control after overnight incubation suggested that a population of myosin molecules demonstrating very slow ATP-dependent precipitation does not exist.

Finally, we determined whether in vitro reconstitution of low ionic strength solubility was feasible by recombining purified myosin with 53K into either depleted extract or dialysis buffer. Whereas 84% of purified myosin precipitates out of depleted extract and more than 99% precipitates out of buffer without the addition of ATP, recombining myosin and 53K into myosin-depleted extract or extract dialysis buffer restored a significant level of solubility to myosin (Table II). In the absence of nucleotide, 25% of the myosin combined with 53K before addition to depleted extract remained soluble versus 16% of myosin added to depleted extract in the absence of exogenous 53K. More dramatically, whereas no purified myosin remains soluble when added to low ionic strength buffer, 57% of myosin remained soluble when first combined with 53K before addition to buffer. Therefore, it is possible to restore limited solubility properties to myosin when purified myosin and an enriched 53K fraction are recombined.

Discussion

The results presented in this study identify a protein in unfertilized sea urchin egg extracts that appears to mediate the low ionic strength solubility of myosin. The protein, which exhibits a subunit molecular mass of 53 kD on SDS-PAGE, is 7 S in native form, and is associated with myosin in nucleotide-induced precipitates. The association of myosin with 53K is demonstrated by their co-sedimentation in sedimentation velocity centrifugation, their co-immunoprecipitation

with an antibody against 53K, their co-precipitation when nucleoside triphosphates are added to extract, and by the finding that 53K binds to a myosin affinity column. The essential role 53K plays in myosin solubility was demonstrated by the behavior of myosin when extract is depleted of 53K; in this case myosin precipitates in a nucleotide-independent manner, exhibiting solubility characteristics typical of purified myosin. Finally, the unusual low ionic strength solubility of myosin seen in cell extracts can be partially restored in vitro in myosin-depleted extract or in low ionic strength buffer by recombining 53K with myosin. To our knowledge, this is the first report of a myosin-binding protein which affects myosin's solubility properties.

The mechanism responsible for the precipitation of myosin from extracts after the addition of nucleoside triphosphates is unknown. However, in the course of this study, much evidence has been accumulated as to what factors are not responsible for myosin precipitation. The 19-kD myosin light chain can be phosphorylated in extracts if ATP or γ-S-ATP is used as a substrate. In the presence of ITP or AMP-PNP, myosin still precipitates but there is no 19-kD light chain phosphorylation. Therefore, light chain phosphorylation is not responsible for myosin precipitation; a finding seemingly contradictory with work showing that thymus myosin light chain phosphorylation enhances bipolar filament formation and precipitation (Craig et al., 1983). It is therefore unlikely that the 53K protein regulates myosin precipitation through a role as a myosin light chain kinase.

It is conceivable that the 53K protein is a myosin heavy chain kinase. Myosin heavy chain kinase II from *Dictyostelium* is inhibited by Ca^{2+}-CaM (Maruta et al., 1983) and its counterparts from *Acanthamoeba* and *Physarum* are insensitive to Ca^{2+} (Collins and Korn, 1980; Ogihara et al., 1983). We note that Ca^{2+} inhibits myosin precipitation from extracts. Other studies on cell extract gelation and contraction have noted that Ca^{2+} interferes with gelation, often causing extensive nonspecific protein precipitation (Stossel and Hartwig, 1976; Condeelis and Taylor, 1977). Heavy chain phosphorylation has been shown to inhibit bipolar filament formation and actin-activated ATPase activity of *Acanthamoeba* myosin II and *Dictyostelium* myosin (Collins and Korn, 1980; Collins et al., 1982; Kuczmarski and Spudich, 1980), consistent with our observation that the myosin that precipitates out of extract does not appear to be in the form of bipolar filaments. Preliminary evidence suggests that egg myosin heavy chain phosphorylation can occur under conditions of myosin precipitation but we do not know if 53K has an accessory role. Furthermore, there are reports that heavy chain kinases can be associated with myosin molecules, remaining enzymatically active even after the myosin is immunoprecipitated with a myosin-specific antibody (Berlot et al., 1985).

