Isolation and Partial Characterization of a 110-kD Dimer Actin-binding Protein

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Abstract. Two Triton-insoluble fractions were isolated from *Acanthamoeba castellanii*. The major non-membrane proteins in both fractions were actin (30–40%), myosin II (4–9%), myosin I (1–5%), and a 55-kD polypeptide (10%). The 55-kD polypeptide did not react with antibodies against tubulins from turkey brain, *paramecium*, or yeast. All of these proteins were much more concentrated in the Triton-insoluble fractions than in the whole homogenate or soluble supernatant.

The 55-kD polypeptide was extracted with 0.3 M NaCl, fractionated by ammonium sulfate, and purified to near homogeneity by DEAE-cellulose and hydroxyapatite chromatography. The purified protein had a molecular mass of 110 kD and appeared to be a homodimer by isoelectric focusing. The 110-kD dimer bound to F-actin with a maximal binding stoichiometry of 0.5 mol/mol of actin (1 mol of 55-kD subunit/mol of actin). Although the 110-kD protein enhanced the sedimentation of F-actin, it did not affect the low shear viscosity of F-actin solutions nor was bundling of F-actin observed by electron microscopy. The 110-kD dimer protein inhibited the actin-activated Mg^{2+}-ATPase activities of *Acanthamoeba* myosin I and myosin II in a concentration-dependent manner. By indirect immunofluorescence, the 110-kD protein was found to be localized in the peripheral cytoplasm near the plasma membrane which is also enriched in F-actin filaments and myosin I.

ACTIN, myosin, and many other proteins that interact with actin or myosin, and hence affect the organized structure and functions of the cytoskeleton, have been purified and characterized from various non-muscle cells and tissues (18, 27, 28, 51, 56). *Acanthamoeba castellanii* is one of the most fully investigated non-muscle cells. In addition to actin (22), it contains two types of myosin isoenzymes. *Acanthamoeba* myosin IA and myosin IB are single-headed, low molecular weight myosins comprised of a single heavy chain (125,000 and 130,000 D, respectively) and a single light chain (17,000 and 27,000 D, respectively) (37, 40, 46). *Acanthamoeba* myosin II is a more conventional myosin comprised of a pair of 185,000-D heavy chains and one pair of 17,500-D and one pair of 17,000-D light chains (11, 12, 16, 39). Immunocytochemical analysis using antibodies specific for the heavy chains of myosin I and myosin II suggested that myosin I is localized close to the plasma membrane while myosin II may be distributed more uniformly in the cytoplasm of the amoeba (21). Many actin-binding proteins in addition to myosin, including several monomer-binding proteins (15, 25, 48), several capping proteins (14, 24), and cross-linking proteins (8, 43, 44), that are thought to regulate the dynamic assembly and disassembly of actin filaments, have also been isolated and characterized from *Acanthamoeba*.

For a complete understanding of the motile functions of the ameba cells, it will be necessary to elucidate the organization of and interaction between all of these proteins in the highly complex cytoskeleton of living cells. As one approach,[1] we undertook to characterize Triton-insoluble fractions isolated from *Acanthamoeba* by differential centrifugation. While it is difficult to verify that such complexes represent real associations in vivo, at a minimum, their isolation and characterization allows the identification of additional potential components of the *Acanthamoeba* cytoskeleton. We describe in this paper a 110-kD homodimer that is a major component of the Triton-insoluble fractions.

Materials and Methods

Proteins

Acanthamoeba myosin I was purified by Drs. Joseph P. Albanesi and Hisao Fujisaki (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute) as described by Maruta et al. (37). *Acanthamoeba* myosin II was purified by Drs. Graham P. Côté and Mark A. L. Atkinson (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute) as described by Collins and Korn (12). Rabbit skeletal muscle F-actin, purified according

1. Preliminary reports of this work were presented at the 24th Annual Meeting of the American Society for Cell Biology (54) and the 29th Annual Meeting of the American Biophysical Society (55).

to Eisenberg and Kielley (19), and plasma gelsolin (17) were gifts of Dr. Lois Greene and Dr. Martine Coüe (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute), respectively. Antisera against *Acanthamoeba* myosin IB and myosin II were prepared according to the method of Gadasi and Korn (21) and supplied by Dr. John Hammer (Laboratory of Cell Biology, National Heart, Lung, and Blood Institute). Antibodies against tubulins from turkey brain and *paramecium* were gifts of Dr. Dominique Pantaloni (Laboratoire d'Enzymologie, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). The monoclonal antibody against yeast tubulin (YOL1/34) described by Kilmartin et al. (26) was a gift of Dr. Eugene R. Katz (State University of New York, Stony Brook, NY).

Reagents

Imidazole, ATP, pepstatin, phenylmethylsulfonyl fluoride, EGTA, and EDTA were obtained from Sigma Chemical Co., St. Louis, MO. Leupeptin and affinity-purified goat anti-rabbit immunoglobulin (horseradish peroxidase-conjugated or fluorescein isothiocyanate-labeled) were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. DE-52 cellulose and hydroxyapatite were from Whatman, Inc., Clifton, NJ, and Bio-Rad Laboratories, Richmond, CA, respectively. Tris and enzyme grade ammonium sulfate were from Schwarz/Mann, Cambridge, MA. [γ-^{32}P]ATP was from New England Nuclear, Boston, MA. Triton X-100 was purchased from Research Products International Corp., Mt. Prospect, IL. All other reagents were of reagent grade and deionized water was used throughout.

Chemical Analyses and Enzymatic Assays

K^+-EDTA and Ca^{2+}-ATPase activities were determined at 30°C as described by Pollard and Korn (46). NH^+_4-EDTA-ATPase activity was measured in a solution containing 2 mM ATP, 0.4 M NH_4Cl, 35 mM EDTA, 25 mM Tris, pH 7.5 (9). Actin-activated Mg^{2+}-ATPase activities of myosin IB and myosin II were assayed at 30°C in a medium containing either 10 mM imidazole, pH 7.5, 2.9 mM $MgCl_2$, 15 mM KCl, 1 mM EGTA, 2 mM ATP (for myosin IB), or 10 mM imidazole, pH 7.0, 6 mM $MgCl_2$, 1 mM ATP (for myosin II). In all these assays, radioactivity released as P_i from [γ-^{32}P]ATP was measured (46). All assays were linear with time and protein concentration. Binding of myosin IB to actin was measured at 25°C as described by Albanesi et al. (2). Unless otherwise stated, protein was determined by the method of Lowry et al. (34) using bovine serum albumin (Armour Pharmaceutical Co., Tarrytown, NY; fraction V) as a standard.

Lipids were extracted by suspending samples in equal volumes of 5 mM imidazole, pH 7.5, 0.15 M NaCl, and extracting twice with chloroform-methanol according to the method of Bligh and Dyer (4). The combined lipid extracts were dried under reduced pressure or by incubation at 65°C for several hours, and then analyzed for phosphorus by the method of Bartlett (3).

