Purification of a High Molecular Weight Actin Filament Gelation Protein from *Acanthamoeba* That Shares Antigenic Determinants with Vertebrate Spectrins

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ABSTRACT I have purified a high molecular weight actin filament gelation protein (GP-260) from *Acanthamoeba castellanii*, and found by immunological cross-reactivity that it is related to vertebrate spectrins, but not to two other high molecular weight actin-binding proteins, filamin or the microtubule-associated protein, MAP-2. GP-260 was purified by chromatography on DEAE-cellulose, selective precipitation with actin and myosin-II, chromatography on hydroxylapatite in 0.6 M KI, and selective precipitation at low ionic strength. The yield was 1–2 μg/g cells. GP-260 had the same electrophoretic mobility in SDS as the 260,000-mol-wt alpha-chain of spectrin from pig erythrocytes and brain. Electron micrographs of GP-260 shadowed on mica showed slender rod-shaped particles 80–110 nm long. GP-260 raised the low shear apparent viscosity of solutions of *Acanthamoeba* actin filaments and, at 100 μg/ml, formed a gel with a 8 μM actin. Purified antibodies to GP-260 reacted with both 260,000- and 240,000-mol-wt polypeptides in samples of whole ameba proteins separated by gel electrophoresis in SDS, but only the 260,000-mol-wt polypeptide was extracted from the cell with 0.34 M sucrose and purified in this study. These antibodies to GP-260 also reacted with purified spectrin from pig brain and erythrocytes, and antibodies to human erythrocyte spectrin bound to GP-260 and the 240,000-mol-wt polypeptide present in the whole ameba. The antibodies to GP-260 did not bind to chicken gizzard filamin or pig brain MAP-2, but they did react with high molecular weight polypeptides from man, a marsupial, a fish, a clam, a myxomycete, and two other amebas. Fluorescent antibody staining with purified antibodies to GP-260 showed that it is concentrated near the plasma membrane in the ameba.

Like many other cells, *Acanthamoeba castellanii* has a high molecular weight protein associated with actin filament gels (1) and actomyosin (2), but since this protein had not been purified, nothing was known about its properties. In particular, there was no information concerning its relation to other high molecular weight actin-binding proteins that include the macrophage ABP/smooth muscle filamin class (3–7), erythrocyte and other spectrins (8–15), and the microtubule-associated protein, MAP-2 (16–18).

Early efforts to purify the high molecular weight protein from *Acanthamoeba* actomyosin-II failed due to smearing of the protein during gel permeation chromatography (2), but several new steps now make it possible to obtain enough highly purified protein, and thus establish some of its properties. Because it forms a gel with actin filaments, it will be called GP-260 (gelation protein with a 260,000-mol-wt subunit polypeptide). This ameba also has a smaller gelation protein called GP-85 (19). Antibodies to GP-260 have been used to localize the protein near the plasma membrane in the ameba and to show that it shares some immunological determinants with vertebrate spectrins.

The existence of a spectrin-like protein in a highly motile cell with a well-characterized contractile protein system (20, 21) will expand the scope of the work possible on the spectrin class of proteins. A brief account of some of these findings was presented in March 1983 at the University of North Carolina Conference on Actin-Membrane Interactions (22).

MATERIALS AND METHODS

Materials: Dr. Susan Hagen generously made the hydroxylapatite by

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using the method of Bernardi (23). Other materials were from the following sources: Sigma Chemical Co., St. Louis, MO (ATP [grade I], imidazole [grade III], dithiothreitol, yeast hexokinase, Triton X-100 and NaN_3); Fischer Scientific Co., Pittsburgh, PA (KI, D-glucose); Whatman Chemical Separation Inc., Clifton, NJ (DEAE-cellulose [DE-52]); Schleicher and Schuell, Inc., Keene, NH (Nitrocellulose paper); Miles Laboratories Inc., Elkhart, IN ("Pentex" bovine albumin solution); Schwarz/Mann, Orangeburg, NY (ultra pure ammonium sulfate).

Acanthamoeba actin was purified by a modification of the method of Gordon et al. (24). Pig erythrocyte spectrin, pig brain spectrin, and affinity-purified antibodies to human erythrocyte spectrin (25) were generous gifts from Vann Bennett and Jonathan Davis of The Johns Hopkins Medical School. Chicken gizzard filamin and an immunoglobulin fraction that contained antibodies to gizzard filamin (26) were kind gifts of Susan Craig of The Johns Hopkins Medical School. Pig brain microtubule protein was supplied by Stephen Rothwell of The Johns Hopkins Medical School.

Cells: *A. castellanii* (Neff) was grown in aerated cultures (21) with half of the proteose peptose in the medium replaced by Difco yeast extract.

Preparation of actomyosin-II: Cells were harvested, washed, lysed by N_2 cavitation, centrifuged, and the extract chromatographed on DE-52 (see Fig. 2) as described by Pollard et al. (2). Fractions that contained myosin-I and -II were identified by ATPase assay (27) and the fractions that contained myosin-II plus the leading edge of the actin peak were warmed to 25°C with 2 mM $MgCl_2$ to polymerize the actin, followed by 1 U/ml of hexokinase and 50 mM D-glucose to precipitate actomyosin-II (2).

Biochemical Methods: Protein concentrations were estimated by the Bradford method (28) using actin as the standard. UV absorption was measured with a Cary 219 spectrophotometer. The apparent viscosity at a low shear rate was measured with a miniature falling ball device (29). Actin polymerization was measured fluorometrically with a 5% pyrene-labeled actin (30). Gel electrophoresis was carried out in 10% polyacrylamide gels with SDS (27). Polypeptides in these gels were transferred electrophoretically to nitrocellulose paper in Tris-glycine buffer with 0.1% SDS and 20% methanol. The gels were stained with Coomassie Brilliant Blue and the nitrocellulose with 0.15% amido black in 45% methanol, 9% acetic acid.

Immunological Methods: Two large white New Zealand rabbits were immunized by injections of 50 μg of purified GP-260 in complete Freund's adjuvant at multiple sites. After 4 wk, the rabbits were boosted by injection of 100 μg GP-260 in incomplete Freund's adjuvant. Immune serum was collected 8–19 d later. Antibodies were purified from 1:10 dilutions of immune serum in STTAB buffer (150 mM NaCl, 10 mM Tris-Cl (pH 7.8), 1 mg/ml Triton X-100, 15 mM NaN_3, 1 mg/ml bovine serum albumin [31]) by adsorption to electrophoretically purified GP-260 transferred to nitrocellulose paper (a modification of the method of Olmsted [32]). The paper was washed successively with (*a*) 4 × 5 ml of STTAB, (*b*) 3 × 5 ml of 150 mM NaCl, 20 mM Tris-Cl (pH 8), (*c*) 3 × 5 ml of 1 M KCl, 20 mM Tris-Cl (pH 8), and (*d*) 3 × 5 ml of 150 mM NaCl, 20 mM Tris-Cl (pH 8). Bound antibodies were eluted with 900 μl of 1 M acetic acid for 10–20 min at 4°C. The eluate was neutralized immediately with 100 μl of 2 M Tris base that contained 5 mg of bovine serum albumin, and then dialyzed against 150 mM NaCl, 20 mM Tris-Cl (pH 8). The nitrocellulose with bound GP-260 was re-used successfully several times for antibody purification. Solid phase antibody binding assay and antibody staining of polypeptides on nitrocellulose were described by Tseng et al. (33).

Fluorescent Antibody Staining: Antibodies to GP-260 that were purified from nitrocellulose blots were used to stain *Acanthamoeba* exactly as described by Tseng et al. (33).