While the binding of 53K to myosin appears to be dependent upon nucleoside triphosphates, the association is also sensitive to ionic strength. High salt has been demonstrated to change the conformation of myosin's tail (Trybus and Lowey, 1984). It is through the polymerization of the tail that myosin forms bipolar filaments, a fact that the spontaneous polymerization of light meromyosin in low salt buffer underscores (Huxley, 1963). After incubation in high salt buffer for an extended period, it is possible that only minimal reformation of the 53K binding site occurs on myosin upon rapid dialysis to low salt. Energetically, the formation of bipolar filaments may be favored so strongly as to prevent 53K from gaining access to a binding site on the tail of myosin. The limited restoration of myosin solubility when 53K and myosin are recombined may also be due to the greater affinity of myosin tails for each other than for another protein. The implication that 53K binds to the tail of myosin also seems plausible given the effect of 53K on myosin solubility and that myosin's tail is the least soluble portion of the molecule. If 53K is a heavy chain kinase, as suggested above, it would be expected to bind to the tail as phosphorylation sites for known heavy chain kinases are at the tip of the tail of myosin (Côté et al., 1981; Trotter et al., 1985). Moreover, although it cannot be ruled out, it seems likely that if 53K binds to the head of the myosin molecule it would not lose its binding capacity when myosin undergoes conformational changes that primarily involve the tail. Investigation of the binding site on myosin for 53K is presently being pursued.

Negative stain electron microscopy shows that purified myosin forms bipolar filaments under extract buffer conditions. These bipolar filaments have a sedimentation coefficient of 21 S, close to the 22 S value for skeletal muscle myosin minifilaments estimated to be composed of eight myosin molecules (Trybus and Lowey, 1985; Reisler et al., 1986). In contrast, repeated examination of ATP-generated precipitates, which has an S value of 24, by negative stain electron microscopy by our lab and others (Kane, R., personal communication) has not revealed bipolar filaments. Therefore, the assembly state of myosin in precipitates associated with 53K is unknown. However, the 24 S form detected for myosin in ATP-treated extracts likely represents myosin coupled with 53K. The fact that myosin is found in two peaks of 10 S and 24 S by sedimentation velocity of ATP-treated extract also suggests that some of the myosin is in the form of 10 S kinked tail monomers. The presence of a major fraction of 53K not associated with myosin and with a 7 S value by sedimentation velocity indicates that this represents the native species of the protein.

Nonmuscle cell myosin-binding proteins that regulate the supramolecular organization of myosin in the cytoplasm can reasonably be expected to exist since myosin undergoes changes in distribution and polymerization in cells. Certainly myosin light chain and heavy chain kinases are part of a myosin regulatory network but it is unclear if the transition in vivo between soluble myosin and bipolar filaments is solely controlled by myosin's phosphorylation state. To date, myosin-binding proteins have been found exclusively in skeletal and cardiac muscle systems. Titin, nebulin, myomesin, the 86-kD protein, and C protein have all been localized to the thick filaments of muscle sarcomeres (Maruyama et al., 1984; Eppenberger et al., 1981; Bahler et al., 1985; Reinach et al., 1982; Jeacocke and England, 1980; Wang, 1985; Horowits et al., 1986). Although assignments of functional roles to these proteins are still tentative, the confinement of each protein to a small morphologically distinct region of the sarcomere suggests that these proteins interact with myosin thick filaments in a regulated and specific manner. Furthermore, myomesin, the 86-kD protein, and C-protein are expressed coincidentally in postmitotic myoblasts, suggesting participation in myosin thick filament organization and alignment (Bahler et al., 1985). The 53 kD protein in egg extract appears to be an authentic myosin-binding pro-

tein. It differs from the skeletal muscle thick filament-binding proteins in that its subunit molecular weight is smaller and, under the conditions in our experiments, it does not appear to bind to myosin bipolar filaments. The 53-kD protein, with a 7 S sedimentation coefficient and apparently made up of disulfide-linked subunits, is unlike titin which has a very high subunit molecular mass. Too little is known about either the skeletal muscle thick filament-binding proteins or 53K to speculate on their relationship.

It is not known if 53K is related to any of the previously described myosin kinases although its capacity to bind to extract myosin in the presence of ATP and to change myosin's solubility properties in extracts could be thought of as properties of a myosin kinase. Alternatively, 53K may be the first in a new class of myosin-binding proteins. Comparable to actin-binding proteins which regulate the length of actin filaments by Ca^{2+}-dependent severing, capping, or binding to monomeric actin, 53K may interact with monomeric or oligomeric forms of myosin in order to regulate the distribution of myosin in nonmuscle cells. Further investigation of 53K should provide more information on its biochemical characteristics, interaction with myosin, and possible role in vivo.

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