SDS PAGE was performed according to Laemmli (31). Proteins were stained with Coomassie Brilliant Blue according to Fairbanks et al. (20). For quantitative densitometry, gels were scanned with the gel scanner attachment of the Beckman DU-8 spectrophotometer at 2 cm/min using a slit width of 0.05 mm at 595 nm. Immunoblot analysis was carried out by the method of Towbin et al. (53) except that 4-chloro-1-naphthol was used as substrate for the horseradish peroxidase conjugate of goat anti-rabbit IgG as described by Hawkes et al. (23).

Sedimentation equilibrium studies and sedimentation velocity experiments were carried out at 5°C in the Beckman Instruments model E analytical ultracentrifuge equipped with a photoelectric scanner. The partial specific volume of the 110-kD dimer protein (0.723 cc/g) was calculated from the amino acid composition by the method of Cohn and Edsall (10). For determination of its amino acid composition, the 110-kD dimer protein was dialyzed against distilled water, hydrolyzed in 6 N HCl at 110°C for 20 h, and analyzed using a Beckman 6300 analyzer.

Isolation of Triton-insoluble Fractions

Acanthamoeba castellanii (Neff Strain) was grown at 29°C in 1-liter flasks as described by Pollard and Korn (46). For large scale preparations, carboys containing 15 liters of medium were inoculated with 1 liter of culture containing about 4×10^6 cells/ml and cells were grown for 3 d at 29°C. Usually, 4 liters of cell suspension at late logarithmic phase (1-2 $\times$ 10^6 cells/ml) were harvested by centrifugation at 2,500 rpm and washed with ice-cold 5 mM imidazole, pH 7.0, 150 mM NaCl. All procedures to prepare the Triton-insoluble fractions were performed at 4°C. The washed cell suspension (50 ml) was diluted in 500 ml of the extraction buffer containing 25 mM imidazole, pH 7.5, 100 mM KCl, 2 mM EGTA, 5 mM dithiothreitol (DTT),[2] 0.5% Triton X-100, 10 μg/ml pepstatin, 1 μg/ml leupeptin, and homogenized with a Dounce homogenizer (five strokes). Immediately after homogenization, phenylmethylsulfonyl fluoride was added to 0.6 mM. Two Triton-insoluble fractions (light and heavy) were separated as follows. The homogenate was centrifuged at 2,500 *g* for 15 min in Sorvall GSA rotor. The pellets were composed of two layers. The smooth white upper layer was suspended in 50 ml of the extraction buffer and centrifuged in a Sorvall SS-34 rotor at 2,500 *g* for 20 min. Again, the upper layer was suspended in 10 ml of 10 mM imidazole, pH 7.0, 0.3 M sucrose (heavy fraction). The supernatant obtained after the first centrifugation was centrifuged in a Sorvall GSA rotor at 23,000 *g* for 20 min. The pellets were suspended in 50 ml of extraction buffer and centrifuged at 23,000 *g* for 20 min. The pellets were suspended in 10 mM imidazole-Cl, pH 7.0, 0.3 M sucrose (light fraction).

Purification of the 110-kD Dimer Actin-binding Protein from the Triton-insoluble Fractions

All purification procedures were performed at 4°C. Light and heavy Triton-insoluble fractions (~1,500 mg of protein) isolated from cells harvested from two carboys were suspended in an equal volume of a buffer containing 20 mM imidazole, pH 7.0, 0.6 M NaCl, and 10 mM DTT, and homogenized with a Dounce homogenizer (three strokes). The homogenate (300 ml) was then centrifuged in a Beckman 30 rotor at 100,000 *g* for 1.5 h. The supernatant (NaCl extract) was carefully removed and filtered through four layers of gauze to remove the floating lipid. Solid ammonium sulfate was added to the NaCl extract to 40% saturation at 4°C. After standing for 15 min, the precipitate was pelleted at 27,000 *g* for 10 min. Ammonium sulfate was added to the supernatant to 60% saturation. The precipitate obtained after centrifugation at 27,000 *g* for 20 min was dissolved in 5 mM Tris, pH 7.5, 0.2 mM DTT, and dialyzed against 4 liters of 5 mM Tris, pH 7.5, and 0.2 mM DTT for 4 h with a change of the buffer. The precipitate that formed during dialysis was removed by centrifugation at 27,000 *g* for 10 min. The supernatant (270 mg of protein) was diluted threefold with 5 mM Tris, pH 7.5, and 0.2 mM DTT, and applied to a DEAE-cellulose column (250 ml) equilibrated with buffer containing 10 mM Tris, pH 7.5, 10 mM NaCl, and 0.2 mM DTT. The column was washed with equilibration buffer until the absorbance at 280 nm became constant and then eluted with 1,600 ml of a linear 10 mM to 300 mM NaCl gradient in 10 mM Tris, pH 7.5, and 0.2 mM DTT. Each fraction was analyzed by SDS PAGE and the major protein peak which was eluted at 60 mM NaCl was pooled. The pooled fractions (110 ml) were loaded on an hydroxyapatite column (25 ml) equilibrated with 10 mM Tris, pH 7.5, 10 mM NaCl, and 0.2 mM DTT. The column was washed with 30 ml of 10 mM Tris, pH 7.5, 0.3 M NaCl, and 0.2 mM DTT, and then eluted with a linear gradient (150 ml) of K_2HPO_4, between 0 and 50 mM in a buffer containing 10 mM Tris, pH 7.5, 0.3 M NaCl, and 0.2 mM DTT. The 110-kD dimer actin-binding protein was eluted as a major protein peak at 25 mM K_2HPO_4. The peak fractions were pooled and dialyzed against 2 liters of 10 mM Tris, pH 7.5, 50 mM KCl, 0.2 mM DTT, and 50% glycerol for 16 h. The purified protein was stored at −20°C.

Antibody against the 100-kD Dimer Actin-binding Protein

The purified 110-kD dimer protein (300 μg) was dissolved in 1 ml of 10 mM Tris, pH 7.5, and 50 mM KCl, and emulsified with an equal volume of complete Freund's adjuvant (Gibco, Grand Island, NY). 1 ml of the mixture was injected into the hind leg muscle of two New Zealand white male rabbits. 3 wk later, the rabbits were boosted with 1.5 ml of an emulsion containing equal volumes of 200 μg of 110-kD dimer in 10 mM Tris, pH 7.5, 100 mM NaCl, and incomplete Freund's adjuvant. The increase in antibody titer was confirmed using an enzyme-linked immunosorbent assay with horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals). The substrate solution (200 μl) contained 25 mM citric acid, 50 mM Na_2HPO_4, 0.5 mg/ml of *o*-phenylene diamine, and 0.03% H_2O_2. The reaction was stopped by adding 80 μl of 5 M H_2SO_4 and the absorbance at 492 nm was measured. The rabbits were bled on the seventh day after the boost. The polyclonal antiserum was affinity purified by adsorption to the 110-kD dimer protein electrophoretically transferred to nitrocellulose paper from a 10% SDS polyacrylamide gel according to the method described by Lehto and Virtanen (33). The purified antibody (15 μg of protein/ml) was stored at 4°C in 50 mM Tris, pH 7.5, 0.15 M NaCl, and 1% bovine serum albumin.