Electron Microscopy: Purified GP-260 was dialyzed against 0.5 M ammonium formate, mixed with an equal part of glycerol, sprayed and dried in vacuo on mica, and rotary-shadowed with platinum (34). The JEOL 100 CX electron microscope was calibrated with skeletal muscle tropomyosin paracrystals.

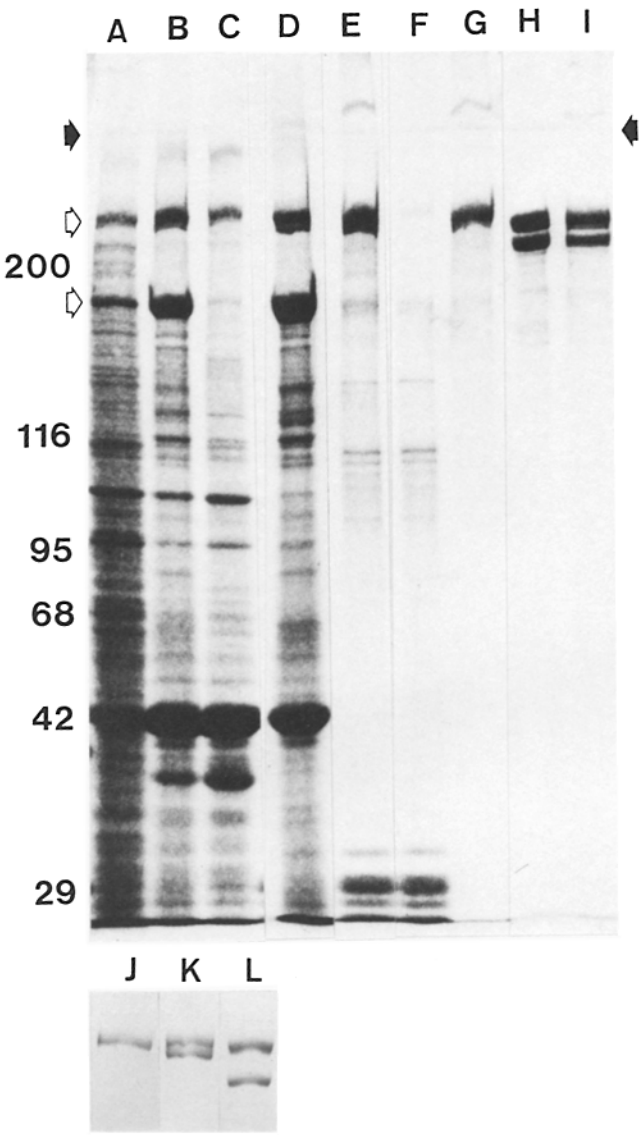

FIGURE 1 Analysis of the polypeptide composition of crude and purified *Acanthamoeba* GP-260 by electrophoresis in 10% polyacrylamide with SDS. Stained with Coomassie Brilliant Blue. (*Left*) The mobilities of molecular weight × 10^{-3} standards. (Filled arrows) The interface of the stacking and separating gels. (Open arrows) The positions of the 260,000-mol-wt pig erythrocyte spectrin band and the 175,000-mol-wt *Acanthamoeba* myosin-II band. (*A*) DEAE pool. (*B*) Actomyosin-II precipitate. (*C*) Buffer G extract of the actomyosin-II. (*D*) Myosin-enriched actomyosin-II (the material insoluble in buffer G). (*E*) GP-260 pool from the hydroxylapatite column. (*F*) Material soluble in 10% sucrose. (*G*) Purified *Acanthamoeba* (GP-260. (*H*) Pig erythrocyte spectrin. (*I*) Mixture of *G* and *H*. (*J–L*) Comparison of *Acanthamoeba* GP-260 (*J*) with pig brain (*K*) and pig erythrocyte (*L*) spectrin on a 5% polyacrylamide gel.

RESULTS

Purification of Acanthamoeba GP-260

For most of this work, I used gel electrophoresis in SDS (Fig. 1) to assay for the high molecular weight polypeptides that co-purify with *Acanthamoeba* myosin-II and actin (2), but the solid phase antibody-binding assay is more convenient now that antibodies are available. This immunological assay is not strictly quantitative for crude samples due to interference by other proteins, but when proper dilutions are made, it can be used to identify which chromatographic fractions contain GP-260 (Figs. 2 and 3).

GP-260 is purified from ~600 g of cells by chromatography on DEAE (Fig. 2) and hydroxylapatite (Fig. 3) and two selective precipitation steps (Fig. 1). An additional fractional precipitation with ammonium sulfate is necessary for some preparations. The polypeptide compositions at each step are shown in Fig. 1 and the yields in Table I.

GP-260 is extracted from the cells in a sucrose buffer and chromatographed on DEAE-cellulose, a step that separates the ameba's contractile proteins into several groups. GP-260

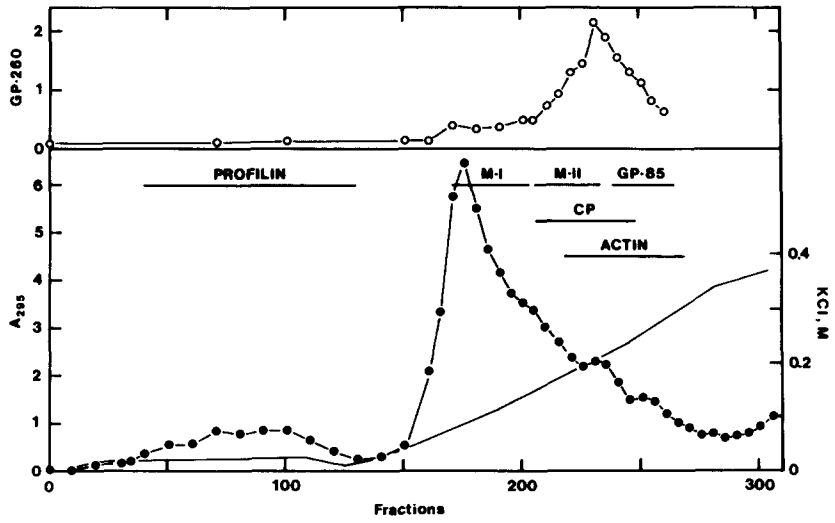

FIGURE 2 Chromatography of a sucrose extract of *Acanthamoeba* on a 5 × 45-cm column of DEAE-cellulose. Fraction size, 20 ml. (●) A_{295}. (——) KCl concentrations. (○) Solid phase antibody binding assay for GP-260 using a 1:100 dilution of column fractions and a 1:100 dilution of an immune serum; units are ^{125}I counts per min × 10^{-3}.

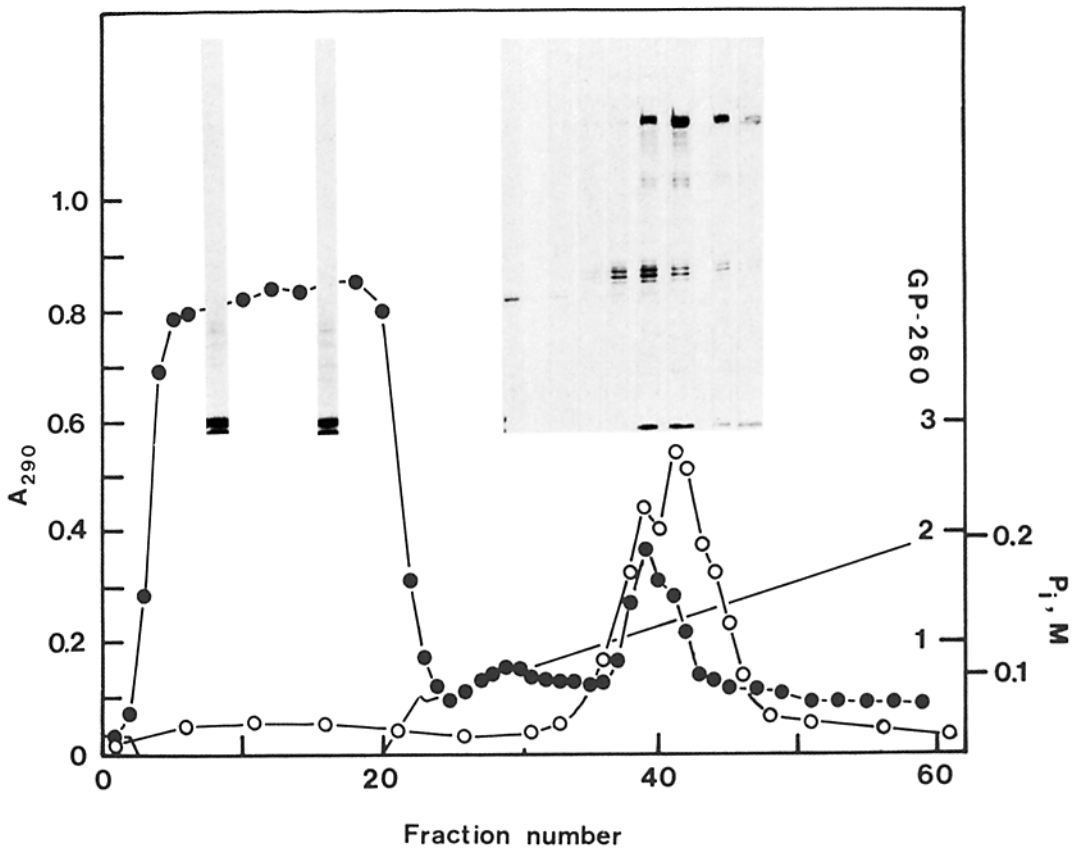

FIGURE 3 Chromatography of a buffer G extract of actomyosin-II on a 1.5 × 7-cm column of hydroxylapatite equilibrated with 0.6 M KI, 60 mM KP_i, 10 mM imidazole (pH 7.5), 1 mM dithiothreitol. The column sample was brought to approximately the same concentrations of KI and KP_i immediately before loading. The sample was followed with ~20 ml of equilibration buffer and then a 200-ml gradient of 60–300 mM KP_i in equilibration buffer. (●) A_{290}. (——) KP_i concentration. (○) Solid-phase antibody binding assay for GP-260 using a 1:12,500 dilution of the fractions and 1:100 dilution of immune serum; units are thousands of ^{125}I counts per min. The high molecular weight polypeptides are shown by SDS PAGE over the peak fractions. Actin runs near the dye front on these gels.

elutes from DEAE-cellulose in a broad zone between 175 and 275 mM KCl that overlaps the peaks of myosin-II (175 mM), GP-85 (275 mM), capping protein (200 mM), and actin(200–300 mM) (Fig. 2). GP-260 is obtained as a by-product of myosin-II purification, so the fractions eluting between 160 and 220 mM KCl that contain myosin-II, GP-260, and the leading part of the actin peak (Fig. 1, lane *A*) are pooled. After polymerization of the actin and removal of ATP with hexokinase and glucose, all of the myosin-II and much of the actin and GP-260 precipitate (Fig. 1, lane *B*) and are separated from the bulk (92%) of the protein by centrifugation. The actomyosin-II precipitate is homogenized in 50–100 ml of a

TABLE I

Purification of Acanthamoeba GP-260

Step	Volume	Total protein
	(ml)	*(mg)*
Cell extract	1,200	12,000 est.
DEAE peak	630	2,520
Actomyosin-II precipitate	57	194
Buffer G extract	56	77
Hydroxylapatite peak	18	4.9
2.0–2.5 M ammonium sulfate precipitate	2	2.3
Sucrose precipitate 1	2	1.1

Protein concentrations estimated by the Bradford method (28). The starting material for this preparation was 600 g of cells estimated to contain 30 g total protein.

low ionic strength actin depolymerizing buffer, buffer G (2 mM imidazole-Cl [pH 7.0], 0.5 mM dithiothreitol, 0.2 mM ATP, 0.2 mM $CaCl_2$), to solubilize about half of the actin and GP-260 (Fig. 1, lane *C*). The fraction of GP-260 extracted depends on the volume of buffer G. A volume of 100 ml is a reasonable compromise, because larger volumes extract more of myosin-II, the most difficult contaminant to remove at later stages in the purification of GP-260. About 50% of the total protein is insoluble in buffer G and is enriched in myosin-II (Fig. 1, lane *D*), but also contains part of the GP-260 and actin. About half of the GP-260 is lost in the pellet at this step. This insoluble GP-260 dissolves in KI but cannot be recovered as a discrete peak when the solubilized, myosin-enriched actomyosin-II is fractionated by gel permeation chromatography in 0.6 M KCl (see Fig. 3 in reference 19).

GP-260 is separated from the actin and most other proteins by chromatography on hydroxylapatite equilibrated with 60 mM KP_i and 0.6 M KI (Fig. 3). GP-260 (but not actin) binds in 60 mM KP_i and is eluted by a gradient at 160–166 mM KP_i. Gel electrophoresis always reveals a single peak of GP-260 at this position (Fig. 3), but the antibody-binding assay reveals that this main peak usually has smaller leading or trailing shoulders of immunoreactive material (Fig. 3). Myosin-II elutes at about the same concentration of KP_i, so it is difficult to purify GP-260 by this method if a sample containing a high concentration of myosin-II, such as the actomyosin, is applied to the column. A peak of material that also absorbs at 290 nm elutes just ahead of the peak of GP-260. It is not associated with any prominent polypeptides (Fig. 3).

The final step in purification of GP-260 is selective precipitation during dialysis against 10% sucrose, 10 mM imidazole, 0.5 mM dithiothreitol. In two of six preparations, the protein did not precipitate in sucrose until it was first concentrated by ammonium sulfate precipitation between 2.0 and 2.5 M. Fortunately, all of the contaminating polypeptides are soluble in sucrose and can be separated from GP-260 by centrifugation at 11,000 *g* for 5 min. The pellet of pure GP-260 is dissolved in 0.5 M KCl, 5% sucrose, 10 mM imidazole (pH 7), 0.5 mM dithiothreitol, and 1 mM NaN_3.

Yield and Purity

The yield of GP-260 from 600 g of cells varied from 250 μg for the first preparation to 1,100 μg for the sixth; with experience there was steady improvement, attributable mainly to the selection of the optimal volume of buffer G to extract protein from the actomyosin. The yield can probably be improved considerably by optimizing for GP-260 at each step, because there are major losses when the DEAE fractions are pooled and during the extraction of the actomyosin. The purity was high (Fig. 1, lane *G*). Although not visible on gels stained with Coomassie Blue, the preparations contained a trace of myosin-II that could be identified by staining gel transfers with antibodies. No other major protein contaminants were detected by gel electrophoresis.

These preparations of GP-260 most likely contain nonprotein material. The UV absorbance spectrum had a maximum at 258 nm and the ratio of A_{280} to A_{260} was 0.56 to 0.72 in three preparations. The material may be a nucleic acid, but it has not yet been characterized or tested for association with GP-260. It may account for the absorbance peak on the hydroxylapatite column that elutes just ahead of the protein. It probably also accounts for why the absorbance of GP-260 is so high at 280 nm—up to a 2 A_{280} per mg/ml of protein in one preparation.

Physical Properties

The purified *Acanthamoeba* polypeptide has approximately the same electrophoretic mobility as the 260,000-mol-wt alpha-chain of spectrin from pig erythrocytes (Fig. 1, lanes *H*, *I*, and *L*) and pig brain (Fig. 1, lane *K*). The electrophoretic mobility in SDS is greater than that of pig brain MAP-2 and less than that of chicken gizzard filamin.