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

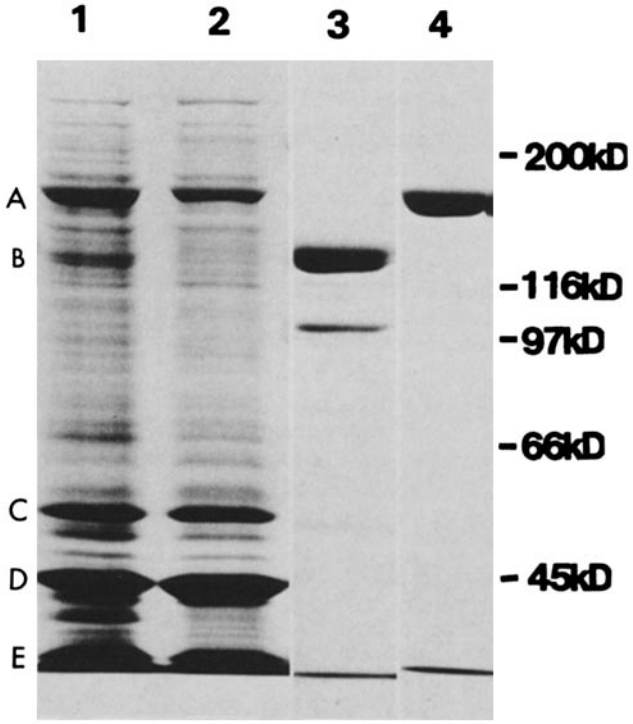


Figure 1. Coomassie Blue–stained SDS polyacrylamide gel (7.5%) of the two Triton-insoluble fractions. (Lane *1*) Light fraction, 50 μg; (lane *2*) heavy fraction, 50 μg; (lane *3*) myosin IA/IB standards, 9.5 μg; (lane *4*) myosin II standard, 7.0 μg. The positions of molecular mass standards are shown on the right: *200 kD*, muscle-myosin heavy chain; *116 kD*, β-galactosidase; *97 kD*, phosphorylase B; *66 kD*, bovine serum albumin; *45 kD*, ovalbumin.

Immunofluorescence Microscopy

Glass coverslips were cleaned, covered with 0.1% polylysine, and treated with 1% glutaraldehyde as described by Cooper et al. (14). *Acanthamoeba castellanii* at middle logarithmic phase were removed from an aerated suspension culture and put on the coverslip. The coverslip was layed on wet filter paper in a petri dish and incubated at 30°C to allow cells to adhere to the coverslip. The cells were fixed with 10% formaldehyde in 0.1 M KH_2PO_4, pH 6.8, for 15 min, washed three times with 0.1 M KH_2PO_4, pH 6.8, and treated with cold (−20°C) acetone for 5 min. Permeabilized cells were incubated for 45 min with either affinity-purified anti-110-kD dimer protein antibody (4 μg/ml) or preimmune serum from the same rabbit (1:100 dilution) in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, and 3% bovine serum albumin, and washed three times with the same buffer. Then, cells were incubated for 30 min with fluorescein-labeled goat anti-rabbit IgG (1:200 dilution) and washed three times with 50 mM Tris, pH 7.5, and 150 mM NaCl. Cells were photographed under phase-contrast and fluorescence microscopy.

F-actin Binding Assay

The binding of 110-kD dimer to F-actin was measured by a high speed centrifugation assay in the presence or absence of Ca^{2+}. F-actin (1 μM) was mixed with various concentrations of 110-kD dimer in 100 μl of a medium containing 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 20 μM ATP, and 0.1 mM $CaCl_2$ or 1 mM EGTA. After incubation at 25°C for 30 min, the solution was centrifuged at 100,000 *g* for 3 h in a Beckman 40 rotor at 20°C. The pellets were suspended in 100 μl of distilled water. Both the supernatants and pellets were dissolved in the same volume of SDS sample buffer. Samples were run on 10% SDS polyacrylamide gels, stained with Coomassie Blue, and quantified by scanning densitometry using purified actin and 110-kD dimer as standards.

F-actin–Bundling Assay

To measure F-actin–bundling activity of the 110-kD dimer, low speed centrifugation was carried out. G-actin (2 μM) was polymerized at room temperature in the presence of varied concentrations of 110-kD dimer in a medium containing 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 0.1 mM $CaCl_2$, and 0.2 mM ATP. After incubation for 3 h the solution was centrifuged at 20,000 *g* for 20 min at 20°C. The supernatants were carefully removed and the protein concentration of the pellets was determined by the Bradford protein assay (5). The pellets were analyzed by SDS PAGE, and actin and the 110-kD dimer were determined by scanning densitometry.

Falling Ball Viscometry

The low shear viscosity of the F-actin/110-kD dimer protein mixture was measured by falling ball viscometry according to MacLean-Fletcher and Pollard (35) with the capillary at an angle of 30° or 50° from the horizontal.

Polymerization Assay of G-actin

Polymerization of G-actin was measured at 25°C by the fluorometric assay (6, 30) using *N*-pyrenyl iodoacetamide–labeled G-actin (5%) in a medium containing 10 mM Tris-Cl, pH 7.5, 2 mM $MgCl_2$, 50 mM or 100 mM KCl, 0.1 mM $CaCl_2$, and 0.2 mM ATP.

Results

Protein Composition of the Triton-insoluble Fractions

The recovery of protein in the combined light and heavy fractions was consistently 2–3% of the total cellular protein, but the distribution of protein between the light and heavy frac-

Table I. Relative Enrichment of Myosin II, Myosin I, 55-kD Polypeptide, and Actin in the Triton-insoluble Fractions

Fraction	Protein	Protein composition*				ATPase activity‡	
		Myosin II	Myosin I	55-kD	Actin	K^+–EDTA	Ca^{2+}
	mg		%			*μmol/min per mg*	
Homogenate	1,914	<1.3			19.5	0.009	0.016
Triton-insoluble fraction							
heavy	25	4.1	0.6	10.4	42.3	0.024	0.026
light	17	9.1	4.5	10.2	27.3	0.121	0.067
23,000 *g* sup	1,672	<1.1			17.3	0.011	0.020

*The content of each protein was determined by quantitative densitometry of Coomassie Blue–stained gels as described in Materials and Methods. It was assumed that each band contained only one protein.

‡ATPase activities were measured at 30°C in a medium containing 10 mM imidazole, pH 7.0, 2 mM EDTA, 0.5 M KC (K^+–EDTA–ATPase) or in a medium containing 10 mM imidazole, pH 7.0, 10 mM $CaCl_2$, 0.5 M KCl (Ca^{2+}–ATPase). ATPase activities of purified myosin I and myosin II are: myosin I, 2.72 (K^+–EDTA) and 0.34 (Ca^{2+}) μmol/min per mg; myosin II, 0.090 (K^+–EDTA) and 0.323 (Ca^{2+}) μmol/min per mg.

tions varied somewhat among different preparations. Both the heavy and light fractions had relatively simple protein compositions (Fig. 1) consisting of four major Coomassie Blue-stained components. The most abundant component (Fig. 1, band *D*) had a molecular mass of 42 kD and co-migrated with purified ameba actin. Since the negatively stained specimens of the Triton-insoluble fractions showed numerous actin filaments in the electron microscope (data not shown), and no other protein is present in such high concentration in the cells, band D must be actin. Band A and the band B doublet were identified as myosin II and myosins IA and IB, respectively, by their molecular masses and by immunoblot analysis using specific antibodies against myosin I and myosin II (data not shown). Band C had a molecular mass of 55 kD by SDS PAGE. As shown later, this polypeptide is a subunit of a 110-kD dimer actin-binding protein.