Electron micrographs of purified GP-260 rotary-shadowed with platinum showed rod-shaped particles 70–110 nm long and ~4 nm wide (Fig. 4*A* and *B*). These particles appear to be very flexible compared with GP-85 (19) and the tail of myosin-II (2), two other rod-shaped actin-binding proteins from *Acanthamoeba* (Fig. 4). The size, shape, and bends of these particles are similar to isolated brain spectrin alpha- and beta-chains (35). These preparations of GP-260 also contain a small number of flexible rod-shaped particles with contour lengths between 140 and 200 nm (Fig. 4*C*), that could be end-to-end dimers of the major species.

At a concentration of 150 μg/ml, the purified GP-260 is insoluble in 5–10% sucrose with NaCl concentrations up to 200 mM, or with 1 mM ATP, or with 30 mM Tris-Cl (pH 8). It is soluble in 5% sucrose with 500 mM KCl, with 500 mM KI, or with 25 mM ATP. Given this insolubility at low ionic strength, it is clear why GP-260 is extracted only partially from the actomyosin-II by the actin depolymerizing buffer.

Interaction with Actin

The purified GP-260 is an actin cross-linking protein capable of forming a gel with purified actin filaments (Fig. 5). The effect on the low shear viscosity of *Acanthamoeba* actin filament solutions is identical in 1 mM EGTA (Fig. 5) and in 0.1 mM $CaCl_2$ (not illustrated). The traces of myosin-II in these preparations are not responsible for the cross-linking of the actin filaments, because concentrations of purified *Acanthamoeba* myosin-II similar to those contaminating the GP-260 have little or no effect on the apparent viscosity of actin under the conditions of this assay. At a concentration of 10 μg/ml, GP-260 had no detectable effect on the time course of polymerization of 7.5 μM *Acanthamoeba* actin in 1 mM $MgCl_2$, 10 mM imidazole-Cl (pH 7.5), 0.1 mM $CaCl_2$, and 0.1 mM ATP measured by fluorescence enhancement, so it

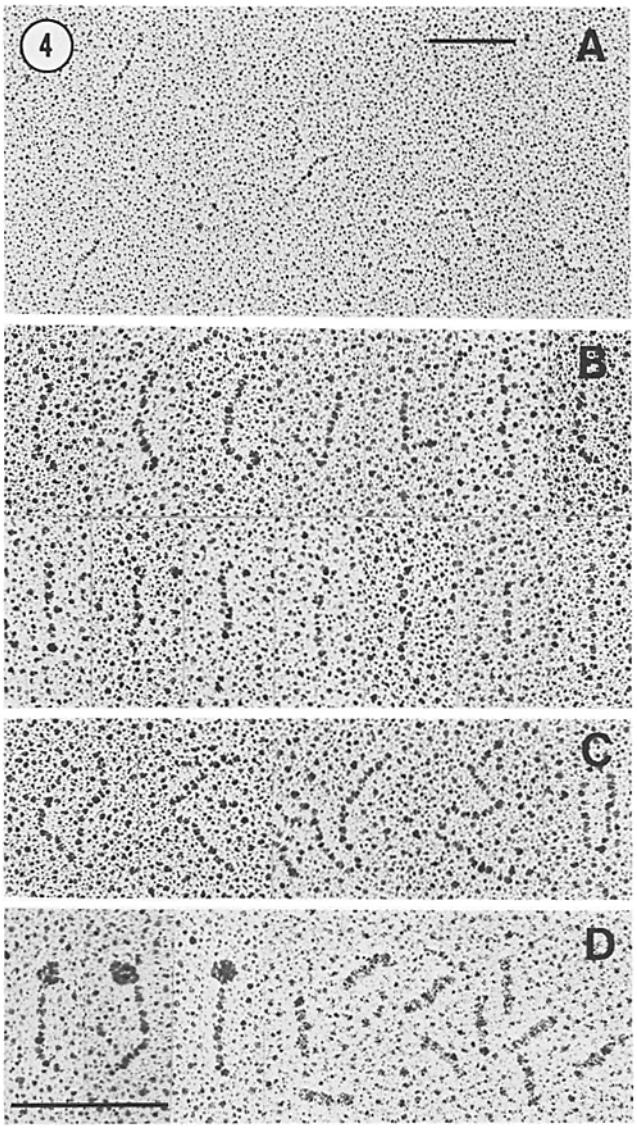

FIGURE 4 Electron micrographs of *Acanthamoeba* GP-260 (*A–C*) compared with *Acanthamoeba* myosin-II and GP-85 (*D*). The purified molecules in 50% glycerol) were sprayed on mica, dried in vacuo, and rotary-shadowed with platinum. (*A* and *B*) Individual GP-260 particles. (C) Possible end-to-end dimers of GP-260. (*D*) (*Left*) Three *Acanthamoeba* myosin-II molecules found as minor contaminants in a preparation of GP-260; (*right*) several purified *Acanthamoeba* GP-85 molecules prepared in the same way. (*A*) Bar, 100 nm. × 116,000. (*B–D*) Bar, 100 nm. × 205,000.

probably has no substantial effect on the nucleation or elongation of actin filaments.

Immunological Studies

Both rabbits produced antibodies to the purified GP-260. When assayed by the solid-phase antibody-binding method with 0.2 μg of GP-260 in each well, these sera were positive at more than 10 times background out to a dilution of 10^4. Purification of these antibodies by binding to and elution from electrophoretically purified GP-260 adsorbed to nitrocellulose yielded small amounts of purified antibody at a concentration 100 times lower than in the original serum.

The sera from rabbits immunized with GP-260 and purified Antibodies to chicken gizzard filamin do not bind to GP-260

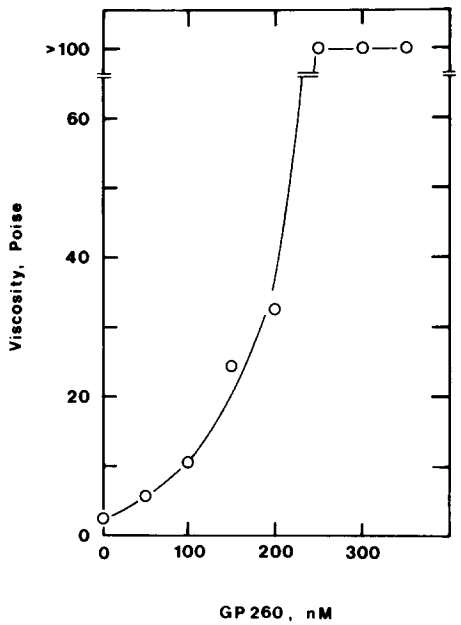

FIGURE 5 Low shear falling-ball viscometry of mixtures of *Acanthamoeba* actin and GP-260. Conditions: 8 μM actin, 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0. 1 mM ATP, 10 mM imidazole (pH 7), 1 mM EGTA; 60 min incubation at 25°C. Apparent viscosities are averages of four measurements at an angle of 80°. A native molecular weight of 520,000 was assumed for GP-260.

antibodies to GP-260 react strongly with polypeptides with molecular weights of 260,000, 240,000, and 100,000 among all of the polypeptides of the ameba that can be separated by gel electrophoresis in SDS (Fig. 6, lane *B-AM*). These same antibodies react with 260,000- and 100,000-mol-wt polypeptides in the soluble extract of *Acanthamoeba* (Fig. 6, lane *B-EX*) and only with the 260,000-mol-wt polypeptide in samples of purified GP-260 (Fig. 6, lane *B-GP*). Pre-immune sera from these rabbits do not bind to any ameba peptides at the dilutions used for the immune sera (Fig. 6*A*). After a second boost with the purified GP-260, both rabbits also produced low levels of antibodies to *Acanthamoeba* myosin-II, the major contaminant in the samples; these sera were not used in the experiments reported here.

The immune sera and purified antibodies from both rabbits also bind to the beta-chains, but not the alpha-chains, of purified spectrin from pig erythrocytes (Fig.6, lane *B-PS*) and pig brain (Fig.7, lane *E*). The reaction is much weaker with pig spectrins than ameba spectrin judging from the intensity of the autoradiograms (cf. Fig. 6, lanes *B-GP* and *B-PS*) and from solid-phase antibody-binding assays. For example, a 100-fold dilution of serum from rabbit JH-20 bound 10 times over background to 500 ng of pig erythrocyte spectrin, while a 10,000-fold dilution of the same antibody bound as well to 200 ng of GP-260. The antibodies to GP-260 do not bind to either chicken gizzard filamin or pig brain MAP-2, two other high molecular weight actin-binding proteins from vertebrates, at least after gel electrophoresis and transfer of the proteins to nitrocellulose (Fig. 6*D, E*).

Purified antibodies to erythrocyte spectrin alpha- and beta-chains (Fig. 6, lanes *C-PS* and *-PS'*) react weakly with 260,000- and 240,000-mol-wt polypeptides in samples of whole amebas (Fig. 6, lane *C-AM*) and purified GP-260 (Fig. 6, lane *C-GP*). In comparison, the reaction with the soluble extract of the ameba is much weaker (Fig.6, lane *C-EX*).

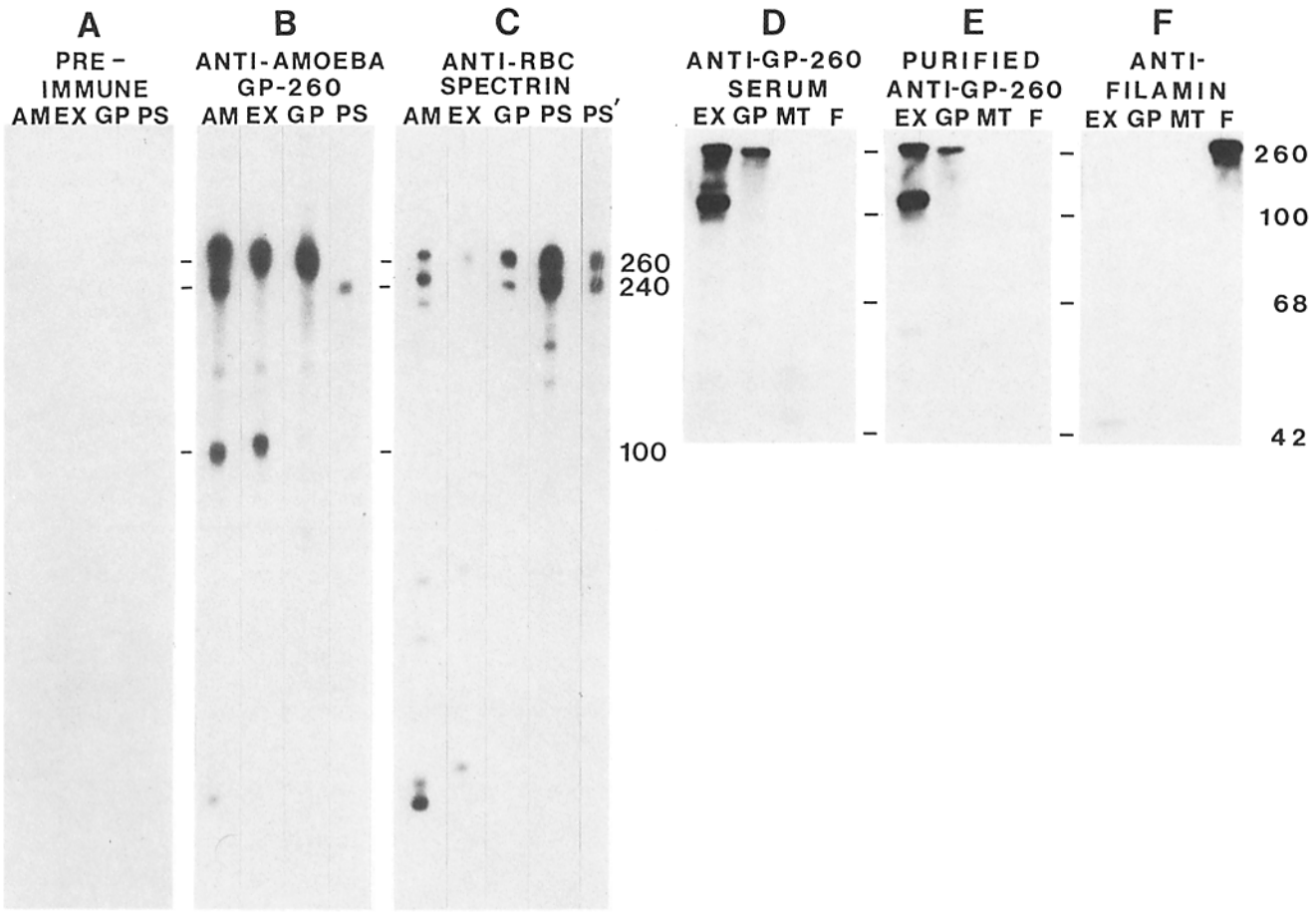

FIGURE 6 Reaction of antisera with polypeptides separated by SDS PAGE and transferred to nitrocellulose paper. *AM*, whole *Acanthamoeba*. *EX*, 140,000 *g* supernatant from *Acanthamoeba* homogenized in 0.34 M sucrose. (The *AM* and *EX* samples were derived from equal numbers of cells so they can be compared directly.) *GP*, purified *Acanthamoeba* GP-260. *PS*, purified pig erythrocyte spectrin. *MT*, pig brain microtubule protein. *F*, purified chicken gizzard filamin. (*D–F* were loaded with about five times more MAP-2 and filamin than GP-260 to look for weak cross-reactivity.) *A* was reacted with a 1:2,000 dilution of pre-immune serum, *B* and *D* with a 1:2,000 dilution of immune anti-*Acanthamoeba* GP-260 serum, *C* with purified antibodies to pig erythrocyte spectrin, *E* with purified antibodies to *Acanthamoeba* GP-260, and *F* with anti-chicken gizzard filamin immunoglobulins. PS' is a short exposure of lane PS to illustrate the positions of the erythrocyte spectrin alpha-chains (molecular weight, 260,000) and beta-chains (molecular weight, 240,000). Antibodies bound to the paper were detected by reaction with ^{125}I-protein A, and autoradiography.

or other peptides in samples of whole amebas fractionated by gel electrophoresis and transferred to nitrocellulose paper (Fig. 6*F*). Antibodies to GP-85 do not react with high molecular weight polypeptides in extracts of the ameba (Tseng, P. C.-H., D. P. Bichell, R. C. Williams, and T. D. Pollard, manuscript in preparation).

Judging from the reactions of the antibodies to GP-260, most of the 260,000-mol-wt polypeptide, but little of the 240,000-mol-wt chain, is extracted into the 140,000 *g* supernatant of cells homogenized in 0.34 M sucrose (Fig. 6, lanes *B-AM* and *-EX*). Likewise, little of the material that reacts with the antibodies to erythrocyte spectrin is extracted into the soluble fraction (Fig. 6, lane *C-AM* and *-EX*).