Electron microscopy of thin sections of both Triton-insoluble fractions revealed numerous membrane structures and many actin filaments (data not shown). We confirmed the presence of membranes by lipid analysis: the fractions contained 0.4–0.6 μmol of lipid phosphorus/mg of protein. In addition, band E had the same mobility on SDS polyacrylamide gels (12%) as the 15-kD protein which is the major protein of *Acanthamoeba* plasma membranes (29) and staining gels with periodic-Schiff reagent revealed the presence of the two lipophosphonoglycans that are characteristic components of the plasma membrane (29).

The major non-plasma membrane components were quantified by scanning densitometry of Coomassie Blue-stained gels (Table I). Myosin I was more enriched in the light fraction than in the heavy fraction, myosin II was also slightly more enriched in the light fraction, while actin was more enriched in the heavy fraction. The relative enrichment of myosin I in the light fraction was also demonstrated by the K^+–EDTA–ATPase activity which was 13 times more concentrated in this fraction than in the homogenate. Myosin I has much higher K^+–EDTA–ATPase activity than Ca^{2+}–ATPase activity (46) while the reverse is true for myosin II (12), and no other ameba enzyme is known to have ATPase activity in the presence of EDTA. Therefore, the K^+–EDTA–ATPase activity is probably a good indication of the myosin I concentration. However, most of the components of the Triton-insoluble fractions were present in greater amounts in the supernatant fraction. Therefore, their enrichment in the insoluble fractions cannot be taken as proof that these fractions represent true cytoskeleton complexes.

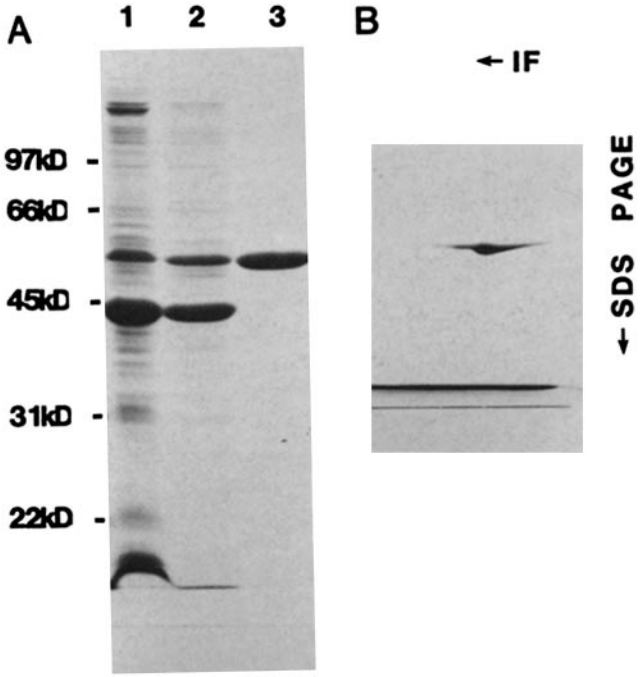


Figure 2. Electrophoretic analysis of the purification of the 55-kD polypeptide. (*A*) SDS PAGE of the Triton-insoluble fraction (lane *1*), 0.3 M NaCl extract (lane *2*), and purified 55-kD (110-kD dimer) polypeptide (lane *3*). The positions of molecular mass standards are shown on the left: *97 kD*, phosphorylase B; *66 kD*, bovine serum albumin; *45 kD*, ovalbumin; *31 kD*, carbonic anhydrase; *22 kD*, soybean trypsin inhibitor. (*B*) Two-dimensional gel electrophoresis-isoelectric focusing. *IF*, isoelectric focusing; *SDS PAGE*, SDS polyacrylamide gel (10%) electrophoresis.

Purification of the 55-kD Polypeptide from the Triton-insoluble Fractions

The 55-kD polypeptide, which comprised 10% of the non-membrane proteins of the Triton-insoluble fractions, was the only one not previously described. This protein co-migrated with calf brain tubulin upon SDS PAGE. However, neither Triton-insoluble fraction bound ^{3}H-colchicine, and antibodies against tubulins from turkey gizzard, *paramecium*, and yeast did not react with any of the proteins by immunoblot assays, indicating that the 55-kD polypeptide was not tubulin.

The 55-kD polypeptide could be extracted from the insoluble fractions with buffer containing >0.2 M NaCl (Fig. 2). The extraction was not affected by ATP. The 55-kD polypeptide in the NaCl extract was fractionated by ammonium sulfate, and purified by DEAE-cellulose column chromatography (Fig. 3 *A*) followed by hydroxyapatite chromatography (Fig. 3 *B*). A minor protein peak, which eluted from hydroxyapatite almost at the beginning of the phosphate gradient, contained several low molecular mass proteins in addition to a small amount of the 55-kD polypeptide (Fig. 3 *B*) and showed strong actin cross-linking activity (Fig. 3 *B*, *inset*). Two protein components in this fraction had molecular masses of 37 and 26 kD, respectively, which are very similar to those of two of the gelactins purified previously in this laboratory (38). Hence, the cross-linking activity of this fraction was probably due to the presence of gelactins.[3] The major peak of the 55-kD polypeptide from the hydroxyapatite column was >98% pure as judged by SDS PAGE (Fig. 2 *A*, lane *3*). Usually, 9–12 mg of purified protein was obtained from 1,500 mg of Triton-insoluble protein.

The purified protein had a molecular mass of 110 kD by sedimentation equilibrium analysis, so the native molecule appeared to be a dimer. By two-dimensional gel electrophoresis-isoelectric focusing analysis, the 55-kD polypeptide contained one major component (Fig. 2 *B*), indicating that the 110-kD protein was a homodimer. The sedimentation coefficient of the protein was 5.5 *s* for a calculated frictional

3. In an abstract (55), we reported incorrectly that the 110-kD dimer protein increased the low shear viscosity of F-actin and accelerated ATP-actin polymerization. We had used a steeper gradient to elute the protein from the hydroxyapatite column and a trace amount of gelation activity contaminated the 110-kD dimer peak.

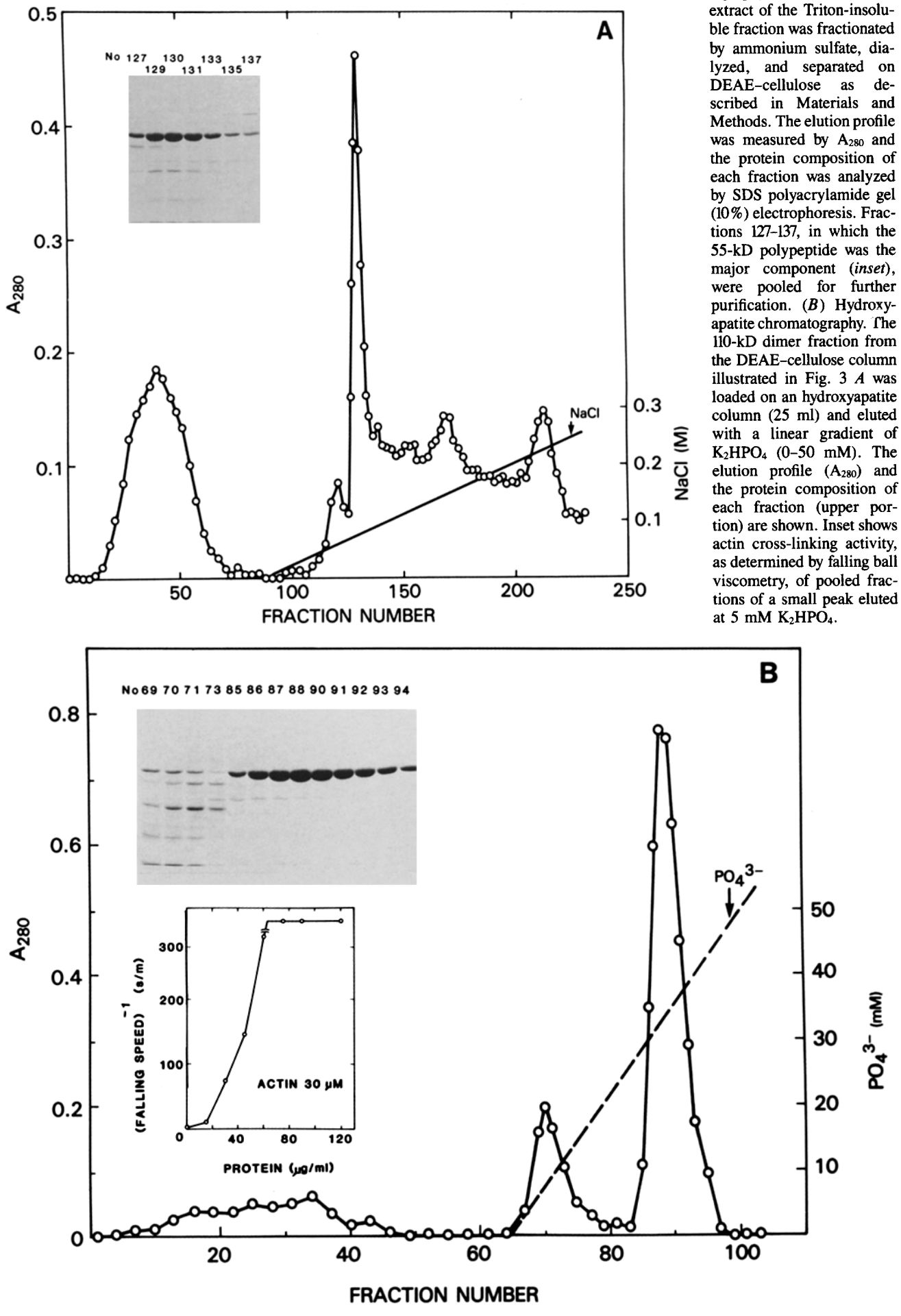


Figure 3. Purification of the 110-kD dimer. (*A*) DEAE-cellulose column chromatography. The 0.3-M NaCl extract of the Triton-insoluble fraction was fractionated by ammonium sulfate, dialyzed, and separated on DEAE-cellulose as described in Materials and Methods. The elution profile was measured by A_{280} and the protein composition of each fraction was analyzed by SDS polyacrylamide gel (10%) electrophoresis. Fractions 127–137, in which the 55-kD polypeptide was the major component (*inset*), were pooled for further purification. (*B*) Hydroxyapatite chromatography. The 110-kD dimer fraction from the DEAE-cellulose column illustrated in Fig. 3 *A* was loaded on an hydroxyapatite column (25 ml) and eluted with a linear gradient of K_2HPO_4 (0–50 mM). The elution profile (A_{280}) and the protein composition of each fraction (upper portion) are shown. Inset shows actin cross-linking activity, as determined by falling ball viscometry, of pooled fractions of a small peak eluted at 5 mM K_2HPO_4.

Table II. Amino Acid Composition of Two Preparations of 110-kD Dimer Actin-binding Protein

Amino acid	Residues	
	Preparation 1	Preparation 2
Asp	11.5	11.8
Glu	10.3	10.0
Lys	7.5	7.6
His	1.7	1.7
Arg	4.9	5.1
Thr	6.3	6.5
Ser	6.7	6.2
Gly	9.8	8.8
Ala	9.1	9.0
Val	7.8	7.7
Met	0.6	1.1
Ile	4.4	4.7
Leu	5.9	5.8
Tyr	3.3	3.4
Phe	4.4	4.4
Pro	5.8	6.2

ratio of 1.50. The amino acid composition of the purified 110-kD dimer protein is given in Table II. The 110-kD dimer was more enriched in basic amino acids than brain tubulin (47) and its amino acid composition is also distinct from those of other actin-binding proteins with similar molecular masses (8, 41).

Binding of the 110-kD Dimer Protein to F-actin

By high speed sedimentation assay (Fig. 4), the 110-kD dimer was shown to bind to F-actin with a maximal binding stoichiometry of 0.5 mol of dimer/mol of actin subunit (1 mol of 55-kD/mol of actin). The binding was not affected by Ca^{2+}. Binding was 50% of saturation at a free dimer concentration of ~0.2 μM, assuming that the 110-kD dimer interacted only with F-actin and that the actin critical concentration was 0.1 μM.

The Effect of 110-kD Dimer on the Sedimentation of F-actin

To test the possibility that the 110-kD dimer may be an actin cross-linking or actin-bundling protein, G-actin was polymerized in the presence of 110-kD dimer and sedimented by low speed centrifugation (Fig. 5). The amount of actin in the low speed pellets increased with increase in 110-kD dimer concentration to a maximum of ~80% of the total actin in the pellets, compared to 30% of the total actin in the pellets in the absence of the 110-kD dimer.

We also measured the effect of the 110-kD dimer on the sedimentation of short gelsolin-capped filaments (F-actin/plasma gelsolin complex, 50:1) by high speed centrifugation. The F-actin/plasma gelsolin complex did not sediment in the absence of the 110-kD dimer (Fig. 5). In the presence of 2.5 μM 110-kD dimer, >40% of the actin sedimented. Since the total actin concentration was 5 μM, and the critical concen-