The 260,000-mol-wt chain in the crude sample is most likely GP-260. The 240,000-mol-wt chain could simply be an insoluble fragment of the 260,000-mol-wt chain, but a more interesting possibility is that it is another polypeptide related to GP-260 (see Discussion). The 100,000-mol-wt polypeptide is not myosin-II or a fragment of myosin-II, because the purified antibodies do not bind to myosin-II and antibodies to myosin-II do not bind to either GP-260 or the 100,000-mol-wt polypeptide. Instead, the 100,000-mol-wt polypeptide is probably a fragment of GP-260, because it has solubility properties similar to GP-260 and varies in amount in different preparations of extract (cf. Figs. 6*B* and 7*A* and *M*).

Purified antibodies to GP-260 also react with high molecular weight polypeptides from a variety of cell types (Fig. 7). The reaction has not been quantitated, but it is strong for *Acanthamoeba, Physarum,* and *Astronyxis*. The reaction with a 260,000-mol-wt polypeptide is weaker for *Naeglaria* (an ameboflagellate), scallop membranes, PtK cells (from a marsupial), fish brain, and human platelets. In the case of *Drosophila* larvae and several chicken striated muscles (but not gizzard), the major reactive species has the same electrophoretic mobility as the myosin heavy chain. Since these antibodies do not react with either myosin-I or myosin-II from *Acanthamoeba* or myosin in samples of platelets, HeLa cells,and PtK cells, it seems likely that they are binding to another polypeptide about the same size as myosin rather than myosin itself.

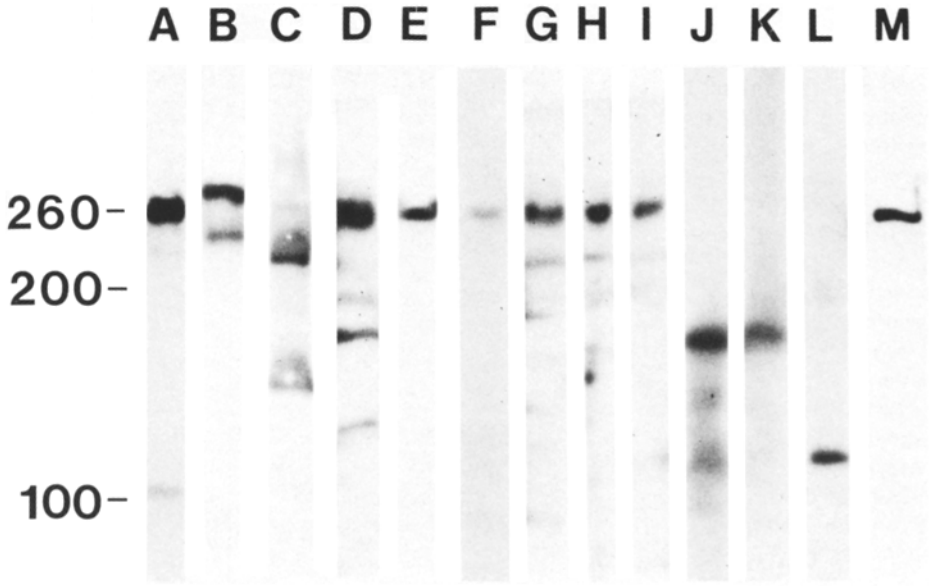

FIGURE 7 Reaction of purified antibodies to *Acanthamoeba* GP-260 with the polypeptides from crude cell samples and purified spectrin from various sources. The samples were prepared by gel electrophoresis and transferred to nitrocellulose paper for reaction with antibodies and ^{125}I-protein A. Autoradiograms localize the sites of antibody binding. (*A*) *Acanthamoeba* extract. (*B*) Whole *Astronyxis*, another *Hartmanellid* ameba. (*C*) Whole *Physarum polycephalum*. (*D*) Whole *Naeglaria gruberi*. (*E*) Purified pig brain spectrin. (*F*) Crude extract of *Fundulus* brain from Mr. Stephen Rothwell (Johns Hopkins Medical School). (*G*) Scallop gill ciliary membranes from Dr. R. E. Stephens (Marine Biological Laboratory, Woods Hole, MA). (*H*) Whole PtK-1 cells. (*I*) Whole human platelets. (*J*) *Drosophila* larvae from Dr. Peter Cherbas (Harvard University). (*K*) Chicken breast muscle. (*L*) Chicken gizzard. (*M*) Another preparation of *Acanthamoeba* extract. The exposures of the autoradiograms were varied to give strong exposures of bands in the high molecular weight region of the gel that are illustrated here. *Left*, Molecular weights $\times 10^{-3}$.

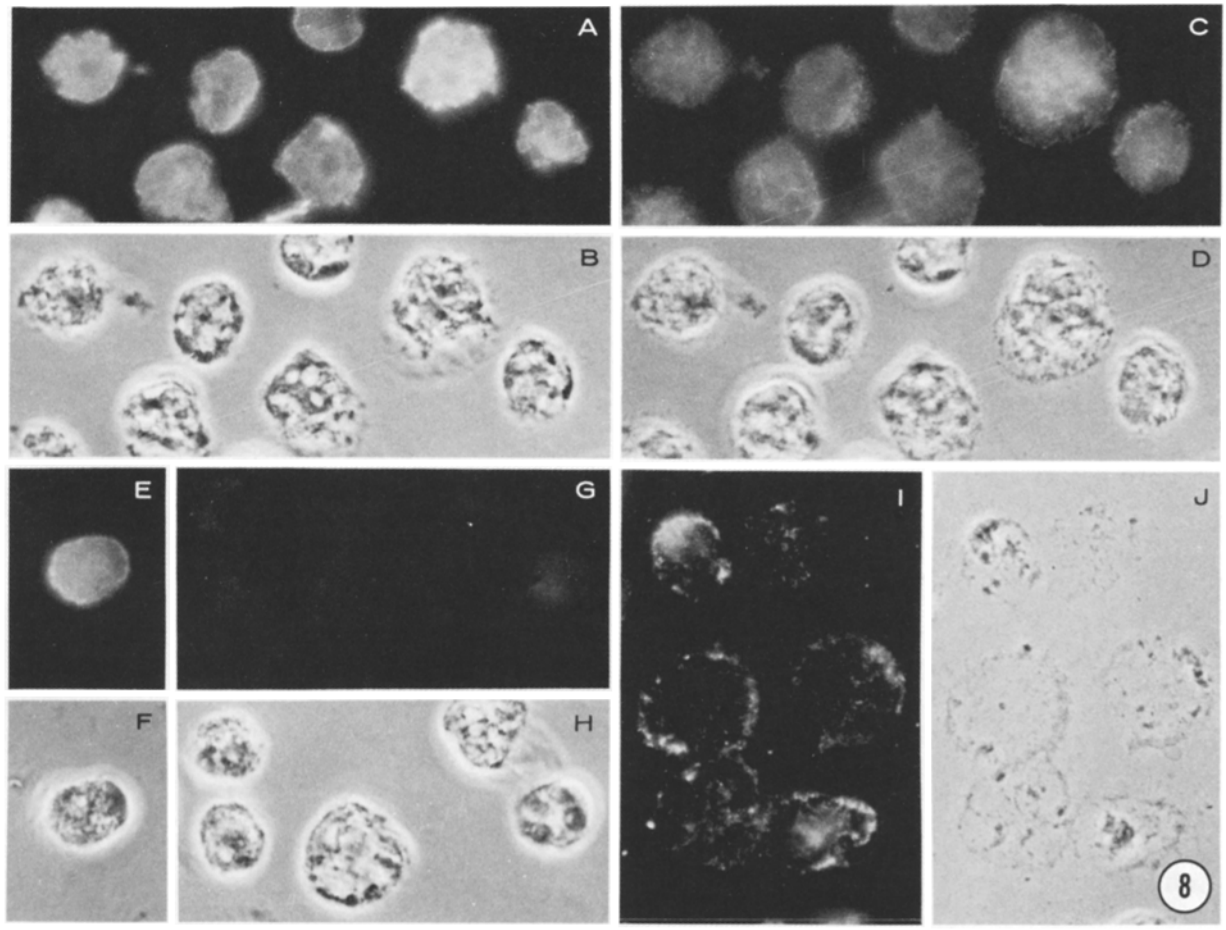

FIGURE 8 Indirect fluorescent antibody staining of *Acanthamoeba* with antibodies to *Acanthamoeba* GP-260. Each field is paired with a corresponding phase-contrast micrograph. (*A, C, I*) Purified antibody. (*E*) A 1:1,500 dilution of immune serum. (*G*) A 1:1,500 dilution of pre-immune serum. (*A–D*), The same field with *A* and *B* focused at the equator of the cells, and *C* and *D* focused at the base of the cells. (*I* and *J*) Six cells where the bulk of the cytoplasm was ripped away during preparation, revealing with greater clarity than in C the pattern of staining of the cortical cytoplasm that has remained attached to the cover slip. × 2,200.