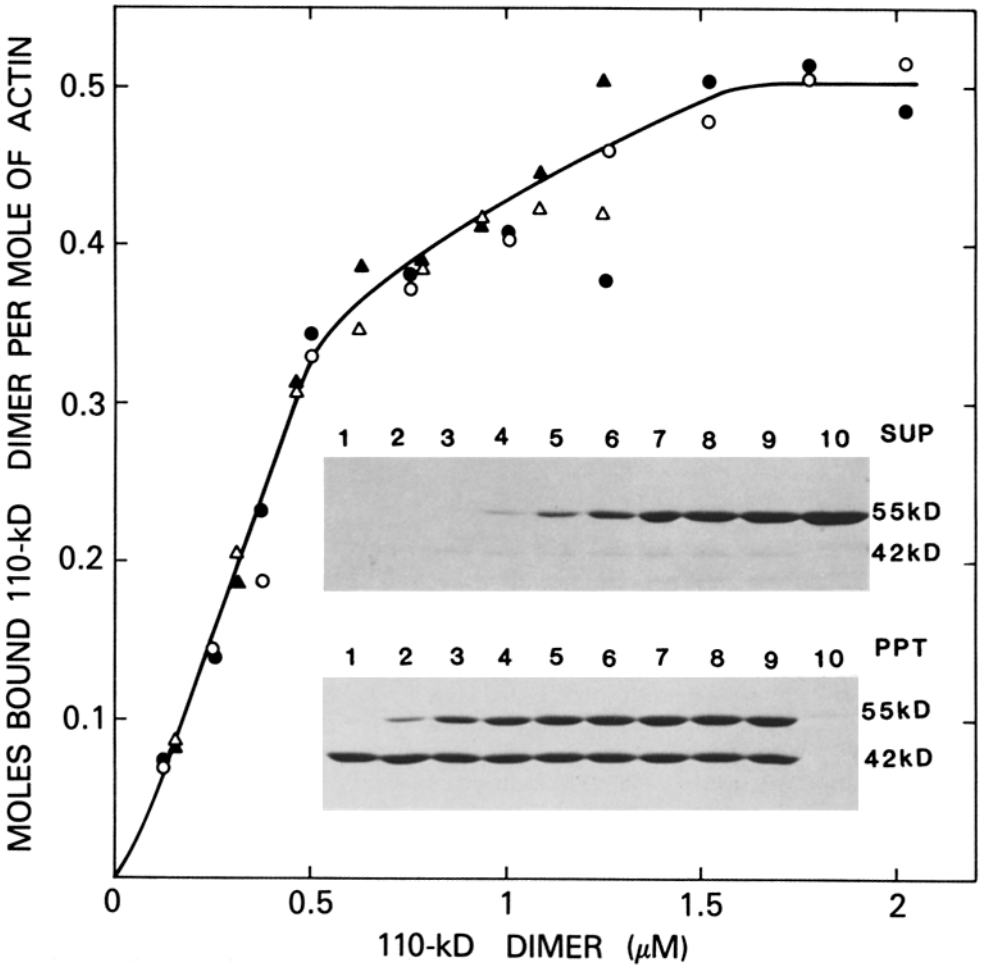


Figure 4. The binding of 110-kD dimer to F-actin. F-actin (1 μM) was mixed with various concentrations of 110-kD dimer in a medium containing 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 20 μM ATP, 0.1 mM $CaCl_2$ (●, ▲), or 1 mM EGTA (○, △). The mixture was centrifuged at 100,000 *g* for 3 h at 20°C. The pellets were analyzed by SDS polyacrylamide gel (10%) electrophoresis, and the 110-kD dimer and actin were quantified by scanning densitometry using purified 110-kD dimer and actin as standards. The data obtained with two different preparations are plotted (circles and triangles). Inset shows the supernatants and pellets. Concentration of 110-kD dimer: (lane *1*) 0; (lane *2*) 0.21 μM; (lane *3*) 0.41 μM; (lane *4*) 0.61 μM; (lane *5*) 0.81 μM; (lane *6*) 1.02 μM; (lane *7*) 1.22 μM; (lane *8*) 1.42 μM; (lane *9*) 1.63 μM. In lane *10*, 1.63 μM 110-kD dimer was added to the medium without F-actin.

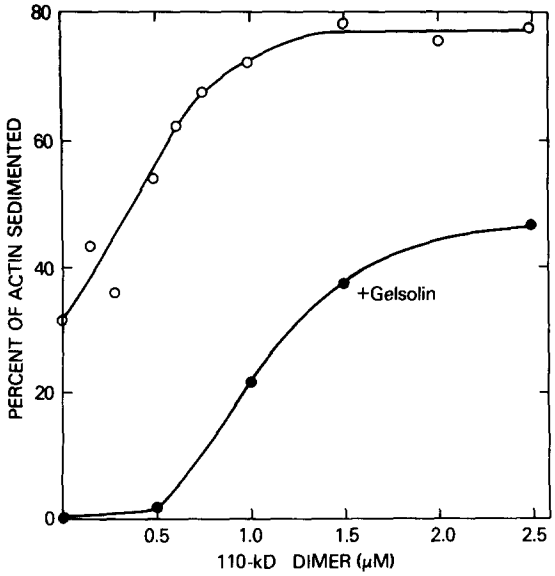


Figure 5. Effect of 110-kD dimer protein on the sedimentation of F-actin. In one experiment (○), 2 μM G-actin was polymerized at room temperature for 3 h in a medium containing 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 0.1 mM $CaCl_2$, 0.2 mM ATP, and various concentrations of 110-kD dimer (0–2.5 μM). The solution was sedimented at 20,000 *g* for 20 min at 20°C. In another experiment (●), short gelsolin-capped F-actin filaments (5 μM F-actin plus 0.1 μM plasma gelsolin) were incubated at room temperature in the same medium with 110-kD dimer. The solution was centrifuged at 100,000 *g* for 3 h at 20°C. The protein in both sets of pellets was analyzed by SDS polyacrylamide gel (10%) electrophoresis as described in Materials and Methods and the actin was quantified by densitometry.

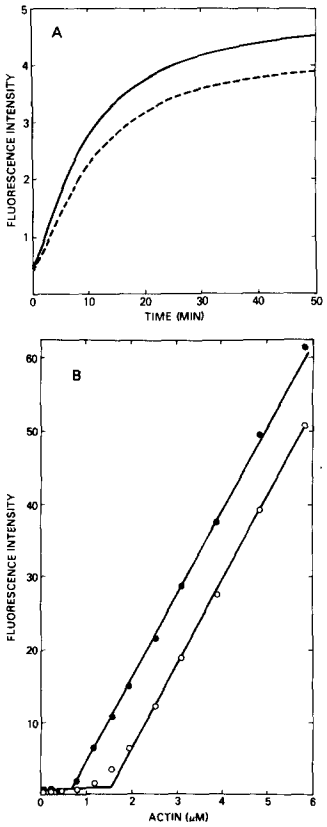


Figure 6. Effect of 110-kD dimer on the rate of polymerization and critical concentration of ADP-actin. (*A*) ADP-G-actin (12 μM, 5% *N*-pyrenyliodoacetamide-labeled actin) was polymerized in buffer containing 5 mM imidazole, pH 7.5, 2 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 0.2 mM ADP, 10 μM diadenosyl pentaphosphate, 0.2 mM DTT, and 0.1 mM NaN_3 in the presence (solid line) or absence (dashed line) of 1.35 μM 110-kD dimer. The increase in fluorescence was measured as a function of time. (*B*) A solution of ADP-F-actin (15.6 μM, 5% *N*-pyrenyl iodoacetamide labeled) in 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 100 mM KCl, 0.1 mM $CaCl_2$, 0.2 mM ADP, 20 μM diadenosyl pentaphosphate, 0.2 mM DTT, and 0.1% NaN_3 was diluted to different actin concentrations in the same buffer with (●) or without (○) 0.4 μM 110-kD dimer. The fluorescence of each sample was measured after 20 h at 25°C, when equilibrium had been reached.

tration in the presence of gelsolin was ~2 μM (17), these results suggest that ~66% of the total F-actin was pelleted as a result of the presence of the 110-kD dimer. Despite these binding data, the 110-kD dimer had no effect on the low shear viscosity of F-actin, as determined by falling ball viscometry, and, under all conditions tested, electron microscopy revealed no consistent, significant differences between actin filaments in the presence and absence of the 110-kD dimer.

Effect of 110-kD Dimer on Actin Polymerization

The effect of the 110-kD dimer on actin polymerization was studied at different G-Actin concentrations under various salt conditions. No significant effect on the rate of polymerization or the critical concentration of ATP-actin was observed. On the other hand, the 110-kD dimer increased the rate of polymerization (Fig. 6 *A*) and decreased the critical concentration (Fig. 6 *B*) of ADP-actin (32).

Effect of 110-kD Dimer on the Actin-activated Mg^{2+}-ATPase Activities of Myosin I and Myosin II

The actin dependence of myosin IB ATPase activity shows an unusual triphasic pattern: an initial hyperbolic activation at low actin concentration is followed by an F-actin–dependent decrease in activity which is then followed by reactivation phase at higher concentrations of F-actin (1). The 110-kD dimer was a more potent inhibitor of the first activation phase than of the activation that occurred at higher actin concentrations (Fig. 7). The ATPase activity at 1 μM F-actin was >90% inhibited by 0.25 μM 110-kD dimer (Fig. 8 *A*) at which concentration the binding of myosin IB to F-actin was only 30% inhibited and 50% inhibition of ATPase activity occurred at a concentration of 110-kD dimer that caused only 10% inhibition of binding. These results are consistent with the fact that at low concentrations of F-actin (high myosin/actin ratio) the ATPase activity exhibits positive cooperative dependence on the myosin concentration (1).

The 110-kD dimer also inhibited the actin-activated ATPase activity of myosin II (Fig. 8 *B*). This inhibition seemed to be greater in the absence of Ca^{2+} than in its presence.

Intracellular Distribution of the 110-kD Dimer

The specific affinity-purified antibody was used for indirect immunofluorescence cytochemistry (Fig. 9 *A*). By immunoblot analysis of a whole extract of ameba, only the 55-kD component was found to be reactive (Fig. 9 *B*). Fluorescent antibody staining showed that the fluorescence was more intense in the peripheral portion of the cytoplasm than in the rest of the cell (Fig. 9 *A*). Filopodia were also fluorescent. No fluorescence was observed in control cells which were incubated with preimmune sera. These results suggest that the 110-kD dimer is concentrated near the plasma membrane which is known to be rich in actin filaments and myosin I (21, 45).

Discussion

Although we do not know if the Triton-insoluble fractions

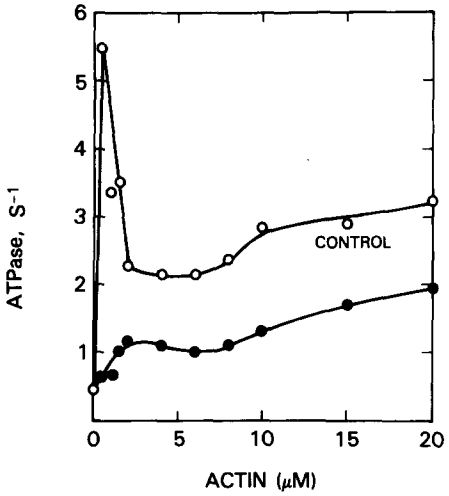


Figure 7. Inhibition of the actin-activated ATPase activity of myosin IB by 110-kD dimer. Actin-activated Mg^{2+}-ATPase activity of myosin IB was measured at 30°C in a medium containing 15 mM imidazole, pH 7.5, 2.7 mM $MgCl_2$, 18 mM KCl, 1 mM EGTA, 2 mM ATP, and the indicated concentrations of F-actin, in the presence (●) or absence (○) of 110-kD dimer. When present, the molar ratio of 110-kD to actin was kept constant at 0.2.