Localization of GP-260 in the Ameba

Both the immune sera (Fig. 8, *E* and *F*) and the purified antibodies (Fig. 8, *A–D, I,* and *J*) to GP-260 stain the cortical cytoplasm next to plasma membrane more strongly than other parts of *Acanthamoeba.* There is also less intense staining of the cytoplasm. The pattern of the fluorescence can be very complex due to convolutions of the plasma membrane, but the plasma membrane staining is obvious to an observer who can focus through the cell in the microscope. Membrane staining is clearest in single photographs focused at the equator, where there is a bright fluorescent ring around the periphery of the cell (Fig. 8, *A* and *E*).

At the base of the cell, a reticular pattern is seen in the plane of the membrane (Fig. 8*C*). Some cells are torn away from their attachments to the cover slip during staining, offering a clear view, unobstructed by overlying cytoplasm, of the reticular pattern of fluorescence associated with the membrane (Fig. 8*I*). A larger relative of *Acanthamoeba* called *Astronyxis* stains in the same way as *Acanthamoeba* (Fig. 9). Controls without anti-GP-260 antibody or with pre-immune serum (Fig. 8, *G* and *H*) gave only very faint general staining, not the distinctive pattern of plasma membrane staining.

DISCUSSION

The GP-260 isolated here is sufficiently pure that one may be confident that it is a component of the actin filament cross-linking system in *Acanthamoeba,* but the mechanism of cross-linking will require a fuller analysis of its physical properties. At high ionic strength, GP-260 is soluble and appears largely as single particles 100 nm long by electron microscopy. Under conditions where the cross-linking experiments were performed, GP-260 aggregates and even precipitates if its concentration is high enough. Thus, it seems likely that multimers of GP-260 are present and participate in the cross-linking of the filaments. The electron microscopic experiments at high ionic strength suggest that end-to-end dimers similar to the ABP/filamin class of gelation proteins (36, 37, 38) might be one type of oligomer of GP-260.

An important factor to be investigated in the future is whether the 240,000-mol-wt polypeptide in the whole cell that reacts with the antibodies to GP-260 might be another component of the native molecule and form some sort of heteromer with GP-260 like the subunits of spectrins. Although not visible on stained gels of purified GP-260 (Fig. 1), there is a trace of a 240,000-mol-wt polypeptide in samples of purified GP-260, because the antibodies to erythrocyte spectrin react with a 240,000-mol-wt species (Fig. 6*C*). Such heteromers might have substantially different properties than the bulk of the GP-260 studied here.

Relationship of GP-260 to Other Actin-binding Proteins of Acanthamoeba

An essential task in characterizing the actin filament system in any cell is to make a complete catalogue of the proteins that bind to actin and affect its polymerization or the organization of actin filaments in the cell. *Acanthamoeba* has been a rich source of actin-binding proteins: two classes of myosin called myosin-I and myosin-II (reviewed in references 1 and 2), two actin monomer-binding proteins called profilin (39) and actophorin (Cooper, J. A., J. D. Blum, and T. D. Pollard, manuscript in preparation), an actin filament capping protein (40) and six actin filament cross-linking proteins (Table II).

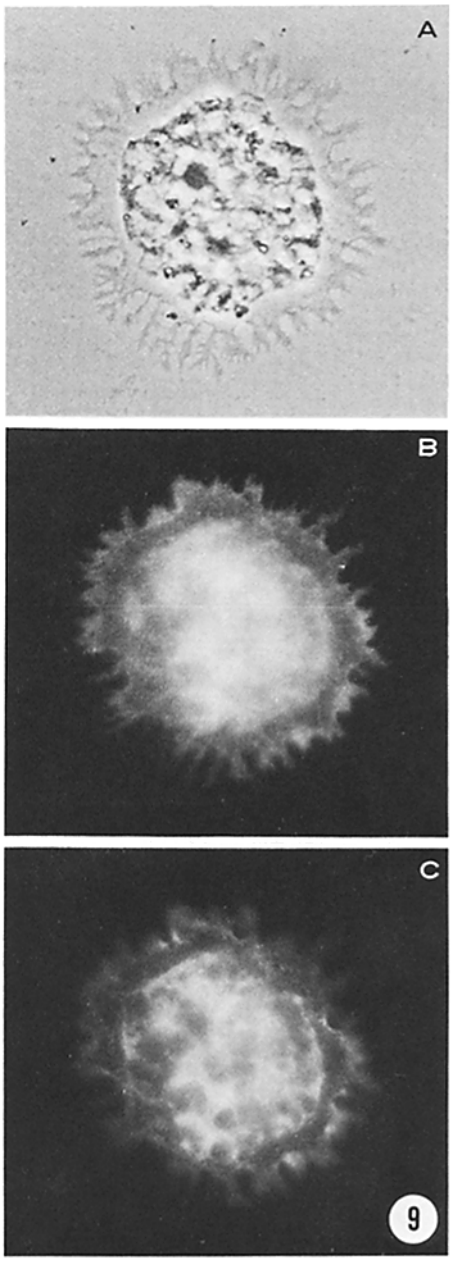

FIGURE 9 Indirect fluorescent antibody staining of *Astronyxis* with purified antibodies to *Acanthamoeba* GP-260. (*A*) Phase-contrast micrograph focused at the base of the cell. (*B*) Corresponding fluorescence micrograph. (*C*) Another fluorescence micrograph focused near the upper surface of the cell showing the complex pattern of cortical staining. × 2,200.

While this list is undoubtedly incomplete, it is already obvious that the actin system in this cell is complex and not likely to be fully understood without characterizing a rather large number of proteins.

GP-260 is the sixth actin filament cross-linking protein to be purified from *Acanthamoeba* (Table II). The others are an alpha-actinin–like protein called GP-85 (19) and four small proteins called gelactins (2, 41). It seems likely that additional cross-linking proteins will also be found. GP-260 is the largest by far and probably present in lower quantities than the others.

It is remarkable that proteins that differ so much in their physical properties (Table II) can have similar effects in the

TABLE II

Comparison of Actin Filament Cross-linking Proteins from Acanthamoeba

Gelation protein	Subunit* composition	Shape	Yield‡, μg/g (μmol/kg)	Critical gelling conc§ μM (actin, μM)	Reference
Gelactin-I	1 × 23,000	Globular	11 (0.48)	0.30 (48)	42
Gelactin-II	2 × 28,000		65 (1.18)	0.45 (48)	42
Gelactin-III	2 × 32,000		16 (0.25)	0.11 (48)	42
				0.16 (12)	43
Gelactin-IV	2 × 38,000		8 (0.10)	0.04 (48)	42
				0.13 (12)	43
GP-85	2 × 90,000	3 × 50-nm rod	100 (0.56)	0.08 (12)	19
GP-260‖	? × 260,000	3 × 100-nm rod	2 (0.004)	0.24 (8)	This report

* Subunit molecular weights by SDS gel electrophoresis. Native molecular weights estimated by gel filtration except for GP-85, where the native molecular weight of 180,000 was measured by sedimentation equilibrium ultracentrifugation.

‡ These yield values are all underestimates of the cellular content of these proteins. For example, the content of GP-85 estimated by antibody binding assay is actually 4.3 μmol/kg of packed cells.

§ The minimal concentration of gelation protein required to form a gel with the given concentration of actin. A tube inversion method was used in reference 42 and falling ball viscometry was used for the other determinations. These two assays are not strictly comparable.

‖ A native molecular weight of 520,000 was used for these calculations.

assays that are commonly used to characterize proteins that bind to actin filaments. In the falling ball assay for the cross-linking of actin filaments, roughly similar molar concentrations of all of these proteins are required to form a gel (Table II). Likewise, all of these proteins form gels with actin filaments equally well in the presence or absence of Ca^{2+}, so that the Ca^{++} sensitivity of gelation of cytoplasmic extracts from *Acanthamoeba* (29) has not yet been explained by the intrinsic properties of the known gelation proteins. (The apparent Ca^{++} sensitivity of GP-85 [19] is now known to be due to an effect of Ca^{++} on the kinetics of actin polymerization, not the cross-linking by GP-85 [Bichell, D. P., D. L. Rimm, P. C.-H. Tseng, and T. D. Pollard, manuscript in preparation].) Similarly, neither GP-85 nor GP-260 has a major effect on the assembly of actin. When polymerization is measured by viscometry, GP-85 appeared to accelerate polymerization (19), but this is not observed when the polymer concentration is measured by the enhancement of the fluorescence of pyrene-actin (Bichell, D. P., D. L. Rimm, P. C.-H. Tseng, T. D. Pollard, manuscript in preparation). Thus, the effect of GP-85 is on the viscosity of the solution, not the kinetics of polymerization.

Why should a cell have so many different actin filament cross-linking proteins? The most likely possibility is that each has a unique function in the cell that may be difficult or impossible to understand from biochemical and biophysical studies on the isolated proteins. For example, although both GP-85 and GP-260 have similar effects on actin in vitro, they most likely have different physiological functions because their distributions in the cell are so different. GP-85 is spread diffusely throughout the cytoplasm and is concentrated in limited regions of the cortex (Tseng, P. C.-H., D. P. Bichell, R. C. Williams, T. D. Pollard, et al., manuscript in preparation), while GP-260 is concentrated near the plasma membrane of the ameba, much like the spectrin in erythrocytes, neurons, and muscle (9, 13, 42, 43). Direct tests of function in the living cell will be required to fully appreciate the sort of subtlety exemplified by the existence of multiple actin filament cross-linking proteins in *Acanthamoeba*.

Relation of GP-260 to Other Actin-binding Proteins

Judging from the cross-reactivity of the antibodies to GP-260 with high molecular weight polypeptides from a wide range of species including protozoa, a clam, an insect, a bird, and man, there are related proteins in many cells and species. This raises intriguing questions, because it is by no means obvious how GP-260 is related to the three well-characterized classes of vertebrate high molecular weight actin-binding proteins: the spectrins, ABP/filamin, and MAP-2. The immunological analysis strongly suggests that GP-260 belongs to the spectrin family, but some of the physical properties of GP-260 suggest instead that it may be in the ABP/filamin group. A clear decision regarding classification will have to await fuller characterization of GP-260, but it is instructive to point out why the available data are ambiguous.

One can argue both for and against classifying GP-260 as a spectrin. This classification is supported by the reciprocal cross-reactivity of antibodies to GP-260 and vertebrate spectrins, the identical electrophoretic mobility of GP-260 and the alpha-chains of spectrins in SDS, the indistinguishable size and shape of GP-260 and isolated alpha- or beta-chains of spectrin, and the common localization of GP-260 and spectrins near the plasma membrane. On the other hand, there are several persuasive arguments against GP-260 belonging to the spectrin family. All known spectrins are composed of two distinct polypeptides that differ in their electrophoretic mobility, peptide maps, and antigenic sites (11, 15, 35, 42). Although each of these chains forms a slender molecule about 100 nm long (like GP-260), the alpha- and beta-chains of spectrin bind tightly enough to each other in parallel heterodimers (44) that denaturing agents are needed to separate them (35). These dimers also associate head to head to form tetramers ~200 nm long (44). Under the high salt conditions used to keep GP-260 in solution, it does not appear to form comparable molecular structures. Further, binding of spectrin to actin filaments requires both alpha- and beta-chains (35), and cross-linking actin filaments requires tetramers (44). Additional features that might identify a spectrin are binding sites for ankyrin and calmodulin (for review see reference 46), but these have not yet been tested with GP-260.

Similarly, one can argue both for and against GP-260 belonging to the ABP/filamin family. Like ABP and filamin (38), the purified GP-260 appears to consist of a single polypeptide that can form a gel with actin filaments. GP-260 also has some tendency to associate end to end like the stable

dimers of ABP and filamin (36). On the other hand, the individual GP-260 particles are somewhat longer than ABP and filamin subunits (38). The absence of cross-reactivity of antibodies to GP-260 and filamin is evidence against relatedness, but like most negative results, this absence is not conclusive. Likewise, ABP and filamin are typically localized in the cortical cytoplasm (47) or deeper in the cell (4) rather than being concentrated near the plasma membrane like GP-260.

There is no evidence that GP-260 is related to the third class of vertebrate high molecular weight actin-binding proteins, MAP-2 (17, 18). The antibodies to GP-260 do not react with any of the polypeptides in pig brain microtuble protein even though they react with spectrin from the same species and organ. Further, antibodies to GP-260 do not stain microtubular structures in the cell. Finally, GP-260 is considerably smaller than the MAP-2 polypeptide.

It is probably fair to conclude that the GP-260, as isolated, is not in the MAP-2 family, and that it does not fit neatly into either the spectrin or ABP/filamin families either. The immunological evidence and cellular localization persuade me to consider GP-260 to be a spectrin, at least as a working hypothesis. The main problem is the apparent absence of a second polypeptide to give GP-260 the structure characteristic of native spectrins. It could be that there are actually two different polypeptides present in the purified GP-260, but that they have the same electrophoretic mobility (like mouse brain spectrin chains [48]) and that they dissociate more readily at high ionic strength than vertebrate spectrins. Alternatively, the missing subunit might be the 240,000-mol-wt polypeptide in whole cell sample that reacts with antibodies to GP-260 and erythrocyte spectrin. This 240,000-mol-wt peptide could simply be an insoluble fragment of GP-260. However, it is attractive to consider that the 240,000-mol-wt peptide might be a second *Acanthamoeba* spectrin chain that is firmly associated with the organelle fraction like the beta-chain of vertebrate spectrins. If this speculation is true, the association between GP-260 and the 240,000-mol-wt peptide must be considerably weaker than the association of alpha- and beta-chains of vertebrate spectrins, and actin binding would have to be a property of GP-260 rather than requiring two chains as in the vertebrate spectrins. Such a model does not coincide with our preconceptions about spectrin based on studies in vertebrate systems, but there is no reason to exclude some variability in other parts of the phylogenetic tree.

Since this work was completed, a report has appeared on a high molecular weight actin-binding protein from *Physarum* (49) that differs in its properties from both GP-260 and the vertebrate actin-binding proteins, illustrating that other proteins may also be difficult to categorize.

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