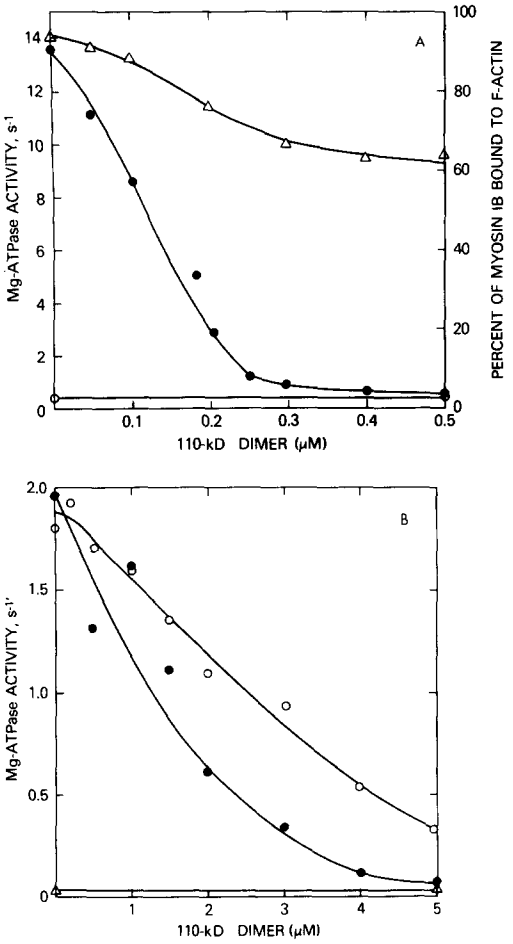


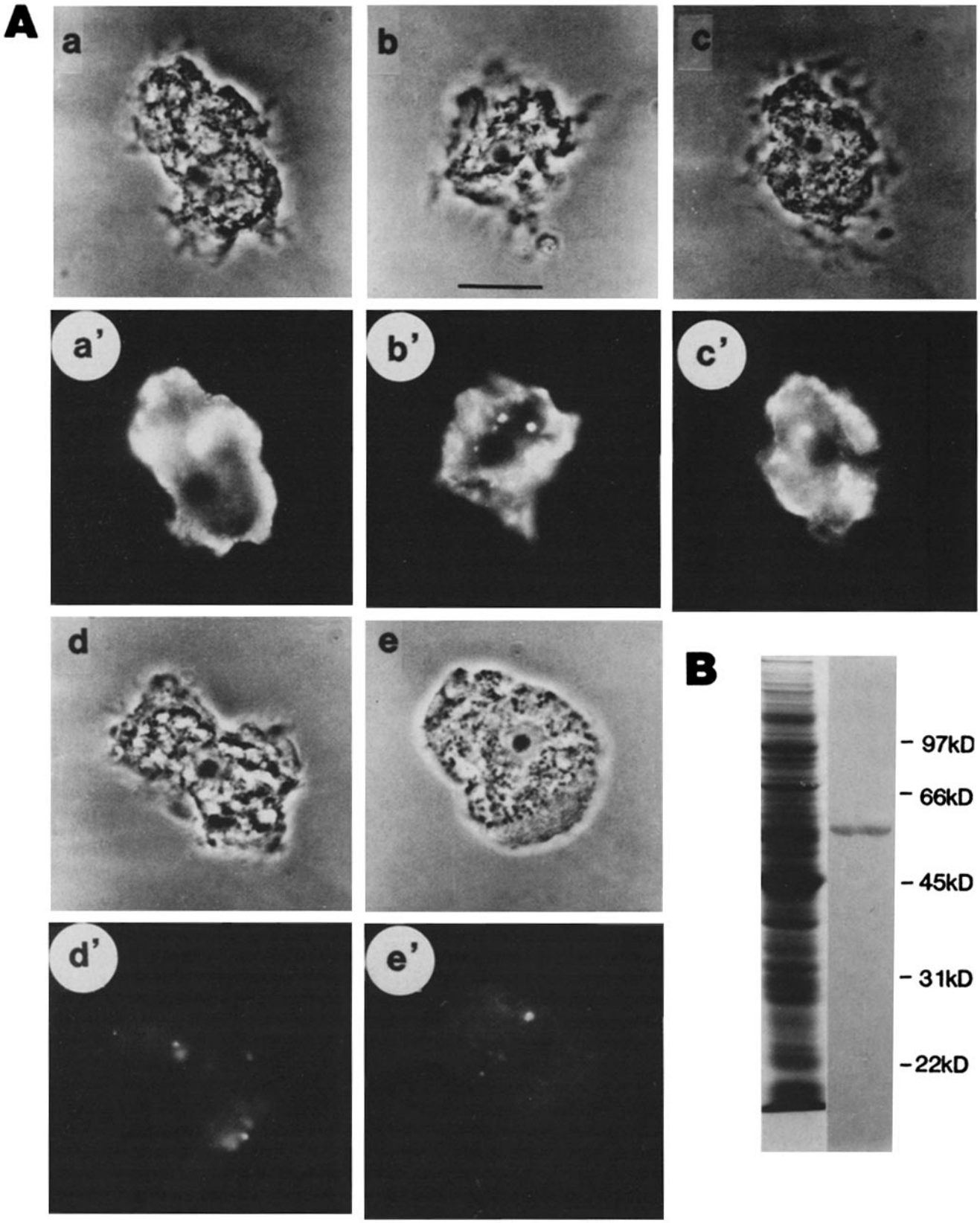
Figure 8. Concentration dependence of the inhibition by the 110-kD dimer of the actin-activated Mg^{2+}-ATPase activities of myosin IB and myosin II. (*A*) Actin-activated Mg^{2+}-ATPase activity of myosin IB was measured at 30°C in a medium containing 10 mM imidazole, pH 7.5, 2.9 mM $MgCl_2$, 15 mM KCl, 1 mM EGTA, 2 mM [γ-^{32}P]-ATP, 0.054 μM phosphorylated myosin IB, and various concentrations of 110-kD dimer in the presence (●) or absence (○) of 1 μM F-actin. For determination of myosin IB binding to F-actin (△), the medium was centrifuged at 100,000 *g* for 1 h at 25°C, and unbound myosin IB in the supernatant was determined by its NH^+_4-EDTA-ATPase activity (9). To prevent myosin IB from adsorbing to the wall of the centrifuge tubes, 1 mg/ml of bovine serum albumin was added to the medium. About 30% of the ATP was found to be hydrolyzed during the centrifugation. (*B*) Actin-activated Mg^{2+}-ATPase activity of myosin II was assayed at 30°C in medium containing 10 mM imidazole, pH 7.0, 6 mM $MgCl_2$, 1 mM [γ-^{32}P]-ATP, 10 μM F-actin, various concentrations of 110-kDa dimer, and 20 μg/ml of dephosphorylated myosin II, in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA. Myosin plus F-actin in the presence of Ca^{2+} (○) or absence of Ca^{2+} (●); myosin alone (△).

are representative of a true membrane–cytoskeletal complex in *Acanthamoeba* they are similar to a concanavalin A–capped plasma membrane complex isolated from *Dictyostelium* (13) (which contained actin, myosin, and three polypeptides with molecular masses of 75,000, 52,000, and 58,000 daltons) and to another *Dictyostelium* cytoskeleton complex (49) (which contained 6.8% myosin).

The 110-kD dimer protein that is greatly enriched in the Triton-insoluble fractions differs from all other actin-binding proteins previously purified from *Acanthamoeba* (14, 15, 24, 25, 38, 43, 44, 48) but a number of actin-binding proteins with similar subunit molecular weights have been purified from various other invertebrate systems including sea urchin egg (7), starfish (36), insect flight muscle (8), and limulus (52), and from mammalian non-muscle cells (41, 42, 50). The binding ratios of these proteins to actin vary between 0.15 and 1. Most of these proteins have been found to bundle or cross-link actin filaments. It is not known if any of them are dimers. Because of its high concentration in the cytoskeletal preparation (one mol/8–10 mol of actin), the magnitude of its binding to actin (0.5 mol/mol of actin), and the fact that it is probably a homodimer, it was expected that the 110-kD protein from *Acanthamoeba* would also cause a structural change in F-actin filaments. The 110-kD dimer did enhance the sedimentation of F-actin filaments, which is usually indicative of filament bundling or cross-linking, but it did not increase the low shear viscosity of F-actin nor could any electron microscopic evidence be obtained for bundling or cross-linking F-actin. We cannot explain the apparent contradictions between these assays.

The function of the 110-kD dimer protein is not known. Our experimental data suggest that it may not be involved principally in the structural organization of the cytoskeleton. Rather, it seems more likely that the 110-kD protein may be involved in the regulation of the actomyosin system. The 110-kD protein inhibited the actin-activated ATPase activity of myosin I (which is localized close to the plasma membrane [21] and is highly enriched in the Triton-insoluble fractions) at substoichiometric ratio to actin. Similarly, the 110-kD

Figure 9. Indirect immunofluorescence analysis of the reaction of *Acanthamoeba castellanii* (Neff Strain) with anti-110-kD dimer antibody. (*A*) Cells were fixed and permeabilized as described in Materials and Methods. They were then treated with either affinity-purified antibody against 110-kD dimer or pre-immune serum in 50 mM Tris-Cl, pH 7.3, 150 mM NaCl, 3% bovine serum albumin followed by FITC-labeled



goat anti-rabbit IgG (1:200 dilution). Cells were photographed under phase-contrast (*a–e*) and fluorescent microscopy (*a'–e'*) at the same magnification. (Bar, 10 μm.) All fluorescence microscopy photographs were taken with 45-s exposure and were printed identically. Cells were treated with either anti-110-kD dimer antibody (*a–c*) or pre-immune serum (*d* and *e*). (*B*) Coomassie Blue-stained gel (10%) showing whole cell extract (60 μg) and corresponding immunoblot showing the reaction of the whole extract with affinity-purified anti-110-kD antibody. The positions of molecular mass markers are shown on the right.

dimer inhibited actin-activated myosin II ATPase activity. In this case, there was a significant difference in the inhibitory action of the 110-kD dimer in the absence and presence of Ca^{2+}. This is the first suggestion of a possibly regulatory role for Ca^{2+} in any of the actomyosin systems of *Acanthamoeba* and it will be of particular interest, therefore, to determine if the 110-kD dimer is truly an actomyosin regulatory protein.

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